HARVARD MEDICAL SCHOOL

MASSACHUSETTS GENERAL HOSPITAL

Martha R. Herbert, Ph.D., M.D.
Assistant Professor, Pediatric Neurology
Director, TRANSCEND Research Program
www.transcendresearch.org
transcend@partners.org

Martinos Center for Biomedical Imaging
149 13th Street, Room 10.018
Boston, Massachusetts 02129
Phone: (617) 724-5920
Fax: (617) 812-6334
mherbert1@partners.org

TRANSCEND

TO: Los Angeles Unified School District
FROM: Martha R Herbert, PhD, MD
RE: Wireless vs. Wired in Classrooms
DATE: February 8, 2013

I am a pediatric neurologist and neuroscientist on the faculty of Harvard Medical School and on staff at the Massachusetts General Hospital. I am Board Certified in Neurology with Special Competency in Child Neurology, and Subspecialty Certification in Neurodevelopmental Disorders.

I have an extensive history of research and clinical practice in neurodevelopmental disorders, particularly autism spectrum disorders. I have published papers in brain imaging research, in physiological abnormalities in autism spectrum disorders, and in environmental influences on ndurodevelopmental disorders such as autism and on brain development and function.

I recently accepted an invitation to review literature pertinent to a potential link between Autism Spectrum Disorders and Electromagnetic Frequencies (EMF) and Radiofrequency Radiation (RFR). I set out to write a paper of modest length, but found much more literature than I had anticipated to review. I ended up producing a 60 page single spaced paper with over 550 citations. It is available at http://www.bioinitiative.org/report/wp-content/uploads/pdfs/sec20_2012_Findings_in_Autism.pdf.

In fact, there are thousands of papers that have accumulated over decades – and are now accumulating at an accelerating pace, as our ability to measure impacts become more sensitive – that document adverse health and neurological impacts of EMF/RFR. Children are more vulnerable than adults, and children with chronic illnesses and/or neurodevelopmental disabilities are even more vulnerable. Elderly or chronically ill adults are more vulnerable than healthy adults.

Current te chnologies were designed and promulgated without taking account of biological impacts other than thermal impacts. We now know that there are a large array of impacts that have nothing to do with the heating of tissue. The claim from wifi proponents that the only concern is thermal impacts is now definitively outdated scientifically.

EMF/RFR from wifi and cell towers can exert a disorganizing effect on the ability to learn and remember, and can also be destabilizing to immune and metabolic function. This will make it harder for some children to learn, particularly those who are already having problems in the first place.

Powerful industrial entities have a vested interest in leading the public to believe that EMF/RFR, which we cannot see, taste or touch, is harmless, but this is not true. Please do the right and precautionary thing for our children

I urge you to step back from your intention to go wifi in the LAUSD, and instead opt for wired technologies, particularly for those subpopulations that are most sensitive. It will be easier for you to make a healthier decision now than to undo a misguided decision later.

Thank you.

Martha Herbert, PhD, MD
Pediatric Neurology
Martinos Center for Biomedical Imaging
Massachusetts General Hospital
Harvard Medical School
Boston, Massachusetts
USA

SECTION 20

Findings in Autism (ASD) Consistent with Electromagnetic Fields (EMF) and Radiofrequency Radiation (RFR)

Martha Herbert, PhD, MD

Harvard Medical School, Assistant Professor of Neurology

Massachusetts General Hospital, Pediatric Neurology

TRANSCEND Research Program, Director

Cindy Sage, MA

Sage Associates

Santa Barbara, CA USA

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Part 1 - INTRODUCTION

The premise of this review is that although scant attention has been paid to possible links between electromagnetic fields and radiofrequency exposures (EMF/RFR) and Autism Spectrum Disorders (ASDs), such links probably exist. The rationale for this premise is that the physiological impacts of EMF/RFR and a host of increasingly well-documented pathophysiological phenomena in ASDs have remarkable similarities. Additional support may be found in the parallels between the rise in reported cases of ASDs and the remarkable increases in EMF/RFR exposures over the past few decades. Reviewing these similarities does not prove that these parallels imply causality – that kind of research has not been done. Moreover, the physiological processes affected by EMF/RFR are also impacted by other environmental factors. Yet EMF/RFR does not need to be a unique contributor to ASDs to add significantly to system overload ('allostatic load') and dysfunction. Even so these pathophysiological overlaps do suggest that the potential for an EMF/RFR-ASD connection should be taken seriously, and that their vulnerable biological features may make many with ASDs more likely to experience adverse EMF/RFR impacts. This is a sufficient basis to recommend that precautionary measures should be implemented and respected, that further research should be prioritized, and that policy level interventions based on existing and emerging data should be designed and pursued. Moreover, pursuing this link could help us understand ASDs better and find more ways to improve the lives of people with ASDs and of so many others.

A. How are biology and behavior related?

Considering a potential link between ASDs and EMF/RFR (or indeed of any potential contributor to incidence or pathogenesis) requires taking account of the evolution that has been occurring in our understanding of the relationship between ASD's behavioral and biological features. ASDs were first labeled as 'autism' in 1943 by Leo Kanner, a child psychiatrist who extracted several key behavioral features, related to communication and social interaction challenges and a tendency toward restricted interests and repetitive behaviors, characteristic of all 11 of the children in his first case series (Kanner 1943). Although in the seven decades since this condition was first constructed as a category there has been some modification of the way these behavioral features have been characterized, ASDs are still defined behaviorally, although sensory issues such as hypo- or hyper-reactivity have recently been included in the diagnostic criteria (Diagnostic and Statistical Manual of Mental Disorders or DSM-V) (American Psychiatric Association 2000, 2013, May).

1. Transduction is fundamental but poorly understood

Yet in considering how an environmental factor such as EMF/RFR could lead to autism and/or influence its severity or incidence, we need to think about how underlying biology is transduced into changes in nervous system electrical activity, and how these in turn generate the set of behaviors we have categorized as 'autism.' {Herbert, 2005 #757} This means not taking behaviors as given, or as purely determined by genetics, but exploring the full range of biology that generates these features and challenges.

2. More than brain

Although 'autism' has long been considered to be a psychiatric or neurological brain-based disorder (Rapin and Katzman 1998; Polleux and Lauder 2004), it has become undeniable that people diagnosed with ASDs often also have a multitude of biological features – including systemic pathophysiological disturbances (such as oxidative stress, mitochondrial dysfunction and metabolic and immune abnormalities) (Ming et al. 2012; Tsaluchidu et al. 2008; Pieczenik and Neustadt 2007; Gonzalez et al. 2011) as well as symptomatic medical comorbidities (such as gastrointestinal distress, recurrent infections, epilepsy, autonomic dysregulation and sleep disruption) (Nikolov et al. 2009; Kotagal and Broomall 2012; Kaartinen et al. 2012; Daluwatte et al. 2012; Tuchman and Cuccaro 2011; Canitano 2007; Malow 2004; Kang and Barnes 2013; Jyonouchi et al. 2011) – in addition to the core defining behaviors – and many of these occur commonly (Kohane et al. 2012). The problem has been that no one such biological feature has turned out to be present in every single person carrying an ASD diagnosis – and they are not specific to ASDs, either. Moreover there has been much variability in many of the features of autism – not only between individuals but in many cases within individuals at different points in time or under different circumstances. Because of this variability, the relevance of many of these biological features has been dismissed as secondary and not intrinsically related to the 'autism.' Instead, many have considered the behavioral features as fundamental not only to how autism manifests and is definedbut also to the core intrinsic nature of ASDs, even though the biological basis of these behaviors has by no means been established.

3. Heterogeneity: More Genetic and Environmental than Physiological

It is not as if this variability is unique to the 'environmental side.' At the present time over 800 genes have been associated with ASDs, and over 100 different rare genetic syndromes are frequently accompanied by ASD, with no clear specific unifying mechanism uniting this remarkable heterogeneity (Trikalinos et al. 2006; Ring et al. 2008; Pelphrey et al. 2011; Mandell 2011; Hall et al. 2012; Bill and Geschwind 2009).

Similarly a large number of potential environmental contributors are under investigation ranging from toxicants and Vitamin D deficiency or failure to take prenatal vitamins to air pollution and stress or infection in pregnancy (Whitehouse et al. 2012; Kocovska et al. 2012; Schmidt et al. 2011; Landrigan 2010; Roberts et al. 2007; Shelton, Hertz-Picciotto, and Pessah 2012; Becerra et al. 2012; Volk et al. 2011). Yet at the physiological level a smaller set of disturbances are showing up as common across substantial numbers of people with ASDs – and in fact not uniquely to ASDs but also in myriad other chronic conditions whose prevalence also appears to be increasing (Bilbo, Jones, and Parker 2012; Knox 2010). Prominent among these are immune disturbances including inflammation, mitochondrial dysfunction, and oxidative stress, as well as toxic body burden. Vulnerability to all of these can be increased mildly or substantially by a variety of often common genetic mutations, but may remain latent without the overlay of environmental triggers. Conversely, with substantial enough environmental input, genetic vulnerability may not be necessary.

4. Mechanism is more than correlation

Just HOW biological features might be related to the behavioral features that have up until now defined ASDs has not been clarified; until recently the main research effort regarding pathophysiology in ASDs has been to establish the presence of these phenomena in the first place. Even so, some correlations between biological and behavioral features have been identified – e.g. a higher level of immune abnormalities correlates with more aberrant behaviors (Wei et al. 2012; Careaga and Ashwood 2012; Jyonouchi et al. 2011; Ashwood et al. 2011; Heuer et al. 2008; Zerrate et al. 2007; Curran et al. 2007). Still, such correlations in themselves do not explain the *mechanisms* by which the *transduction of pathophysiology into behavior* might actually occur. In order to do that, an important component would be to study the relationship between systemic pathophysiology and nervous system electrophysiology.

5. EMF/RFR research may help us understand how ASDs 'work'

Assessing the potential contribution of EMF/RFR to ASDs puts this question of the nature of the pathophysiology-behavior transduction into an interesting and provocative light since the brain is simultaneously a tissue-based physical organ that can be compromised by cellular pathophysiology as well as altered developmental processes, and an information processing system that operates through networks of synchronized electrical oscillations (brain waves) – and EMF/RFR impacts may occur directly at both of these levels. To date the emphasis in ASD research has largely been on 'structure-function' relationships that have been anatomy-centered. This research has generated correlations between brain structures and behaviors, and has found some genetic correlates as well, but it has made assumptions that these phenomena are rooted in genetics and genetically perturbed molecular structures and substances. This leads to

targeting the molecular level with pharmaceuticals, but not to the broader agenda of understanding environmental or physiological contributions or dynamic features of brain and behavior. Thus, exploring how EMF/RFR impacts ASDs may help to force the question of how these pathophysiological and electrophysiological/information processing levels actually interact, and how anatomy may in many ways be a product rather than a cause of physiology.

B. Time courses of mechanisms

For the most part, researchers have looked for causes of autism in mechanisms that occur early and create permanent change or damage. This approach is logical if one assumes that genetic influences are overwhelmingly predominant, and 'autism' is a fixed lifelong trait. However evidence is emerging that ASDs may in many respects be more state-like and variable than trait-like and fixed.

1. Plasticity

One of the remarkable shifts in conceptual thinking about ASDs is an appreciation of its brain plasticity (Helt et al. 2008). Growing numbers of reports of improvement and loss of diagnosis, reversal of neurological symptoms in a growing number of mouse models of genetic syndromes that in humans prominently feature autism (Cobb, Guy, and Bird 2010; Ehninger et al. 2008; Goebel-Goody et al. 2012; Henderson et al. 2012; Kaphzan et al. 2012; Liu, Huang, and Smith 2012; Mehta, Gandal, and Siegel 2011; Paylor et al. 2008; Rotschafer et al. 2012; Sato et al. 2012; Suvrathan et al. 2010), short-term pharmaceutically induced improvement in brain connectivity (Narayanan et al. 2010), and transient reversal or abeyance of symptomatology under various circumstances (including fever, fluid-only diet, and certain antibiotic treatments (Sandler et al. 2000; Curran et al. 2007)) – all of these throw into question the long-standing assumption that we are simply dealing with a 'broken brain.' Indeed, how could a 'broken brain' produce markedly improved function with such a short turnaround time? (Herbert 2009) Such a time frame cannot possibly be accounted for by remodeling of the brain's anatomical substrate. 'Brain waves' and their synchronization, on the other hand, could easily vary over short time periods. Looking into physiological and environmental modulators not only of brain development but also of everyday brain function becomes increasingly imperative.

In addition, documentation of average to superior intelligence in most people with autism (Edelson 2006; Dawson et al. 2007), as well as of domains of perceptual superiority (Soulieres, Zeffiro, et al. 2011; Soulieres, Dawson, et al. 2011; Samson et al. 2011; Soulieres et al. 2010; Soulieres et al. 2009; Mottron et al. 2006; Mottron 2004; Bertone et al. 2005; Perreault et al. 2011), call into question the long-standing assumption that ASDs are intrinsically or for the most part associated with cognitive deficits – another strike against the outdated 'deficit' or 'broken brain' model.

2. Mechanisms that operate actively throughout the lifecourse

One particularly valuable lesson about ASDs that can be learned from looking at how EMF/RFR affects underlying biology is that these impacts are by no means confined to early development. We already have clinical reports of 'intermittent autism' – for example, some children with mitochondrial disease who have ups and downs of their bioenergetics status 'have autism' on their bad days but don't display autistic features on their good days (Korson 2007). These children with their vulnerable, barely compensated mitochondria seem to be teetering right at the brink of the interface of metabolic and electrophysiological dysfunction, tipping back and forth on this knife edge. It makes one wonder what everyday exposures – allergens, infection, pesticide on the school playground, even perchance EMF/RFR – might contribute to the bad days (with their loss of electrophysiological optimization, probably on account of insufficient energy to drive fully integrated brain function), and conversely how many choices exist in everyday life that could tilt things in the direction of more good days (by helping to stabilize more optimal nervous system performance) (Herbert and Weintraub 2012).

The short time course needed for biologically effective EMF/RFR 'doses' to lead to observable impacts reflects that these exposures can affect cells without obstruction (unlike many chemical agents), and create impacts within minutes. This type of mechanism may also give us fresh and important ways of understanding the short-term variability – the good days and the bad days – that are so common in ASD even in those who do not have a formal diagnosis of mitochondrial disease.

3. Pathophysiology and Allostatic Load

Based on these considerations, the strategy to be pursued in this examination of a potential EMF/RFR - ASD link is to review the many parallels between underlying biology, or pathophysiology, in ASDs and the impacts of EMF/RFR on living organisms. EMF/RFR exposures have demonstrated impacts at just about every level at which biology and physiology have been shown to be disrupted in ASDs. EMF/RFR has been shown to potentiate the impact of various toxicants when both exposures occur together (Juutilainen, Kumlin, and Naarala 2006); this may be additive or more than additive. This suggests that EMF/RFR may synergize with other contributors and make things worse. With many different environmental factors piling on to a much smaller number of environmentally vulnerable physiological mechanisms (Herbert 2010), one must consider that the model of 'allostatic load' – the sum total of stressors and burdens – may be central to understanding how the many risk factors interact to create autism – and to create a spectrum of levels of severity across so many of ASD's associated features. A cascade of exposures interacting with vulnerabilities can potentially lead to a tipping point for an individual, such as the phenomenon of autistic regression experienced by a substantial subset of people with ASDs. When exposures increase at the population

level, we are likely to see trends of increase in the number of people passing that tipping point and getting diagnosed. EMF/RFR exposures have increased several thousand-fold or more in the past two decades from wireless technology innovations that have unplanned side effects from pulsed RFR, a newly classified human carcinogen (Baan et al, 2011). Nearly six billion people globally own wireless phones, for example. Many hundreds of thousands more are exposed to wireless whole-body transmissions from wireless antenna facilities (Sage and Carpenter, BioInitiative 2012 Report, Section 24). For this as well as for physiological reasons allostatic loading as a viable concept for the study of ASDs should reasonably address EMF/RFR as one of the collection of exposures of relevance to the overall stress load, since it is now a chronic and unremitting exposure in daily life at environmentally relevant levels shown to cause bioeffects from preconception and pregnancy through infancy, childhood and the whole lifecourse.

In an article entitled "Unrelenting Stress is Toxic,: The New Scientist (28 July 2012) describes stress in an eloquent way:

> "*Unrelenting stress is toxic because it can turn the body's defense system against itself. Neuroendocrinologist Bruce McEwen at Rockefeller University in New York says the stress response that evolved to protect us from harm can be hijacked and actually cause harm when the stress level never abates. In a normal situation, the introduction of stress causes the body to deliver a boost of energy – by sending a surge of glucose to the muscles – and to increase heart rate, blood pressure and breathing to get oxygen to the muscles in hurry. At the same time, blood vessels constrict and clotting factors increase – ready to slow bleeding in case you are wounded. These responses are a part of a fight-or-flight survival kit, and once the stress has passed, these should subside. But for people under unrelenting stress, this response never quite switches off – leaving sugar levels unregulated, high blood pressure, increate risk of blood clots, depressed sex drive and an immune system buckling under the strain. Prolonged exposure to stress hormones can have other effects as well, including affecting the brain by altering the structure of the neurons and their connections, which in turn can influence behaviour and hormonal processes."*

This passage refers to effects on the hypothalamo-pituitary-adrenal axis {Aldad, 2012 #2034}, but as will be discussed in the Part II, equally important is cellular stress from stress proteins (heat shock protein HSP) and from oxidative stress generated at very low-intensity EMF and RFR levels as detailed in the BioInitiative 2012 Update, Section 7 by Martin Blank, PhD) {Blank, 2012 #2467}. Both are significant kinds of stress that can add body-burdens via allostatic loading.

Part II - PARALLELS IN PATHOPHYSIOLOGY

This section will review parallels in pathophysiology between ASDs and impacts of EMF/RFR. It will begin with a review of mechanisms of direct impact at the level of molecules, cells, tissues and genes. It will then move on to consider how these levels of damage lead to degradation of the integrity of functional systems including mitochondrial bioenergetics, melatonin, immune function and nervous system physiology. The review of parallels will conclude with a discussion of electromagnetic signaling and synchronized oscillation from membranes to nervous system, treating 'aberrant' neural systems and somatic function and behaviors as consequences or 'outputs' of disturbed underlying physiology to which EMF/RFR is a plausible contributor.

A. DAMAGE: MEANS AND DOMAINS

ASDs have been conceptualized as 'neurodevelopmental' which has focused attention on how genes and environment could alter brain development. This leads to the unstated presumption that virtually everything important about the brain in ASDs has to do with differences in the way it was formed. In genetics this has led to a hunt for neurodevelopmental genes. There is no question that environmental impacts can alter brain development, and impact brain function across the lifespan. This chapter begins the work to systematically rectify the omission of EMF/RFR as one environmental contributor in ASDs.

However the influence of the environment on neurodevelopmental conditions such as ASDs does not stop there. Evidence is accumulating showing that increased expression of genes associated with physiological dysregulation, as well as single-nucleotide polymorphisms (SNPs) associated with these issues, may be if anything more prominent than alterations of 'neurodevelopmental' genes (Lintas, Sacco, and Persico 2012).In a study of gene expression in ASDs, Down syndrome and Rett syndrome, these authors state, *"Our results surprisingly converge upon immune, and not neurodevelopmental genes, as the most consistently shared abnormality in genome-wide expression patterns. A dysregulated immune response, accompanied by enhanced oxidative stress and abnormal mitochondrial metabolism seemingly represents the common molecular underpinning of these neurodevelopmental disorders."* Others have also found pathophysiology-related genes as figuring most prominently in alterations of gene expression in ASD (Kong et al. 2012; Jung, Kohane, and Wall 2011; Voineagu et al. 2011; Waly et al. 2012). SNPs associated with methylation abnormalities, impaired glutathione synthesis and mitochondrial dysfunction also have been identified as significant risk factors.

Genetics may create risk, but the actual nervous system and health consequences probably come from dysfunction at the physiological level. Evidence for pathophysiological dysfunction in ASDs increasingly abounds. In particular, a growing body of literature documents immune aberrations, low total and reduced glutathione levels, lower activity of the anti-oxidative stress system and mitochondrial dysfunction. These phenomena may be both genetically and environmentally modulated. As will be discussed further below, they are certainly pertinent to the neurodevelopment of the brain, which has been by far the dominant focus autism research, but it does not stop there as they can significantly modulate brain function in real time, as well as shape the function of the entire organism, including the autonomic system, the cardiovascular, endocrine, immune, gastrointestinal and reproductive systems and more.

1. Cellular Stress

Oxidative Stress

Autism (ASD) research indicates that oxidative stress may be a common attribute amongst many individuals with autism. In the past decade the literature on this has moved from a trickle to a flood. Studies document reduced antioxidant capacity, increased indicators of oxidative stress and free radical damage, alterations in nutritional status consistent with oxidative stress, altered lipid profiles, and pertinent changes not only in blood but also in brain tissue. Associations of ASDs with environmental exposures such as air pollution and pesticides are indirectly supportive as well, since such exposures are linked in other literature to oxidative stress (Kanthasamy et al. 2012; Roberts et al. 2010; Knox 2010; Rose, Melnyk, Trusty, et al. 2012; Rose, Melnyk, Pavliv, et al. 2012; Ghanizadeh et al. 2012; Frustaci et al. 2012; Rossignol and Frye 2011; Adams et al. 2011, 2011; Mostafa et al. 2010; Zecavati and Spence 2009; Yao et al. 2006; Naviaux 2012; Chauhan and Chauhan 2006; Chauhan, Chauhan, and Brown 2009).

Reactive oxygen species are produced as a normal consequence of mitochondrial oxidative metabolism as well as other reactions, but when their number exceeds the cell's antioxidant capacity a situation of oxidative stress develops. It is certainly the case that oxidative stress can be a consequence of exposures to chemical toxicants, or of the interactive impacts of toxicants, nutritional insufficiencies and genetic vulnerabilities. This set of risk factors has received considerable attention for the potential roles each component and various possible combinations could play in causing or exacerbating autism.

Less often mentioned in the ASD pathophysiology literature is that it is also well established that EMF/RFR exposures can be associated with oxidative damage. Published scientific papers that demonstrate the depth of EMF and RFR evidence reporting oxidative damage in human and animal models are profiled in Section 6 (Genotoxicity) of this BioInitiative 2012 Report and in the BioInitiative Report (2007),

both by Henry Lai, PhD {Lai, 2012 #2548}{Lai, 2007 #2549}. These cellular effects can occur at low-intensity, legal levels of exposure that are now 'common environmental levels' for pregnant women, the fetus, the infant, the very young child, and the growing child as well as for adults. Electromagnetic fields (EMF) can enhance free radical activity in cells (Lai and Singh 2004; De Iuliis et al. 2009) particularly via the Fenton reaction, and prolonging the effect causes a larger increase, indicating a cumulative effect. The Fenton reaction is a catalytic process of iron to convert hydrogen peroxides, a product of oxidative respiration in the mitochondria, into hydroxyl free radical, which is a very potent and toxic free radical (Lai, in the BioInitiative Report 2007) {Lai, 2007 #2549}. Free radicals damage and kill organelles and cells by damaging macromolecules, such as DNA, protein and membrane components.

Further indications of a link to oxidative stress are findings that EMF and RFR at very low intensities can modulate glutamate, glutathione and GABA, and affect mitochondrial metabolism. Alterations in all these substances and processes have been documented in ASDs (Bristot Silvestrin et al. 2012; Brown et al. 2012; Choudhury, Lahiri, and Rajamma 2012; Essa et al. 2012; Oberman 2012; Yang and Pan 2012; Chauhan, Audhya, and Chauhan 2012; Frustaci et al. 2012; Main et al. 2012; Pecorelli et al. 2012; Rose, Melnyk, Pavliv, et al. 2012; Rose, Melnyk, Trusty, et al. 2012; Waly et al. 2012; Banerjee et al. 2012; Coghlan et al. 2012; Enticott et al. 2012; Kang and Barnes 2013; Mendez et al. 2012; Piton et al. 2012; Anitha, Nakamura, Thanseem, Matsuzaki, et al. 2012; Anitha, Nakamura, Thanseem, Yamada, et al. 2012; Gargus 2008; Giulivi et al. 2010; Hadjixenofontos et al. 2013; Napolioni et al. 2011; Rossignol and Frye 2011). Campisi et al (2010) report that increased glutamate levels from 900 MHz cell phone frequency radiation on primary rat neocortical astroglial cell cultures induced a significant increase in ROS levels and DNA fragmentation after only 20 min with pulsed RFR at non-thermal levels (Campisi et al. 2010).

Fragopoulou et al (2012) conducted proteomics analysis of proteins involved in brain regulation in mice as a consequence of prolonged exposure to EMF(Fragopoulou et al. 2012). They identified altered expression of 143 proteins, ranging from as low as 0.003 fold downregulation up to 114 fold overexpression with affected proteins including neural function-related proteins including Glial Fibrillary Acidic Protein (GFAP), alpha-synuclein, Glia Maturation Factor beta (GMF), apolipoprotein E (apoE)), heat shock proteins, and cytoskeletal proteins (i.e., neurofilaments and tropomodulin), as well as proteins of brain metabolism such as aspartate aminotransferase and glutamate dehydrogenase. The authors pointed out that oxidative stress was consistent with some of these changes.

Aberrations in glutathione metabolism and deficiencies in reserves of reduced glutathione are increasingly associated with ASDs, both systemically and in the brain. The parallel with EMF/RFR impacts here is strong, since glutathione reduction associated with

EMF/RFR is reported in at least twenty three relevant research studies in both human and animal studies since 1998, including the following citations (Shapiro et al. 2012; Ozgur, Guler, and Seyhan 2010; Ozguner et al. 2005; Moustafa et al. 2001; Kesari, Kumar, and Behari 2011; Jelodar, Akbari, and Nazifi 2012; Hoyto et al. 2008; Guney et al. 2007; Esmekaya, Ozer, and Seyhan 2011; Atasoy et al. 2012){Al-Demegh, 2012 #2624}{Kumaf, 2010 December #2619}{Meral, 2007 #2627}{Oktem, 2005 #2074}{Ozguner, 2006 #2625} It is increasingly appreciated that glutathione is a final common pathway, a critical piece of environmentally vulnerable physiology, as glutathione reserves are compromised by an enormous number of environmental stressors, so that the cumulative impact upon glutathione may be far greater than could be predicted by the magnitude of any specific exposure (Lee, Jacobs, and Porta 2009), which supports an allostatic loading model.

Also of note are studies showing that the effects of EMF/RFR can be reduced by supplementation with antioxidants and radical scavengers. As an example, Vitamins E and C reduced adverse impacts on rat endometrium from 900MHz EMR exposure (Guney et al. 2007). Gingko bioloba has also prevented mobile phone-induced increases in malondialdehyde and nitric oxide levels in brain tissue as well as decreases in brain superoxide dismutase and glutathione peroxidase activities and increases in brain xanthin oxidase and adenosine deaminase activities, and treated rats were spared the histopathological cell injury found in the untreated rats (Ilhan et al. 2004). Substantial further literature on antioxidants and radical scavengers is reviewed in Section 15 in Belyaev's contribution to the Bioinitiative 2012 Report (Belyaev 2012).

Stress Protein (Heat Shock Protein) Responses

Another well-documented effect of exposure to low- intensity ELF and RFR is the creation of stress proteins (heat shock proteins) that signal a cell is being placed under physiological stress) (Weisbrot et al. 2003; Velizarov, Raskmark, and Kwee 1999; Leszczynski et al. 2004; Leszczynski et al. 2002; de Pomerai et al. 2000; Daniells et al. 1998; Blank and Goodman 2004). Heat shock proteins are in a family of inducible proteins that are initiated when any increased need for protection from stray electrons occurs (Padmini 2010; Bottoni, Giardina, and Scatena 2009). The HSP response is generally associated with heat shock, exposure to toxic chemicals and heavy metals, and other environmental insults. HSP is a signal of cells in distress. Plants, animals and bacteria all produce stress proteins to survive environmental stressors like high temperatures, lack of oxygen, heavy metal poisoning, and oxidative stress. It should also be noted that the generation of HSP stress proteins can have constructive medical applications, such as protection from reperfusion of the heart following ischemic injury (George et al. 2008). Another concomitant impact of cellular stress can be protein misfolding, which has been documented in association with exposure to EMF/RFR. (Bohr and Bohr 2000; Mancinelli et al. 2004)

Although a number of papers have demonstrated increases in HSPs in people with ASDs (El-Ansary and Al-Ayadhi 2012; Evers, Cunningham-Rundles, and Hollander 2002; El-Ansary, Ben Bacha, and Kotb 2012; Walker, Segal, and Aschner 2006; Vojdani et al. 2004), it has been investigated far less often than oxidative stress. Part of the research needed to study possible influences of EMF/RFR on ASDs would be to study this more carefully.

2. Membranes and Channels

Cell membranes and Lipid peroxidation

Cell and organelle membranes play roles in partitioning cells from the extracellular milieu as well as in sustaining boundaries and regulating flow of materials between cellular compartments needing different metabolic parameters for their activities. They also play critical roles in maintaining electrical differences and the flow of electricity.

Adey (2002) summarized studies that report cell membranes as the site of initial field transductive coupling.

> *"Collective evidence points to cell membrane receptors as the probable site of first tissue interactions with both ELF and microwave fields for many neurotransmitters (Mironova et al. 1994), hormones (Liburdy 1995; Ishido, Nitta, and Kabuto 2001), growth- regulating enzyme expression (Byus, Pieper, and Adey 1987; Chen et al. 2000; Litovitz et al. 1993) (Penafiel et al. 1997), and cancer-promoting chemicals (Cain, Thomas, and Adey 1993; Mevissen, Haussler, and Loscher 1999). In none of these studies does tissue heating appear involved causally in the responses. Physicists and engineers have continued to offer microthermal, rather than athermal, models for these phenomena (Barnes 1996; Astumian, Weaver, and Adair 1995), with views that exclude consideration of cooperative organization and coherent charge states, but it is difficult to reconcile experimental evidence for factors such as modulation frequency-dependence and required duration of an amplitude-modulated signal to elicit a response (coherence time) (Litovitz et al. 1993) with models based on the equilibrium dynamics of tissue heating."* (Adey 2002)

Membranes are well-known targets of oxidative stress. Membrane damage is a major route through which free radical damage proliferates through the cellular system. Lipid peroxidation of membranes most often affects polyunsaturated fatty acids such as EPA and DHA which are the most abundant and vulnerable lipids in the brain where the damage they sustain can have serious impacts – DHA is 40% of brain tissue. Lipid peroxidation of membranes has been identified as an effect of EMF/RFR in multiple studies (Desai, Kesari, and Agarwal 2009; Phelan et al. 1992). A variety of other mechanisms for membrane alteration related to EMF/RFR have been intimated in the

literature. Physicochemical properties of membranes such as phase transition of phosphatidylcholine can be shifted by nonthermal effects of microwave radiation (Beneduci et al., 2012). Membrane potential and currents may also be impacted by pulsed radiofrequency fields (Linz et al., 1999). This has been observed graphically in altered cellular movement in Paramecium caudatum, with these cells becoming broader, with a broader-appearing cytopharynx, with their pulse vesicles having difficult in expelling their content outside the cell, and with less efficient movement of cilia (Cammaerts et al (2011) which the authors suggested might be due to targeting of the cellular membrane. The impacts on this unicellular organism may help us imagine what the impact of EMF/RFR might be on cells with some structural similarities, such as columnar epithelial cells and ciliated cells in mucosal surfaces in the respiratory system, digestive tract, uterus and fallopian tubes and central spinal cord.

Indications of lipid peroxidation of membranes has been documented in ASDs, including malonaldehyde and isoprostanes, as well as alteration of membrane phospholipids and prostaglandins (Pecorelli et al. 2012; El-Ansary et al. 2010; El-Ansary, Ben Bacha, and Kotb 2012; Zhang, Sun, et al. 2012; Yao et al. 2006; Al-Gadani et al. 2009; Chauhan and Chauhan 2006; Ming, Stein, et al. 2005; Zoroglu et al. 2004) In one study the iosoprostane levels showed a biomodal distribution with the majority of ASD subjects showing moderate increase but a smaller group showing dramatic increases (Ming, Stein, et al. 2005). Thromboxane, reflecting platelet activation, was also elevated in one study (Yao et al. 2006). Given that this phenomenon has been identified in many people with ASDs, it is plausible that such individuals will likely be more vulnerable to having such cellular injuries caused, worsened or both by EMF/RFR exposures.

Calcium channels

Of particular prominence in the EMF/RFR physiological impact literature is the impact on calcium channels and signaling. Calcium signaling is ubiquitous in biological systems ranging from single-celled organisms to the most sophisticated functioning of our nervous and immune systems. This signaling takes place through a myriad of mechanisms within and between cells. The exquisite tuning of organisms is influenced by the precision of functioning of these systems, with even subtle disturbances having the potential to ramify in a nonlinear fashion through a system causing larger-scale disturbances elsewhere. EMF/RFR exposures have been shown to create disturbances in calcium signaling through a variety of mechanisms, including membrane leakage (Nesin et al. 2012), alteration of calcium-binding proteins and GFAP reactivity (Maskey et al. 2012; Maskey et al. 2010), and altered ultrastructural distribution of calcium and calcium-activated ATPases after exposure (Kittel et al. 1996).. Adey (2002) provided an overview of key studies on calcium efflux and the importance of calcium in cell signalling. *"Early studies described calcium efflux from brain tissue in response to ELF exposures (Bawin and Adey 1976; Blackman et al. 1985), and to ELF-modulated RF*

fields (Bawin and Adey 1976) (Blackman 1979) (Blackman et al. 1985; Dutta, Ghosh, and Blackman 1989). Calcium efflux from isolated brain subcellular particles (synaptosomes) with dimensions under 1.0 μm also exhibit an ELF modulation frequency-dependence in calcium efflux, responding to 16 Hz sinusoidal modulation, but not to 50 Hz modulation, nor to an unmodulated RF carrier (Lin-Liu and Adey 1982). In the same and different cell culture lines, the growth regulating and stress responsive enzyme ornithine decarboxylase (ODC) responds to ELF fields (Byus et al. 1988; Litovitz et al. 1993) and to ELF-modulated RF fields (Byus, Pieper, and Adey 1987) (Litovitz et al. 1993) (Penafiel et al. 1997) ." (Adey 1994)

Dutta et al (1992) reported:

> *"Radio-frequency electromagnetic radiation (RFR) at 915 and 147 MHz, when sinusoidally amplitude modulated (AM) at 16 Hz, has been shown to enhance release of calcium ions from neuroblastoma cells in culture. The dose-response relation is unusual, consisting of two power-density "windows" in which enhanced efflux occurs, separated by power-density regions in which no effect is observed. To explore the physiological importance of these findings, we have examined the impact of RFR exposure on a membrane-bound enzyme, acetylcholinesterase (AChE), which is intimately involved with the acetylcholine (ACh) neurotransmitter system. Neuroblastoma cells (NG108), exposed for 30 min to 147-MHz radiation, AM at 16 Hz, demonstrated enhanced AChE activity, as assayed by a procedure using 14C-labeled ACh. Enhanced activity was observed within a time window between 7.0 and 7.5 h after the cells were plated and only when the exposure occurred at power densities identified in a previous report as being effective for altering the release of calcium ions. Thus RFR affects both calcium-ion release and AChE activity in nervous system-derived cells in culture in a common dose-dependent manner."* (Dutta et al. 1992)

The prominence of these calcium signaling impacts of EMF/RFR are striking when considered in relation to ASD pathophysiology, where such alterations have been proposed as of central importance. Calcium channels play an important role in regulating neuronal excitability, whose disturbance during development has been thought by many to be potentially contributory to the development of ASDs, as well as to the often associated vulnerability to seizures. Gene alterations have been identified associated with a number of voltage-gated calcium channels in ASDs Smith, 2012 #1451}(Krey and Dolmetsch 2007; Pasca et al. 2011; Gargus 2009; Lu et al. 2012). However, based on an examination of patient laboratory and phenotype data it has been argued that aberrant calcium signaling could be downstream: Palmieri and Persico (2010) suggest that *"an abnormal neuroimmune response as a relevant player in elevating intracellular Ca2+ levels, deranging neurodevelopment, driving oxidative stress, and ultimately affecting synaptic function and neural connectivity especially in long-range neuronal pathways*

physiologically responsible for integrated information processing." (Palmieri and Persico 2010) Peng and Jou (2010) have in turn shown how increased intracellular calcium can cause oxidative stress, and a vicious circle: *"...mitochondrial ROS [reactive oxygen species]rise can modulate Ca2+ dynamics and augment Ca2+ surge. The reciprocal interactions between Ca2+ induced ROS increase and ROS modulated Ca2+ upsurge may cause a feedforward, self-amplified loop creating cellular damage far beyond direct Ca2+ induced damage." (Peng and Jou 2010)*

Environmental as well as genetic routes to calcium signaling dysfunction have been identified (Pessah and Lein 2008) including chemicals such as the polyaromatic hydrocarbons. PCB-95 in particular modulates the calcium-dependent signaling pathway responsible for activity-dependent dendritic growth {Wayman, 2012 #2550;Wayman, 2012 #2551}. In fact, once a genetic mutation has been associated with altering a critical signaling pathway and conferring risk for autism, chemicals or other environmental agents can be identified that target the same pathways and also confer ASD risk. Stamou et al. (2012) have reviewed this strategy of identifying multiple mechanisms converging on common signaling pathways regarding Ca(2+)-dependent mechanisms as well as extracellular signal-regulated kinases (ERK)/phosphatidylinositol-3-kinases (PI3K) and neuroligin-neurexin-SHANK (Stamou et al. 2012). From this point of view, there may be no particular reason to privilege genetic mutations in their contribution to a disturbance of calcium signaling, since whether this function becomes derailed due to a genetic mutation, from a chemical toxin or from EMF/RFR perturbation of calcium signaling, the functional effect is comparable. Moreover if a person is subject to multiple triggers all of which have calcium signaling impacts, the gene-environment interactions may lead to impacts that could be less, the same as or more than any one contributor alone might create.

3. Junctions and Barriers

The damage discussed so far has been at the molecular and subcellular level. However impacts from this level reverberate up to larger scales in the system. Where membranes create boundaries between cells and subcellular compartments, barriers do this at a larger scale. Cells become capable of forming barriers between each other through tight junctions which block substances and cells from 'slipping through the cracks,' so to speak, between the cells. Conversely, gap junctions are subcellular structures providing openings that allow physical passage of materials between cells otherwise separated by membranes.

It appears that such connections between cells can also be altered by electromagnetic fields and radiofrequency exposures, at least under certain circumstances. High frequency magnetic fields have been observed to be associated with a sharp decrease in

intercellular gap junction-like structures, in spite of increased gene expression for pertinent proteins {Cervellati, 2009 #1449}. Changes in tight junctions have been observed upon exposure to microwave and x-ray irradiation {Palfia, 2001 #1458}.

A number of papers in the ASD research field document problems pertinent to junctions. Connexin abnormalities have been documented in neuropathological studies (Fatemi et al. 2008). and MacFabe and colleagues identified lipid alterations associated with oxidative stress, membrane fluidity and the modulation of gap junction coupling (Thomas et al. 2012). Decrease in platelet endothelial cell adhesion molecule-1 were reduced and this reduction correlated with repetitive behavior and abnormal brain growth; achesion molecules modulate permeability and signaling at the blood-brain barrier as well as leukocyte infiltration into the central nervous system (Onore et al. 2012).

EMF and RFR might also compromise biologically important barrier structures that separate blood flow from organs like the brain (Salford et al, BioInitiative Report 2012, Section 10) {Salford, 2012 #2477}. This raises important questions regarding whether other 'barriers' that keep blood flow separate from the gut (gut-blood barrier), or the placenta (blood-placenta barrier) or the eye (ocular-blood barrier) may also be rendered pathologically leaky, and allow albumin, toxins, pro-inflammatory cytokines and infectious agents to cross this barrier into the intestines (invoking immune responses) and impacting the developing fetus {Somosy, 1993 #1470}. While there are a fair number of negative studies, there are also many studies showing and association between EMF/RFR and pathological leakage of the blood-brain barrier (BBB), as well as evidence in animal studies of damage to brain cells and damage to or death of neurons. Such leakage has been shown to be potentiated by physiological factors such as diabetes and insulin (Gulturk et al 2010) and has also potentiated viral lethality in a dose-dependent fashion (Lange et al, 1991). Many of the positive findings were associated with non-thermal exposures comparable to normal cell phone radiation exposure {Salford, 1994 #2553;Salford, 2003 #2552}{Salford, 2007 #2629;Salford, 1992 #2628}{Eberhardt, 2008 #1428}{Nittby, 2009 #2307;Nittby, 2008 #2556}. There are scattered reports of increased permeability across other membranes and barriers, such as the blood-testicle barrier in mice (Wang, 2008; wang et al., 2010 and the rat liver canalicular membrane {Lange, 1993 #2557}. A 1992 study by Kues et al. reported that *"studies in our laboratory have established that pulsed microwaves at 2.45 GHz and 10 mW/cm2 are associated with production of corneal endothelial lesions and with disruption of the blood-aqueous barrier in the non-human primate eye."* (Kues et al. 1992) A recent study showing impact of high-frequency electromagnetic fields on trophoblastic connexins (Cervellati et al. 2009) may indicate the vulnerability of the placenta and placental barrier function to electromagnetic fields. A thorough review and methodological discussion of literature regarding EMF/RFR impacts on the BBB is provided by Salford in Section 10 of the BioIniative 2012 Report {Salford, 2012 #2477}.

According to a review by Zlokovic, *"BBB breakdown, due to disruption of the tight junctions, altered transport of molecules between blood and brain and brain and blood, aberrant angiogenesis, vessel regression, brain hypoperfusion, and inflammatory responses, may initiate and/or contribute to a "vicious circle" of the disease process, resulting in progressive synaptic and neuronal dysfunction and loss in disorders such as Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis, multiple sclerosis, and others."* (Zlokovic 2008). The integrity of the BBB can be compromised by oxidative stress which can lead to increased permeability (Parathath, Parathath, and Tsirka 2006). The resultant extravasation of albumin into brain parenchyma can be excitotoxic and neurotoxic (Hassel, Iversen, and Fonnum 1994; Eimerl and Schramm 1991).

The evidence suggesting possible existence of barrier function compromise in people with ASDs is largely indirect. The existence of brain neuroinflammation in ASDs has been documented in a growing number of studies (Boso et al. 2006; El-Ansary and Al-Ayadhi 2012; Young et al. 2011), and this is known to be associated with BBB permeability(Erickson, Dohi, and Banks 2012; Janigro 2012; Takeshita and Ransohoff 2012). In a review of clinical MRI findings in ASDs 19/59 showed white matter signal abnormalities (Boddaert et al. 2009), which in other settings have been associated with cerebral hypoperfusion, though not necessarily in the same locations as the hyperintensities (Vardi et al. 2011) {Brickman, 2009 #2581}. Blood flow abnormalities, predominantly hypoperfusion, documented in a few dozen PET and SPECT studies, could also be caused by and/or associated with physiological phenomena associated with vascular permeability as will be revisited below. Increased intestinal permeability has been documented (although its absence has also been documented) (de Magistris et al. 2010; Lucarelli et al. 1995; D'Eufemia et al. 1996; Horvath and Perman 2002; White 2003; Robertson et al. 2008; Souza et al. 2012) and discussed in the context of food exposures, particularly gluten (Silva et al. 2012; Sapone et al. 2011; Visser et al. 2009; Simpson et al. 2009; Fasano 2009; Lammers et al. 2008; De Angelis et al. 2006). The reactivity to large numbers of different foods clinically observed in many children with autism has been framed by some as a manifestation of indiscriminate exposure of the immune system and the brain to food proteins on account of intestinal permeability as well as BBB permeability (Theoharides and Doyle 2008). This reactivity could in turn feed in to aberrant immune responsivity which in turn could further amplify barrier vulnerability{Fasano, 2009 #654}.

A number of studies have made an association between an increased risk of having a child with autism and maternal infection during pregnancy. This phenomenon looks like it is a result of the maternal immune system response rather than being due to an impact deriving from a specific infectious agent; but the potential for an accompanying compromise of the placental barrier is also conceivable in this setting. Under these

circumstances the fetal risk of exposure to maternal blood toxins, cytokines and stress proteins in-utero could potentially be increased if placenta barrier (BPB) function were impaired. The integrity, or compromise thereto, of the maternal-fetal interface via the placenta is an important modulator of brain development (Hsiao and Patterson 2012).

4. Genetic Alterations and Reproductive Impacts

Because of the high heritability of autism that was calculated from the concordance rates of monozygotic (identical) vs. dizygotic (fraternal) twins found in by a series of small twin studies performed some decades ago, the overwhelming emphasis in recent decades in autism research has been on genetics, and on finding linkages between genes, brain and behavior. As mentioned earlier, this point of view also promotes more of a structural/anatomical orientation than a bioelectric/physiological orientation. Along with this emphasis it has seemed obvious to people just looking at the stubborn persistence of symptoms in affected individuals that ASDs are inborn, lifelong brain defects. From this vantage point there would be no reason to think about the transduction of pathophysiology – whether acquired or genetic or some combination – to brain and hence behavior (or, more broadly, neurocognitive function). Thus the research agenda of looking for gene-brain-behavior correlations has seemed both self-evident and sufficient.

In recent years the genetic premises of this seemingly obvious framing of autism as overwhelmingly genetic have been undermined at several levels. (The undermining of the brain premises will be discussed beyond what was covered in Part I in later sections.) First the number of reported cases is increasing, making it more difficult to maintain that ASDs are purely genetic because these increases can only be partly explained away by greater awareness or other data artifacts (King and Bearman 2009; Hertz-Picciotto and Delwiche 2009). Second, the complexity of the ways we understand how genes might relate to autism has grown, from an expectation a decade ago that a small number of genes (even less than a dozen) would explain everything to an identification of close to a thousand genes associated with autism, as well as 'de novo' mutations present in ASD children but not their parents and even 'boutique' mutations not shared beyond an individual family. Out of over a hundred genetic syndromes in which autism commonly occurs, it is unclear what the pertinent genetic mutations and rearrangements have in common to account for the shared association with ASDs (Anney et al. 2010; Betancur 2011). Moreover, a recent twin study that was much larger than any of the prior such studies identified a modest genetic role but a substantial environmental role (Hallmayer et al. 2011). Also of interest, a Swedish study of identical twins and schizophrenia grouped into monochorionic (shared placenta) and dichorionic (each had its own placenta) showed 60% concordance for schizophrenia diagnosis for monochorionic twins but only 10.7% concordance for dichorionic twins (Davis, Phelps, and Bracha 1995); though this work has not yet been replicated in ASD twins, in principle it opens the door to non-genetic

interpretations of any concordance figures that have generally been assumed to be indicators of heritable genetics. The authors of this study interpreted their findings as consistent with data on viral infection as a contributor to schizophrenia risk (a possibility also entertained in ASDs (Patterson 2012; Teixeira and Barichello 2012; Atladottir et al. 2012, 2012; Hornig et al. 1999)), but one could also consider the possibility of differences in the dichorionic cases in the integrity of the placental barrier.

All of this calls into question the idea that genetics can be presumed to be the 'cause' of autism simply based upon heritability calculations, and upgrades the importance of looking not only at the environment and environmentally vulnerable physiology, but also at acquired mutations. There is certainly progress being made through genetic research to the identification of networks of genes and mechanisms on which genes converge (Voineagu et al. 2011), but environmental mechanisms converge on these mechanisms too (Stamou et al. 2012), and the mechanisms are what drive the impacts.

Genotoxicity

One route through which environmental impacts may influence an organism's status is by changing genes through mutation – that is, by genotoxicity. This has been proposed as a mechanism for the generation of 'de novo' mutations (found in children but not their parents) being found in ASDs (Kinney et al. 2010) and increasingly in other settings as well, making mutations something that needs to be accounted for rather than simply assuming tey are associated with normal, stable variation. Reviews and published scientific papers on genotoxicity and EMF report that both ELF-EMF and RFR exposures can be considered genotoxic – i.e., damaging to DNA – under certain conditions of exposure, including under conditions of intermittent and/or chronic ELF and RFR exposure that are of low-intensity and below current world safety standards (Ruediger 2009; Ivancsits et al. 2005; Diem et al. 2005; Blank and Goodman 2011; Phillips, Singh, and Lai 2009; REFLEX 31 May 2004; Sage and Carpenter 2009; Lai and Singh 2004). Types of genetic damage reported have included DNA fragmentation and single- and double-strand DNA breaks, micronucleation and chromosome aberrations, all of which indicate genetic instability. Genotoxic impacts of EMF/RFR are further reviewed in the BioInitiative Working Group 2007 contribution by Lai as well as in Section 6 of the present Bioinitiative Report {Lai, 2007 #2549;Lai, 2012 #2548}.

The European research program REFLEX (Risk Evaluation of Potential Environmental Hazards From Low-Energy Electromagnetic Field Exposure Using Sensitive in vitro Methods – a 5FP EU project) documented many changes in normal biological functioning in tests on DNA at exposure levels below existing public safety standards(REFLEX 31 May 2004). Some of the key findings included:

- Gene mutations, cell proliferation and apoptosis which are caused by or result in altered gene and protein expression profiles. The convergence of these events is required for the development of all chronic diseases.
- Genotoxic effects and a modified expression of numerous genes and proteins after EMF exposure could be demonstrated with great certainty.
- Genotoxic effects produced by RF-EMF in fibroblasts, HL- 60 cells, granulosa cells of rats and neural progenitor cells derived from mouse embryonic stem cells.
- Response of cells to RF exposure between SAR levels of 0.3 and 2 W/Kg with a significant increase in single- and double-strand DNA breaks and in micronuclei frequency.
- A clear demonstration of increase in intracellular generation of free radicals in HL-60 cells accompanying RF-EMF exposure.
- The observation that the induced DNA damage was not based on thermal effects, which raises concerns about the thermal-based environmental safety limits for ELF-EMF exposure.

These impacts could be contributors to a role for genetics in ASDs that does not derive from only inheritance but also from environmental and epigenetic influences. Moreover, in the light of the great heterogeneity of genetic findings in ASD alongside the documented impacts of EMF/RFR upon many other levels of pathophysiology than simply genetics, it becomes worth reflecting whether genetics might not be the primary problem but instead, in many cases at least, just one of many levels of collateral damage from environmental impacts. Whatever genetic variants a person carries may bias their system toward specific vulnerability, or may contribute more generically by increasing entropy and molecular disorder; in either capacity they may aggravate the situation but may not be part of the main cause.

Contributors to Genotoxicity

Oxidative Stress and free radical damage to DNA

Oxidative stress and excessive free radical production are very well known to be potentially genotoxic. They can be a consequence of myriad environmental factors, including but by no means limited to EMF/RFR. The DNA damage that can result could very well be one cause of 'de novo' mutations. Although there is not a consensus at this time about the rates or causes of *de novo* mutations in ASDs, and using present methods of detection are only found in a small percentage of individuals with ASDs, given the potential contribution of environmentally triggered oxidative stress and free radical damage that we know is present in at least large numbers of people with ASDs, a serious investigation of the potential contribution of EMF and RFR to de novo mutations in ASD seems warranted, given the large increase in exposure to these phenomena accompanying the massively increased non-ionizing radiation exposures in daily life due to

electrification and the global saturation of RFR from wireless technologies (BioInitiative 2012 Report, Section 24, Public Health Implications, Sage and Carpenter) (Sage and Carpenter 2012).

Challenge to DNA repair mechanisms

Reduced DNA repair may contribute to increased risk of cancers, but it may also contribute to a variety of other diseases and disturbances of growth and development. When the rate of damage to DNA exceeds the rate at which DNA can be repaired, there is the possibility of retaining mutations and initiating pathology. Failure to trigger DNA damage repair mechanisms, or incomplete or failed repair, may be a consequence of a variety of commonplace stressors, including EMF/RFR exposure. A decrease in DNA repair efficiency has been reported to result from exposure to low-intensity RFR in human stem cells, and other cells. Mobile phone frequency GSM exposure at the frequency of 915 MHz consistently inhibited DNA repair foci in lymphocytes (Markova et al. 2005; Belyaev et al. 2005; Belyaev, Markova, and Malmgren 2009). Belyaev, Markova and colleagues (2005) Markova et al. (2009) reported that very low-intensity microwave radiation from mobile phones inhibits DNA repair processes in human stem cells. A significant reduction in 53BP1 ((tumor suppressor p53 binding protein 1) foci was found in cells exposed to microwave radiofrequency radiation within one hour of exposure. Fibroblast cells were impacted in this fashion but adapted over time, whereas stem cells were similarly affected (inhibited 53BP1 foci) but did not adapt to microwave radiation during chronic exposure (Markova et al. 2005; Belyaev et al. 2005). Additional challenges to DNA repair mechanisms include not only toxicants and other damaging inputs but also nutritional insufficiencies of substances important to the proper functioning of DNA repair mechanisms, including Vitamin D, essential fatty acids, and minerals such as selenium and molybdenum (Christophersen and Haug 2011). The high possibility that various such contributors may combine supports an 'allostatic load' model of environmental injury and genotoxicity. Also note the overlap between nutritional risk factors for oxidative stress and for impaired DNA repair mechanisms. This supports a vicious circle model where the more oxidative damage to the genome, the less the cells will be prepared to deal with it successfully. It can also work the other way around – nutrients can attenuate the degree of damage; instances of this will be discussed in the Melatonin section below.

Chromatin condensation

Chromatin condensation is another hallmark of damage from EMF and RFR. Orderly chromatin condensation is a normal part of cell division, but it can also be provoked pathologically. The work of Markova, Belyaev and others has repeatedly shown that RFR exposure can cause chromatin condensation. Belyaev (1997) reported that super-low intensity RFR resulted in changes in genes, and chromatin condensation of DNA at intensities comparable to exposures from cell towers (typically at RFR levels of 0.1 to 1.0

uW/cm2) (Belyaev, Alipov, and Harms-Ringdahl 1997). Significant microwave-induced changes in chromatin conformation were observed when rat thymocytes were analyzed in-between 30-60 min after exposure to MW (Belyaev and Kravchenko 1994). This effect nearly disappeared if the cells were incubated more than 80 min between exposure and analysis.

In recent studies, human lymphocytes from peripheral blood of healthy and hypersensitive to EMF persons were exposed to non-thermal microwave radiation NT MW) from the GSM mobile phones (Belyaev et al. 2005; Markova et al. 2005). NT MW induced changes in chromatin conformation similar to those induced by heat shock, which remained up to 24 h after exposure. The same group has reported that contrary to human fibroblast cells, which were able to adapt during chronic exposure to GSM/UMTS low intensity RFR exposure, human stem cells did not adapt (Belyaev, Markova, and Malmgren 2009).

Researchers have recently identified large numbers of "spontaneous genetic glitches," or de novo mutations, more likely to be transmitted by fathers than by mothers to their children (Neale et al. 2012; O'Roak et al. 2012; Sanders et al. 2012). These glitches are widely distributed across the genome, with their location rather than their size conferring risk. The Eichler team at the University of Washington found that 39% of the 126 most severe or disruptive mutations map to a network associated with chromatin remodeling that has already been ranked as significant amongst autism candidate genes (O'Roak et al. 2012). Whether the prominence of chromatin-related gene mutations can be related in any meaningful way to the impacts of EMF/RFR on chromatin condensation is not possible to say at this point in time and this apparent parallel between ASDs and EMF/RFR may be a pure coincidence, though an intriguing one worth looking into further, including regarding how these mutations and the chromatin-remodeling impacts of EMF/RFR exposure may interact.

Gonadal and germline impacts

De novo mutations have been shown to be more of a problem related to paternal age (O'Roak et al. 2012; Paul, Nagano, and Robaire 2011; Iossifov et al. 2012; Cantor et al. 2007; Alter et al. 2011), and this may be related to the impact of environmental factors such as EMF/RFR on the stem cell genome, particularly in sperm which have no DNA repair capacity. Vulnerability of testes and ova, and of sperm and egg cells, relates to the tissue milieu in which damage to the germline can take place, as well as on the greater vulnerability of stem cells. Several international laboratories have replicated studies showing adverse effects on sperm quality, motility and pathology in men who use and particularly those who wear a cell phone, PDA or pager on their belt or in a pocket (Agarwal et al. 2008; Agarwal et al. 2009; Wdowiak, Wdowiak, and Wiktor 2007; De Iuliis et al. 2009; Fejes et al. 2005; Aitken et al. 2005) Kumar, 2012). Other studies

conclude that usage of cell phones, exposure to cell phone radiation, or storage of a mobile phone close to the testes of human males affect sperm counts, motility, viability and structure (Aitken et al, 2004; Agarwal et al, 2007; Erogul et al., 2006). Animal studies have demonstrated oxidative and DNA damage, pathological changes in the testes of animals, decreased sperm mobility and viability, and other measures of deleterious damage to the male germ line (Dasdag et al. 1999; Yan et al. 2007; Otitoloju et al. 2010; Salama et al. 2009) Behari et al, 2006; Kumar et al, 2012). Of note, altered fatty acids consistent with oxidative stress have been found in sperm cells in male infertility (Zalata et al. 1998; Zalata, Hafez, and Comhaire 1995).

There are fewer animal studies that have studied effects of cell phone radiation on female fertility parameters. Panagopoulous et al. 2012 report decreased ovarian development and size of ovaries, and premature cell death of ovarian follicles and nurse cells in *Drosophila melanogaster* (Panagopoulos 2012). Gul et al (2009) report rats exposed to stand-by level RFR (phones on but not transmitting calls) caused decrease in the number of ovarian follicles in pups born to these exposed dams (Gul, Celebi, and Ugras 2009). Magras and Xenos (1997) reported irreversible infertility in mice after five (5) generations of exposure to RFR at cell phone tower exposure levels of less than one microwatt per centimeter squared (μW/cm2) (Magras and Xenos 1997).

Implications of genotoxicity

The issue of genotoxicity puts the contribution of genetic variation into a different light – as something that needs to be accounted for, not necessarily assumed as the starting point. In this regard it has been speculated that the apparent higher rates of autism in Silicon Valley, discussed in the past as related to 'geek genes' (Silberman 2001), might be conditioned by higher levels of exposure to EMF/RFR. The relationship between the greater vulnerability of male sperm than of female eggs to adverse effects of EMF/RFR exposure and the marked (4:1) predominance of paternal origin of de novo point mutations (4:1 bias), also deserves further careful attention (O'Roak et al. 2012).

5. Implications of Damage

We have reviewed parallels between ASD and EMF/RFR in molecular, cellular and tissue damage, including cellular stress (oxidative stress, the heat shock response and protein misfolding), injury of membranes, aberrant calcium signaling, and compromise of junctions and barriers. The genotoxicity of EMF/RFR was reviewed in relation to issues of environmental contributions to autism and of the phenomenon of de novo mutations. The compromise of the tissue substrate appears to have many commonalities in ASDs and in EMF/RFR exposures. Also notable was the possibility of attenuating some of the damage through increasing antioxidant status.

These commonalities come to mind in considering the implications of a recent study documenting arrest of symptomatology in a mouse model of Rett syndrome through a bone marrow transplant of wild-type microglia (Derecki et al. 2012; Derecki, Cronk, and Kipnis 2012). The introduction of these competent microglia cells did not directly target the neuronal defect associated with the MECP2 gene mutation; instead the benefits of the transplant were diminished through inhibition of phagocytosis. Phagocytosis involves removing debris. This suggests that while research has focused on how specific molecular defects, particularly in the synapse, may contribute to Rett pathophysiology, there may also be an important contribution from cellular debris, misfolded proteins and other disordered cellular structure and function. Such disorder could be accumulating in cells under the conditions of pathophysiological disarray reviewed above. This has potentially broad implications for other genetic disorders, as well as for conditions like ASDs which are for the most part idiopathic. Based on this study as well as on the levels of damage just reviewed, problems in cells that are pertinent to ASDs most likely go beyond any specific defect introduced by a mutation. Additionally it is conceivable that many of the mutations may be not part of normal background variation but instead collateral damage from the same environmental factors that are also driving the damage to the pathophysiology. It is also encouraging that at least some of the damage and dysfunction was reversible by a generic cellular mechanism (phagocytosis), and this could have broad significance for idiopathic ASDs as well, along with other conditions involving related pathophysiological challenges.

B. DEGRADATION OF SYSTEM INTEGRITY

In the setting of molecular, cellular and tissue damage, one would predict that the organization and efficiency of a variety of organelles, organs and systems would also be degraded. EMF/RFR exposures yield a stressful situation of chronically interrupted homeostasis. Here we will review disturbances from EMF/RFR in systems (including include oxidative and bioenergetics metabolism, immune function and electrophysiological oscillations) that include molecular and cellular components subject to the kinds of damage discussed in the previous section. We will review disturbances that have been associated with EMF/RFR, and consider the parallel disturbances that have been documented in ASDs.

1. Mitochondrial Dysfunction

Mitochondria are broadly vulnerable, in part because the integrity of their membranes is vital to their optimal functioning – including channels and electrical gradients, and their membranes can be damaged by free radicals which can be generated in myriad ways. Moreover, just about every step in their metabolic pathway can be targeted by environmental agents, including toxicants and drugs, as well as mutations (Wallace and Starkov 2000). This supports an allostatic load model for conditions in which

mitochondrial dysfunction is an issue, which includes ASDs as well as myriad other chronic conditions.

Mitochondria are commonly discussed in terms of the biochemical pathways and cascades of events by which they metabolize glucose and generate energy. But in parallel with this level of function there also appears to be a dimension of electromagnetic radiation that is part of the activity of these organelles. For example, electromagnetic radiation can be propagated through the mitochondrial reticulum, which along with the mitochondria has a higher refractive index than the surrounding cell and can serve to propagate electromagnetic radiation within the network (Thar and Kuhl 2004). It is also the case that *"The physiological domain is characterized by small-amplitude oscillations in mitochondrial membrane potential (delta psi(m)) showing correlated behavior over a wide range of frequencies…. Under metabolic stress, when the balance between ROS [reactive oxygen species, or free radicals] generation and ROS scavenging [as by antioxidants] is perturbed, the mitochondrial network throughout the cell locks to one main low-frequency, high-amplitude oscillatory mode. This behavior has major pathological implications because the energy dissipation and cellular redox changes that occur during delta psi(m) depolarization result in suppression of electrical excitability and Ca2+ handling…"* (Aon, Cortassa, and O'Rourke 2008). These electromagnetic aspects of mitochondrial physiology and pathophysiology could very well be impacted by EMF/RFR.

There are also a variety of types of mitochondrial damage that have been documented in at least some of the studies that have examined the impacts of EMF/RFR upon mitochondria. These include reduced or absent mitochondrial cristae (Khaki et al. 2006; Lahijani, Tehrani, and Sabouri 2009; Esmekaya et al. 2011), mitochondrial DNA damage (Xu et al. 2010), swelling and crystallization (Lahijani, Tehrani, and Sabouri 2009), alterations and decreases in various lipids suggesting an increase in their use in cellular energetics (Chernysheva 1987), damage to mitochondrial DNA (Xu et al. 2010), and altered mobility and lipid peroxidation after exposures (Wang et al. 2002). Also noted has been enhancement of brain mitochondrial function in Alzheimer's transgenic mice and normal mice (Dragicevic et al. 2011). The existent of positive as well as negative effects gives an indication of the high context dependence of exposure impacts, including physical factors such as frequency, duration, and tissue characteristics; these are intensively reviewed in Belyaev's contribution to BioInitiative 2012 in Section 15 (Belyaev 2012).

The idea that mitochondrial dysfunction might be common in ASDs met with a fair bit of consternation, and many professionals have preferred to limit their consideration to mitochondrial disorders with proven genetic mutations. However the concept of mitochondrial dysfunction is better established in other areas of medicine, with thousands

of papers and hundreds of reviews carrying "mitochondrial dysfunction" in their titles. By now there is a large amount of evidence for biochemical and other abnormalities in a large portion of children with autism that are consistent with mitochondrial dysfunction (Giulivi et al. 2010; Palmieri et al. 2010; Pastural et al. 2009). Recently published postmortem brain tissue studies that have added a new dimension of evidence for mitochondrial abnormalities in ASDs will be reviewed in the section on alteration of brain cells below.

Some have called the mitochondrial issues most commonly seen in ASDs 'secondary mitochondrial dysfunction' (Zecavati and Spence 2009; Rossignol and Frye 2011) to indicate that it results from environment insults and/or other pathophysiological dysfunction rather than directly from genetics (Hadjixenofontos et al. 2012); the already discussed potential for EMF/RFR to damage channels, membranes and mitochondria themselves could contribute in a number of ways to degrading mitochondrial function without a basis in genetic mutation, as could toxicant exposures and immune challenges. In a meta-analysis of studies of children with ASD and mitochondrial disorder, the spectrum of severity varied, and 79% of the cases were identified by laboratory not associated with genetic abnormalities (Rossignol and Frye 2011). "*Substantial percentages of autistic patients display peripheral markers of mitochondrial energy metabolism dysfunction, such as (a) elevated lactate, pyruvate, and alanine levels in blood, urine and/or cerebrospinal fluid, (b) serum carnitine deficiency, and/or (c) enhanced oxidative stress……In some patients, these abnormalities have been successfully explained by the presence of specific mutations or rearrangements in their mitochondrial or nuclear DNA. However, in the majority of cases, abnormal energy metabolism cannot be immediately linked to specific genetic or genomic defects."* (Palmieri and Persico 2010)

2. Melatonin Dysregulation

Melatonin, mitochondria, glutathione, oxidative stress

Melatonin is well-known for its role in regulation of circadian rhythms, but it also plays important metabolic and regulatory roles in relation to cellular protection, mitochondrial malfunction and glutathione synthesis. (Leon et al. 2005; Luchetti et al. 2010; Limon-Pacheco and Gonsebatt 2010) *"It is known that melatonin scavenges oxygen and nitrogen-based reactants generated in mitochondria. This limits the loss of the intramitochondrial glutathione and lowers mitochondrial protein damage, improving electron transport chain (ETC) activity and reducing mtDNA damage. Melatonin also increases the activity of the complex I and complex IV of the ETC, thereby improving mitochondrial respiration and increasing ATP synthesis under normal and stressful conditions."* (Leon et al. 2005) It also helps prevent the breakdown of the mitochondrial membrane potential, decrease electron leakage, and thereby reduce the formation of

superoxide anions. (Hardeland 2005) Pharmacological doses of melatonin not only scavenge reactive oxygen and nitrogen species, but enhance levels of glutathione and the expression and activities of some glutathione-related enzymes. (Limon-Pacheco and Gonsebatt 2010; Gupta, Gupta, and Kohli 2003)

Melatonin can attenuate or prevent some EMF/RFR effects

Melatonin may have a protective effect in the setting of some EMF/RFR exposures, apparently in relation to these functions just described. EMF/RFR can impact melatonin; one example is exposure to 900-MHz microwave radiation promoted oxidation, which reduced levels of melatonin and increased creatine kinase and caspase-3 in exposed as compared to sham exposed rats (Kesari, Kumar, and Behari 2011).

Further types of adverse impacts can be seen in the next set of examples, but what is interesting is that melatonin can attenuate or prevent them. In an experiment exposing rats to MW from a GSM900 mobile phone with and without melatonin treatment to study renal impacts(Oktem et al. 2005), the untreated exposed rats showed increases of lipid peroxidation markers as reduction of the activities of superoxide dismutase, catalase and glutathione peroxidase indicating decrement in antioxidant status. However these negative effects were inhibited in the exposed rats treated with melatonin. Melatonin also inhibited the emergence of preneoplastic liver lesions in rats exposed to EMFs (Imaida et al. 2000). The development of DNA strand breaks was observed in RFR exposed rats; this DNA damage was blocked by melatonin (Lai and Singh 1997). Exposure of cultured cortical neurons to EMF led to an increase in 8-hydroxyguanine in neuronal mitochondria, a common biomarker of DNA oxidative damage, along with a reduction in the copy number of mitochondrial DNA and the levels of mitochondrial RNA transcripts; but these effects could all be prevented by pretreatment with melatonin (Xu et al. 2010). In a study of skin lesion induced by exposure to cell phone radiation, the skin changes in the irradiated group (which included thicker stratum corneum, epidermal atrophy, papillamatosis, basil cell proliferation, increased epidermal granular cell layer and capillary proliferation, impaired collagen tissue distribution and separation of collagen bundles in dermis) were prevented (except for hypergranulosis) by melatonin treatment (Ozguner et al. 2004). Melatonin as well as caffeic acid phenyethyl ester (an antioxidant) both protected against retinal oxidative stress in rates exposed long-term to mobile phone irradiation (Ozguner, Bardak, and Comlekci 2006). Nitric oxide (NO) was increased in nasal and sinus mucosa in rats after EMF exposure, with this NO possibly acting as a defense mechanism suggesting tissue damage; but this was prevented by pretreatment with melatonin (Yariktas et al. 2005). Melatonin treatment significantly prevented the increase in the MDA (malondyaldehyde, a marker of lipid peroxidation) content and XO (xanthine oxidase) activity in rat brain tissue after 40 days of exposure, but it was unable to prevent the decrease of CAT activity and increase of carbonyl group contents (Sokolovic et al. 2008).

Of note, the melatonin production of infants in isolettes in neonatal intensive care units appears to be impacted by the high ELF-EMF environment, in that when infants were removed from those exposures they showed an increase in melatonin levels (Bellieni, Tei, et al. 2012). There is an increased prevalence of ASDs in children who were born prematurely (Indredavik et al. 2010; Indredavik et al. 2008; Johnson et al. 2011; Johnson et al. 2010; Johnson and Marlow 2011; Lampi et al. 2012; Limperopoulos 2009, 2010; Limperopoulos et al. 2008; Matson, Matson, and Beighley 2011; Pinto-Martin et al. 2011). There are many potential prematurity-associated factors that could contribute to increased risk for ASDs, but electromagnetic exposure might be one of them worthy of further consideration, as it could be modified; conversely, such exposures in vulnerable infants are likely to have much broader impacts beyond reducing melatonin synthesis.

Melatonin and autism

Based on the commonality of both sleep disorders and low melatonin levels, Bourgeron (2007) proposed that synaptic and clock genes are important in ASDs, and that future studies should investigate the circadian modulation of synaptic function (Bourgeron 2007). A number of melatonin-related genetic variants have been identified as associated with ASDs. Polymorphisms, deletions and polymorphisms in the ASMT gene, which encodes the last enzyme of melatonin synthesis, have been found (Pagan et al. 2011; Jonsson et al. 2010; Melke et al. 2008), and variations have been found as well for melatonin receptor genes (Chaste et al. 2010; Pagan et al. 2011; Jonsson et al. 2010). CYP1A2 polymorphisms have been found in slow melatonin metabolisers, in whom melatonin levels are aberrant and initial response to melatonin for sleep disappeared in a few weeks (Braam et al. 2012).

Regarding melatonin status in people with ASDs, a recent meta-analysis summarized the current findings as indicating that *"1) Physiological levels of melatonin and/or melatonin derivatives are commonly below average in ASD and correlate with autistic behavior, 2) Abnormalities in melatonin-related genes may be a cause of low melatonin levels in ASD, and 3) …treatment with melatonin significantly improves sleep duration and sleep onset latency in ASD."* (Rossignol and Frye 2011) The meta-analysis also showed that polymorphisms in melatonin-related genes in ASD could contribute to lower melatonin concentrations or an altered response to melatonin, but only in a small percentage of individuals, since pertinent genes were found in only a small minority of those screened.

Autism AND Melatonin AND Glutathione

Whereas PubMed searches for "autism AND melatonin" and "autism AND glutathione" each coincidentally yielded 72 citations, and "melatonin AND glutathione" yielded 803 citations, the search for "autism AND melatonin AND glutathione" yielded zero citations. This is interesting given the strong connection of melatonin and glutathione metabolically, as discussed above, alongside of the strongly established interest in both

glutathione and melatonin in ASD research and increasingly in clinical practice. Hopefully one contribution of an investigation of EMF/RFR links to ASDs will be to help bring attention to this relationship, which may help identify potential environmental and physiological causes for low melatonin in those without pertinent mutations. Of pertinence, tryptophan hydroxylase (TPH2) – the rate limiting enzyme in the synthesis of serotonin, from which melatonin is derived – is extremely vulnerable to oxidation, and tends to misfold when its cysteine residues are oxidized, with the enzyme being converted to a redox-cycling quinoprotein (Kuhn and Arthur 1999; Kuhn and Geddes 1999; Kuhn et al. 2011; Kuhn and Arthur 1997).

3. Disturbed Immune Function

There is by now a broad appreciation of the presence of immune disturbances in ASDs, to the point where there is an emerging discussion of ASDs as neuroimmune disorders (Bilbo, Jones, and Parker 2012; Persico, Van de Water, and Pardo 2012). Research identifying immune features in ASDs spans from genetics where risk genes have been identified to epigenetics where altered expression of immune genes is being reported as prominent in ASD epigenetics (Kong et al. 2012; Waly et al. 2012; Lintas, Sacco, and Persico 2012), and also includes prenatal infectious and immune disturbances as risk factors for autism as well as other neurodevelopmental and neuropsychiatric diseases as well as other conditions such as asthma (Patterson 2011; Smith et al. 2007; Fox, Amaral, and Van de Water 2012). Immune disturbances in infants and children with ASD are heterogeneous, with some but not all manifesting autoimmunity (Soumiya, Fukumitsu, and Furukawa 2011; Martin et al. 2008). Anecdotally, recurrent infection is common while on the other hand some get sick less often than their peers. It is common for people with autism to have family members with immune or autoimmune diseases (Croen et al. 2005). The immune system is turning out to have an important role in brain development (Bilbo and Schwarz 2012; Schwarz and Bilbo 2012; Boksa 2010). As mentioned, glial activation associated with brain immune response has been identified in a growing number of studies. Whether or not EMF/RFR contributes to these features of ASDs causally, based on the evidence below regarding immune impacts of EMF/RFR exposure (which is also reviewed much more thoroughly by Johansson in Section 8 of the present Bioinitiative Report) (Blank 2012), it is certainly plausible that such exposures could serve as aggravating factors.

Low-intensity exposures

It is clear that the body's immune defense system responds to very low-intensity exposures. Chronic exposure to factors that increase allergic and inflammatory responses on a continuing basis is likely to be harmful to health, since the resultant chronic inflammatory responses can lead to cellular, tissue and organ damage over time. We are increasingly appreciating the extent to which many chronic diseases are related to chronic

immune system dysfunction. Disturbance of the immune system by very low-intensity electromagnetic field exposure is discussed as a potential underlying cause for cellular damage and impaired healing (tissue repair), which could lead to disease and physiological impairment (Johansson 2009; Johannson 2007).

Both human and animal studies report that exposures to EMF and RFR at environmental levels associated with new technologies can be associated with large immunohistological changes in mast cells as well as other measures of immune dysfunction and dysregulation. Mast cells not only can degranulate and release irritating chemicals leading to allergic symptoms; they are also widely distributed in the body, including in the brain and the heart, which might relate to some of the symptoms commonly reported in relation to EMF/RFR exposure (such as headache, painful light sensitivity, and cardiac rhythm and palpitation problems).

Consequences of immune challenges during pregnancy

As mentioned, infection in pregnancy can also increase the risk of autism and other neurodevelopmental and neuropsychiatric disorders via maternal immune activation (MIA). Viral, bacterial and parasitic infections during pregnancy are thought to contribute to at least 30% of cases of schizophrenia (Brown and Derkits 2010). The connection of maternal infection to autism is supported epidemiologically, including in a Kaiser study where risk was associated with psoriasis and with asthma and allergy in the second trimester (Croen et al. 2005), and in a large study of autism cases in the Danish Medical registry (Atladottir et al. 2010) with infection at any point in pregnancy yielding an adjusted hazard ration of 1.14 (CI: 0.96-1.34) and when infection occurred during second trimester the odds ratio was 2.98 (CI: 1.29-7.15). In animal models, while there is much variation in study design, mediators of the immune impact appear to include oxidative stress, interleukin-6 and increased placental cytokines (Smith et al. 2007; Patterson 2009; Boksa 2010). Garbett et al. (2012) commented on several mouse models of the effects of MIA on the fetal brain that *"The overall gene expression changes suggest that the response to MIA is a neuroprotective attempt by the developing brain to counteract environmental stress, but at a cost of disrupting typical neuronal differentiation and axonal growth."* (Garbett et al. 2012). Maternal fetal brain-reactive autoantibodies have also been identified in some cases (Braunschweig et al. 2012; Braunschweig and Van de Water 2012; Fox, Amaral, and Van de Water 2012; Goines et al. 2011; Wills et al. 2009; Wills et al. 2011; Zimmerman et al. 2007).

Although we have evidence of immune impacts of EMF/RFR, the impact of repeated or chronic exposure to EMF and RFR during pregnancy is poorly studied; could this trigger similar immune responses (cytokine production) and stress protein responses, which in turn would have effects on the fetus? Although this has been poorly studied, we do have data that very low cell phone radiation exposures during both human and mouse

pregnancies have resulted in altered fetal brain development leading to memory, learning, and attention problems and behavioral problems (Aldad et al. 2012).

Potential immune contributions to reactivity and variability in ASDs

Immune changes in ASDs appear to be associated with behavioral change (Shi et al. 2003; Ashwood et al. 2008; Ashwood et al. 2011; Breece et al. 2012; Heuer et al. 2008), but the mechanisms are complex and to date poorly understood (Careaga and Ashwood 2012) and likely will need to be elucidated through systems biology methods that capture multisystem influences on the interactions across behavior, brain and immune regulation (Broderick and Craddock 2012), including electrophysiology.

Two of the particularly difficult parts of ASDs are the intense reactivity and the variability in assorted symptoms such as tantrums and other difficult behaviors. Children with ASDs who also have gastrointestinal symptoms and marked fluctuation of behavioral symptoms have been shown to exhibit distinct innate immune abnormalities and transcriptional profiles of peripheral blood monocytes (Jyonouchi et al. 2011). It is worth considering EMF/RFR exposures could be operating through related mechanisms so as to add to allostatic loading in ways that exacerbate behavior. In Johansson 2006 and 2007 a foundation is provided for understanding how chronic EMF/RFR exposure can compromise immune function and sensitize a person to even small exposures in the future (Johannson 2007; Johansson et al. 2006). Johansson discusses alterations of immune function at environmental levels resulting in loss of memory and concentration, skin redness and inflammation, eczema, headache, and fatigue. Mast cells that degranulate under EMF and RFR exposures and substances secreted by them (histamine, heparin and serotonin) may contribute to features of this sensitivity to electromagnetic fields (Johansson et al. 2006). Theoharides and colleagues have argued that environmental and stress related triggers might activate mast cells, causing inflammatory compromise and leading to gut-blood-brain barrier compromise, seizures and other ASD symptoms (Theoharides et al. 2012, 2010), and that this cascade of immune response and its consequences might also be triggered in the absence of infection by mitochondrial fragments that can be released from cells in response to stimulation by IgE/anti-IgE or by the proinflammatory peptide substance P (Zhang, Asadi, et al. 2012).

Seitz et al. (2005) reviewed an extensive literature on electromagnetic hypersensitivity conditions reported to include sleep quality, dizziness, headache, skin rashes, memory and concentration impairments related to EMF and RFR {Seitz, 2005 #2582}. Some of these symptoms are common in ASDs, whether or not they are due to EMF/RFR exposure, and the experience of discomfort may be hard to document due to difficulties with self-reporting in many people with ASDs.

Johansson (2007, 2009) also reports that benchmark indicators of immune system allergic and inflammatory reactions occur under exposure conditions of low-intensity non-

ionizing radiation (immune cell alterations, mast cell degranulation histamine-positive mast cells in biopsies and immunoreactive dendritic immune cells) (Johannson 2007; Johansson 2009). In facial skin samples of electro- hypersensitive persons, the most common finding is a profound increase in mast cells as monitored by various mast cell markers, such as histamine, chymase and tryptase (Johansson et al. 2001). In ASDs, infant and childhood rashes, eczema and psoriasis are common, and they are common in family members as well (Bakkaloglu et al. 2008).

4. Alteration of and damage to cells in the brain

Brain cells have a variety of ways of reacting to environmental stressors, such as shape changes, metabolic alterations, upregulation or downregulation of neurotransmitters and receptors, other altered functionality, structural damage, production of un-metabolizable misfolded proteins and other cellular debris, and apoptosis; these range along a spectrum from adaptation to damage and cell death. These types of alterations can be looked at in animals under controlled conditions, but in human beings direct cellular examination can only be done on surgical biopsy tissue – which is hardly ever available in people with ASDs – or after death, at which point there has been a whole lifetime of exposures that are generally impossible to tease apart if there were even motivation to do so. This complicates the comparison of brain cell and tissue-related pathophysiology between what is seen in ASDs and what is associated with EMF/RFR exposures.

Brain cells

Impact of EMF/RFR on cells in the brain has been documented by some of the studies that have examined brain tissue after exposure, although the interpretation of inconsistencies across studies is complicated by sometimes major differences in impact attributable to differences in frequencies and duration of exposure, as well as to differences in resonance properties of tissues and other poorly understood constraints on cellular response. These studies and methodological considerations have been reviewed in depth in Belyaev, 2012 in section 15 of the 2012 BioInitiatve Report (Belyaev 2012), as well as by Salford et al. (2012) in Section 10 (Salford, Nittby, and Persson 2012). A few examples of observations after exposure have included dark neurons (an indicator of neuronal damage), as well as alteration of neuronal firing rate (Bolshakov and Alekseev 1992), and upregulation of genes related to cell death pathways in both neurons and astrocytes (Zhao, Zou, and Knapp 2007). Astrocytic changes included increased GFAP and increased glial reactivity (Chan et al. 1999; Ammari et al. 2008; Ammari et al. 2010; Brillaud, Piotrowski, and de Seze 2007), as well as astrocyte-pertinent protein expression changes detected by Fragopoulou et al, 2012 as mentioned above. Also observed has been a marked protein downregulation of the nerve growth factor glial maturation factor beta (GMF) which is considered as an intracellular signal transduction regulator in astrocytes, which could have significant impact on neuronal-glial interactions as well as

brain cell differentiation and tumor development. Diminution of Purkinje cell number and density has also been observed, (Ragbetli et al. 2010)including in two studies of the impacts of perinatal exposure {Albert, 1981 #2584;Albert, 1981 #2583}. Promotion of pro-inflammatory responses in EMF-stimulated microglial cells has also been documented (Yang et al. 2010).

Neuropathology findings in ASDs have been varied and have been interpreted according to various frameworks ranging from a regionalized approach oriented to identifying potential brain relationships to ASD's behavioral features (Amaral, Schumann, and Nordahl 2008)to identifying receptor, neurotransmitter and interneuron abnormalities that could account for an increased excitation/inhibition ratio {Levitt, 2009 #551}{Geschwind, 2007 #2586}{Anney, 2010 #423}{Casanova, 2006 #2587}{Rubenstein, 2003 #809}. Studies have documented a range of abnormalities in neurons, including altered cellular packing in the limbic system, reduced dendritic arborization, and reductions in limbic GABAergic systems. Over the past decade a shift has occurred from presuming that all pertinent brain changes occurred prior to birth, to an acknowledgement that ongoing cellular processes appear to be occurring not only after birth but well into adulthood. (Bauman and Kemper 2005) One of the reasons for this shift was the observation that head size (as well as brain weight and size) was on average larger in children with autism, and the head sizes of children who became diagnosed with autism increased in percentile after birth {Herbert, 2005 #642}.

Neuroinflammation, glial activation and excitotoxicity

Although much attention has been paid in ASD brain literature to specific regions manifesting differences in size and activity in comparison to those without ASDs, there are other observations that are not strictly regional in nature, such as more widely distributed scaling differences (e.g. larger brains, wider brains, increased white matter volume, along with altered functional connectivity and coherence to be discussed below). Recently more studies have appeared identifying pathophysiological abnormalities such as neuroinflammation, mitochondrial dysfunction and glutathione depletion in brain tissue. Neuroinflammation was first identified in a study of postmortem samples from eleven individuals aged 5-44 who had died carrying an ASD diagnosis, in which activated astrocytes and microglial cells as well as abnormal cytokines and chemokines were found. Other research has identified further astrocyte abnormalities include, altered expression of astrocyte markers GFAP abnormalities including elevation, antibodies, and altered signaling {Laurence, 2005 #1729;Singh, 1997 #1730}(Fatemi et al. 2008). Increased microglia activation and density as well as increased myeloid dendritic cell frequencies have also been documented. (Vargas et al. 2005; Breece et al. 2012; Tetreault et al. 2012), as has abnormal microglial-neuronal interactions (Morgan et al. 2012). Recently through use of the PET ligand PK11105 microglial activation was found to be significantly higher in multiple brain regions in young adults with ASDs (Suzuki et al.

2013). Genes associated with glial activation have been documented as upregulated. Garbett et al measured increased transcript levels of many immune genes, as well as changes in transcripts related to cell communication, differentiation, cell cycle regulation and chaperone systems (Garbett et al. 2008). Voineaugu and colleagues performed transcriptomic analysis of autistic brain and found a neuronal module of co-expressed genes which was enriched with genetically associated variants, and an immune-glial module showing no such enrichment for autism GWAS signals (Voineagu et al. 2011).

Neuroinflammation also does not appear to be strictly localized in a function-specific fashion, and it may contribute both to more broadly distributed and more focal features for tissue-based reasons. It may be that brain regions with particular prominence in ASDs may have distinctive cellular characteristics – e.g. the amygdala (Baron-Cohen et al. 2000; Dziobek et al. 2010; Hall et al. 2010; Mercadante et al. 2008; Nordahl et al. 2012; Otsuka et al. 1999; Schulkin 2007; Schumann and Amaral 2006; Schumann et al. 2009; Truitt et al. 2007; Zirlinger and Anderson 2003), which may have a larger or more reactive population of astrocytes (Johnson, Breedlove, and Jordan 2010) or the basal ganglia which may have greater sensitivity to even subtle hypoxia or perfusion abnormalities. In this case it may be the histology of these areas that makes them vulnerable to environmental irritants, and this may contribute to how environmental factors such as EMF/RFR might trigger or aggravate some of ASD's features. More widely distributed brain tissue pathology be part of what leads to differences in ASDs in brain connectivity. However these types of tissue-function relationships have been poorly investigated. The contribution of tissue differences is one of the physical considerations covered by Belyaev (2012) in Section 15 of the 2012 BioInitiative Report {Belyaev, 2012 #2324}.

Various signs of mitochondrial dysfunction and oxidative stress have also been identified in the brain. Findings include downregulation of expression of mitochondrial electron transport genes (Anitha, Nakamura, Thanseem, Matsuzaki, et al. 2012) or deficit of mitochondrial electron transport chain complexes (Chauhan et al. 2011), brain region specific glutathione redox imbalance (Chauhan, Audhya, and Chauhan 2012), and evidence of oxidative damage and inflammation associated with low glutathione redox status (Rose, Melnyk, Pavliv, et al. 2012). Oxidative stress markers were measured as increased in cerebellum (Sajdel-Sulkowska, Xu, and Koibuchi 2009).

Additional support for the presence of tissue pathophysiology-based changes in brains of people with ASDs comes from the various studies documenting reduction in Purkinje cell numbers (Whitney et al. 2009; Whitney et al. 2008; Bauman and Kemper 2005; Shi et al. 2009; Blatt and Fatemi 2011; Fatemi et al. 2002; Fatemi et al. 2012), possibly due to oxidative stress and an increased excitation/inhibition ratio that could potentially be acquired (Fatemi et al. 2012). Also of note are changes in the glutamatergic and GABAergic systems, which when imbalanced can disturb the excitation/inhibition ratio

and contribute to seizure disorders; reductions in GABA receptors as well as in GAD 65 and 67 proteins that catalyse the conversion of glutamate into GABA have been measured. (Yip, Soghomonian, and Blatt 2007, 2008, 2009) A consensus statement on the cerebellum in ASDs stated that, *"Points of consensus include presence of abnormal cerebellar anatomy, abnormal neurotransmitter systems, oxidative stress, cerebellar motor and cognitive deficits, and neuroinflammation in subjects with autism."* (Fatemi et al. 2012)

Some indirect corroboration for these findings has come from neuroimaging, where the initial hypothesis regarding the tissue basis of the larger size of brains in so many people with autism – that it was due to a higher density of neurons and more tightly packed axons – came under question with the emergence of contradictory findings, well reviewed a few years ago by Dager and colleagues (Dager et al. 2008). These include reduced rather than increased density of NAA (n-acetylaspartate, a marker of neuronal integrity and density that is produced in the mitochondria), reduced rather than increased fractional anisotropy suggesting less tightly packed axonal bundles (Bode et al. 2011; Cascio et al. 2012; Mak-Fan et al. 2012; Travers et al. 2012; Walker et al. 2012; Wolff et al. 2012){Sundaram, 2008 #2588} and greater rather than lower diffusivity, all of which may be more consistent with lower density of tissue and tissue metabolites and more fluid, which could be consistent with neuroinflammation and/or oxidative stress. The early postnatal development of such lower fractional anisotropy and increased diffusivity was measured in the process of occurring recently, in the first large prospective longitudinal imaging study of infants, who trended from 6 months to 2 years in the direction of these findings becoming more pronounced – but still with substantial overlap with those infants who did not develop autism (Wolff et al. 2012). This trend was consistent with prior studies showing increase in head size after birth, and added some information about what was happening in the brain to drive this size increase, although due to its methods it could only indirectly address the possibilty that emergence during the first few years of life of tissue pathophysiology disturbances such as neuroinflammation might be contributing to these trends (Herbert 2012).

There is also substantial variability across many different types of brain findings. Of interest is that a number of functional brain imaging and electrophysiology studies have identified greater heterogeneity in response to stimuli between individuals in the ASD group than individuals in the neurotypical control group (Muller et al. 2003; Dinstein et al. 2012). This may make more sense from the point of view of non-linear response – i.e. a disproportionality between output and input (as well as state and context sensitivity), in a pathophysiologically perturbed brain system. Nonlinearity has also been a significant methodological issue in EMF/RFR research because linear methods of study design and data analysis have often been insensitive to effects, whereas nonlinear methods have been argued to show greater sensitivity (Carrubba and Marino 2008; Marino, Wolcott,

Chervenak, Jourd'heuil, Nilsen, Frilot, et al. 2001; Marino and Frilot 2003; Carrubba et al. 2006; Carrubba et al. 2012; Marino, Nilsen, and Frilot 2003; Marino, Wolcott, et al. 2001, 2001; Carrubba et al. 2007; Marino et al. 2000){Bachmann, 2005 #2072} .

The presence of various types of tissue pathophysiology both in findings in postmortem tissue from individuals with ASDs and in documented impacts of EMF/RFR exposure are intriguing and suggest overlap in processes involved. But it is not really possible to infer any specific agent of injury from cellular responses since for the most part these are not specific but rather are stress or repair responses generic to a variety of triggers. It is important to entertain how environmental agents could contribute to brain changes in ASDs, and how these changes may develop over progress over time after the earliest periods in brain development. EMF/RFR exposures could be preconceptional, prenatal or postnatal – or all of the above; it is conceivable that this could be the case in ASDs as well.

Altered development

There is some evidence for altered brain and organism development in relation to EMF/RFR exposure. Aldad et al. 2012 exposed mice in utero to cellular telophones, with resultant aberrant miniature excitatory postsynaptic currents, dose-responsive impaired glutamatergic synaptic transmission onto layer V pyramidal neurons of the prefrontal cortex (Aldad et al. 2012). Lahijani exposed preincubated chicken embryos to 50 Hz EMFs, and made the following morphological observations: *"exencephalic embryos, embryos with asymmetrical faces, crossed beak, shorter upper beak, deformed hind limbs, gastroschesis, anophthalmia, and microphthalmia. H&E and reticulin stainings, TEMS, and SEMs studies indicated EMFs would create hepatocytes with fibrotic bands, severe steatohepatitis, vacuolizations, swollen and extremely electron-dense mitochondria, reduced invisible cristae, crystalized mitochondria with degenerated cristae, myelin-like figures, macrophages engulfing adjacent cells, dentated nuclei, nuclei with irregular envelopes, degenerated hepatocytes, abnormal lipid accumulations, lipid droplets pushing hepatocytes' nuclei to the corner of the cells, abundant cellular infiltrations cellular infiltrations inside sinusoid and around central veins, disrupted reticulin plexus, and release of chromatin into cytosol,, with partially regular water layers,"* and attributed cell damage to elevated free radical induced cell membrane disruptions (Lahijani, Tehrani, and Sabouri 2009).

Although it is of great interest to characterize the changes in development associated with ASDs, it is also difficult to do in human beings because at present diagnosis is not possible until at least 2-3 years after birth. By now there have been a lot of prospective studies of infants at high risk for autism, but the in vivo brain imaging and electrophysiology data from these studies is only starting to be published, and so the for now the main sources of information are still inference backwards from post-mortem or

imaging data, and animal models, both of which have clear limitations. Thus it is impossible to seek precise parallels here between what we know about the development of ASDs compared with the impacts of EMF/RFR exposures.

Nevertheless it is of real concern that such exposures have elicited some of the brain tissue changes that have been documented, both in early development and subsequently. Already noted above is the question of whether high exposures of neonates to monitoring equipment may affect the melatonin levels of neonates (Bellieni, Tei, et al. 2012); these exposures also impact heartrate variability . There are no studies yet on infants exposed to baby surveillance monitors or DECT wireless phones. However there are good laboratory testing studies yielding actual measurements of these devices that conclude: "*Maximum incident field exposures at 1m can significantly exceed those of base stations (typically 0.1 - 1 V/m). At very close distances the derived or reference exposure limits are violated" for baby surveillance monitors and DECT phones.* Further, the authors conclude that, based on very strictly controlled laboratory testing of everyday devices like baby monitors and some cordless phones *"(W)orse case peak spatial SAR values are close to the limit for the public or uncontrolled environments, e.g., IEEE802.11b and Bluetooth Class I".*(Kuhn et al. 2012) Even exposure of the fetus to laptop computer wireless emissions through the pregnant mother's use of them may on her lap involve induction of strong intracorporeal electric current densities from the power supply possibly even more than the device itself (Bellieni, Pinto, et al. 2012).

Brain Blood Flow and metabolism

Cerebral perfusion and metabolism abnormalities have been identified in close to 2 dozen papers studying autistic cohorts. Cerebral perfusion refers to the quantity of blood flow in the brain. Abnormal regulation of cerebral perfusion is found in a range of severe medical conditions including tumors, vascular disease and epilepsy. Cerebral hypoperfusion has also been found in a range of psychiatric disorders (Theberge 2008). Neurocognitive hypotheses and conclusions, as well as localization of perfusion changes, have been heterogeneous across these papers. Hypoperfusion or diminished metabolism has been identified in frontal regions {George, 1992 #2565}{Gupta, 2009 #2575}{Degirmenci, 2008 #2563}{Wilcox, 2002 #2578}{Galuska, 2002 #2564}{Ohnishi, 2000 #2571}, temporal lobes {Boddaert, 2002 #2558}{Burroni, 2008 #2559}{Degirmenci, 2008 #2563}{Galuska, 2002 #2564}{George, 1992 #2565}{Hashimoto, 2000 #2566}{Ohnishi, 2000 #2571}{Ryu, 1999 #2573}{Starkstein, 2000 #2576}{Zilbovicius, 2000 #2579}, as well as a variety of subcortical regions including basal ganglia {Degirmenci, 2008 #2563}{Ryu, 1999 #2573}{Starkstein, 2000 #2576}, cerebellum {Ryu, 1999 #2573}, limbic structures {Ito, 2005 #2568}{Ohnishi, 2000 #2571} and thalamus {Ito, 2005 #2568}{Ryu, 1999 #2573}{Starkstein, 2000 #2576} – i.e., in a widely distributed set of brain regions. It is interesting to note that even with this regional variation in localization, most of these publications showed that

cerebral perfusion was *reduced*; in the only one of those studies reporting some areas of localized hyperfusion, these areas were found in the middle of areas in the frontal pole and temporal lobe that were hypoperfused {McKelvey, 1995 #2570}, Only one study showed no difference in perfusion between autistic and control subjects {Herold, 1988 #2567}. Possibly because virtually all of these studies were oriented toward testing neuropsychological rather than pathophysiological hypotheses, there were no probes or tests reported to unearth the tissue level alterations that might be underlying these reductions in blood flow in these brains.

While a large number of animal studies have documented BBB abnormalities from EMF/RFR exposures, only a few PET studies have been performed evaluating EMF exposure effects upon brain glucose metabolism. Volkow et al. performed PET scans both with and without EMF exposure (50 min of GSM-900 with maximum SAR of 0.901 W/kg), and the participants were blinded to the exposure situation (Volkow et al. 2011). A 7% increase in metabolism in the exposure situation compared to controls was identified regionally on the same side of the head as where the mobile phone was placed, in the right orbitofrontal cortex and in the lower part of the right superior temporal gyrus . The strength of the E-field from the phones correlated positively with the brain activation, which the authors hypothesized was from an increase in brain neuron excitability. . A subsequent smaller study by Kwon et al. demonstrated not increased but decreased brain ^{18}FDG uptake after GSM-900 exposure, this time in the temporoparietal junction (Kwon et al. 2011).

Many possible mechanisms could be involved in the metabolic and perfusion abnormalities identified, ranging from altered neuronal activity that was hypothesized in the Volkow et al. (2011) ^{8}FDG PET study to narrowing of vascular lumen in the setting of reduced perfusion. Underlying tissue pathophysiology-based phenomena could influence the measurable metabolism and perfusion abnormalities, via mechanisms such as excitotoxicity, cell stress response, constriction of capillary lumen by activated astrocytes, volume effects of vascular extravasation, subtle alterations in blood viscosity due to immune or oxidative stress-associated blood chemical changes, with other possibilities as well. Given the types of damage at the cellular level covered in this pathophysiology section so far – including oxidative stress, membrane and barrier function damage and poorly functioning channels, which occur both in ASDs as a consequence of EMF/RFR exposure, and given the heterogeneity of localization of abnormalities in the autism perfusion papers as well as considerations of nonlinearity, it may not be so surprising that the results in the two PET studies of human impacts of EMF exposure were not consistent.

6. Electrophysiology perturbations

At this stage the argument we hit a key pivot point, where we look at how the alterations in molecular, cellular and systems physiological function, which occur in the brain as well as in the body, impact the transduction into the electrical signaling activities of the brain and nervous system. Certainly the cells and tissues whose physiological challenges we have already discussed provide the material substrate for the electrical activity. Although ASD behaviors are influenced by many factors, they must in principle be mediated through nervous system electrophysiology.

If the cells responsible for generating synapses and oscillatory signaling are laboring under cellular and oxidative stress, lipid peroxidation, impaired calcium and other signaling system abnormalities, then mitochondrial metabolism will fall short, all the more so because of the challenges from the immune system which in turn be triggered to a major extent by environment. How well will synapses be generated? How well will immune-activated and thereby distracted glial cells be able to modulate synaptic and network activity? (Tasker et al. 2012; Eroglu and Barres 2010; Bilbo and Schwarz 2009; Fields 2006)

At present we are in the early stages of being able to formulate these questions well enough to address them. We do know that microglial activation can impact excitatory neurotransmission mediated by astrocytes (Pascual et al. 2012). We do know that the cortical innate immune response increases local neuronal excitability and can lead to seizures (Rodgers et al. 2009; Gardoni et al. 2011). We do know that inflammation can play an important role in epilepsy (Vezzani et al. 2011). We know less about lower levels of chronic or acute pathophysiological dysfunction and how they may modulate and alter the brain's electrophysiology.

Seizures and Epilepsy

EEG signals in ASDs are abnormal on a variety of levels. At the most severe level, EEGs show seizure activity. In addition to the association of some severe epilepsy syndromes (e.g. Landau Kleffner, tuberous sclerosis) with autism, the risk of epilepsy is substantially higher in people with ASDs than in the general population, with a large subset of these individuals experiencing seizure onset around puberty, likely in relation to aberrations in the dramatic and brain-impactful hormonal shifts of that phase of life. Although less than 50% of people clearly have seizures or epilepsy, a much larger number have indications of epileptiform activity, and an even larger percent have subclinical features that can be noted by a clinical epileptologist though not necessarily flagged as of clinical concern.

Epileptic seizures can be both caused by and cause oxidative stress and mitochondrial dysfunction. Seizures can cause extravasation of plasma into brain parenchyma (Mihaly and Bozoky 1984; Librizzi et al. 2012; Marchi et al. 2010; van Vliet et al. 2007; Yan et al. 2005) which can trigger a vicious circle of tissue damage from albumin and greater

irritability, as discussed above. Evidence suggests that if a BBB is already disrupted, there will be greater sensitivity to EMF/RFR exposure than if the BBB were intact (Tore et al. 2002; Tore et al. 2001), suggesting that such exposures can further exacerbate vicious circles already underway.

The combination of pathophysiological and electrophysiological vulnerabilities has been explored in relation to the impact of EMF/RFR on people with epilepsy – which, as discussed above, is a lot more common in ASDs than in the general population.. EMF/RFR exposures from mobile phone emissions have been shown to modulate brain excitability and to increase interhemispheric functional coupling (Vecchio et al. 2012; Tombini et al. 2012). In a rat model the combination of picrotoxin and microwave exposure at mobile phone-like intensities led to a progressive increase in neuronal activation and glial reactivity, with regional variability in the fall-off of these responses three days after picrotoxin treatment (Carballo-Quintas et al. 2011), suggesting a potential for interaction between a hyperexcitable brain and EMF/RFR exposure.

One critical issue here is nonlinearity and context and parameter sensitivity of impact. In one study, rat brain slices exposed to EMF/RFR showed reduced synaptic activity and diminution of amplitude of evoked potentials, while whole body exposure to rats led to synaptic facilitation and increased seizure susceptibility in the subsequent analysis of neocortical slices (Varro et al. 2009). Another study unexpectedly identified enhanced rat pup post-seizure mortality after perinatal exposure to a specific frequency and intensity of exposure, and concluded that apparently innocuous exposures during early development might lead to vulnerability to stimuli presented later in development (St-Pierre et al. 2007)

Sleep

Sleep involves a profound change in brain electrophysiological activity, and EEG abnormalities including disrupted sleep architecture figure in sleep challenges in ASD. Sleep symptoms include bedtime resistance, sleep onset delay, sleep duration and night wakings, and sleep architecture can involve significantly less efficient sleep, less total sleep time, prolonged sleep latency, and prolonged REM latency (Buckley et al. 2010; Giannotti et al. 2011), with these sleep problems being worse in children with ASDs who regressed than in those who did not regress into their autism {Giannotti, 2011 #1611}. EEG abnormalities have also been associated with EMF/RFR exposure, including disrupted sleep architecture as well as changes in sleep spindles and in the coherence and correlation across sleep stages and power bands during sleep {Borbely, 1999 #2165}{Huber, 2003 #2166}.

Sleep disturbance symptoms are also common in both situations. Insomnia is commonly reported in people who are chronically exposed to low-level wireless antenna emissions. Mann (1996) reported an 18% reduction in REM sleep, which is key to memory and

learning functions in humans. In ASDs sleep difficulties are highly pervasive and disruptive not only to the affected individual but also to their whole family due to the associated problems such as noise and the need for vigilance.

The multileveled interconnections involved in the modulation of sleep exemplify the interconnectedness of the many levels of pathophysiology reviewed here: *"Extracellular ATP associated with neuro- and glio-transmission, acting via purine type 2 receptors, e.g., the P2X7 receptor, has a role in glia release of IL1 and TNF. These substances in turn act on neurons to change their intrinsic membrane properties and sensitivities to neurotransmitters and neuromodulators such as adenosine, glutamate and GABA. These actions change the network input-output properties, i.e., a state shift for the network."* (Clinton et al. 2011) With disturbance simultaneously at so many of these levels, it is not surprising that sleep dysregulation is nearly universal in ASDs, and common in the setting of EMF/RFR exposures.

Quantitative electrophysiology

While clinical reading of EEG studies is done visually, a growing number of studies are examining EEG and MEG data using digital signal processing analysis, and often using data collected in controlled research settings with high density array equipment and carefully designed stimuli paradigms. In these settings a variety of abnormalities have been identified other than epileptic. These include abnormalities in the power spectrum, i.e. the distribution of power over the different frequencies present, with some studies showing impaired or reduced gamma-and activity (Sun et al. 2012; Rojas et al. 2008){Rippon, 2007 #2585} and others showing reduction of spectral power across all bands (Tierney et al. 2012) while still others showed increased high-frequency oscillations (Orekhova et al. 2007) Abnormalities in coherence and synchronization between various parts of the brain have been found (Muller 2008; Muller et al. 2011; Wass 2011), comparable to abnormal functional connectivity measured by fMRI (Just et al. 2004) but measurable using EEG or MEG with higher temporal resolution {Duffy, 2012 #2593}{Isler, 2010 #1421}{Murias, 2007 #2591;Murias, 2007 #2590}{Coben, 2008 #2592}. Several studies have identified reduced complexity and increased randomness in EEGs of people with autism (Lai et al. 2010; Catarino et al. 2011), as well as an increase in power but a reduction in coherence (Isler et al. 2010; Mathewson et al. 2012). Some electrophysiological metrics are emerging as potential discriminators between brain signal from individuals with ASDs and those who are neurotypical, such as a wavelet-chaos-neural network methodology applied to EEG signal (Ahmadlou, Adeli, and Adeli 2010).

EMF/RFR also has impacts at levels of brain function measurable by these techniques. At various frequencies and durations of exposure it has been noted to impact alpha and beta rhythms (Hinrikus et al. 2008), to increase randomness (Marino, Nilsen, and Frilot 2003;

Marino and Carrubba 2009), to alter power, to modulate interhemispheric synchronization (Vecchio et al. 2007), to alter electrical activity in brain slices (Tattersall et al. 2001) and to alter the patterns of coordination (spectral power coherence) across the major power bands (Hountala et al. 2008). Bachman et al. (2006) showed statistically significant changes in EEG rhythms and dymanics occurred in between 12% and 20% of healthy volunteers {Bachmann, 2006 #2069}. In children, exposures to cell phone radiation have resulted in changes in brain oscillatory activity during some memory tasks [97,102].

Sensory processing

At the symptomatic level issues with sensory processing are highly prevalent in ASDs. Phenomenology can include hypersensitivity to external stimuli, hyposensitivity to internal sensations and difficulty localizing sensation including pain, and difficulty processing more than one sensory channel at one time. (Robledo, Donnellan, and Strandt-Conroy 2012; Perry et al. 2007; Sacco et al. 2010) There is now electrophysiological evidence of abnormalities at early (brainstem) stages of sensory processing, as well as in later stages of processing that occur in the cortex. Some studies have shown lower and some longer latencies of response to an auditory stimulus. Domains of perception where the performance of people with ASDs is superior to that of neurotypical individuals have been identified. (Marco et al. 2011) *"It is obvious…that sensory processing abnormalities in ASD are distributed rather than localized; sensory abnormalities in ASD obviously span multiple dimensions of latency, adaptation, magnitude and behavior abnormalities, with both enhanced and impaired behavior associated with aberrant cortical responses. Given this diversity in findings, the heterogeneity of ASD, and broad variability seen over and over again in the ASD groups almost irrespective of the study, it is hard to imagine that one single theory could account for all of these observations…. It is therefore probable that several mechanisms and neuronal abnormalities, most likely at multiple levels (from single neurons through to inter-area connections), all contribute to varying degrees to the abnormal sensory processing observed in ASD. It is also likely that no single mechanism is unique to one sensory modality, which is why we see such a widely distributed range of abnormalities across modalities."* (Kenet 2011)

It is also possible that the mechanisms may not simply be neural – they may also be modulated by glial, metabolic, immune, perfusional and other physiological processes and physical properties as well. Yet although there is some consideration of the pathophysiology-sensory function interaction (Kern et al. 2010), it has basically not been fleshed out in studies of ASDs with experimental designs integrating pathophysiological and electrophysiology.

Kenet et al. (2010) demonstrated environmental vulnerability of sensory processing in the brain by the exposure of rat dams to noncoplanar polychlorinated biphenyls (PCBs), during gestation and for three subsequent weeks of nursing{Kenet, 2011 #1852}. *"Although the hearing sensitivity and brainstem auditory responses of pups were normal, exposure resulted in the abnormal development of the primary auditory cortex (A1). A1 was irregularly shaped and marked by internal nonresponsive zones, its topographic organization was grossly abnormal or reversed in about half of the exposed pups, the balance of neuronal inhibition to excitation for A1 neurons was disturbed, and the critical period plasticity that underlies normal postnatal auditory system development was significantly altered. These findings demonstrate that developmental exposure to this class of environmental contaminant alters cortical development."* (Kenet et al. 2007). This study may be particularly relevant for EMF/RFR exposures, as the noncoplanar PCBs were discussed above as targeting calcium signaling as do EMF/RFR exposures – i.e. they both converge upon a common cellular mechanism (Pessah and Lein 2008; Stamou et al. 2012), justifying exploring the hypothesis that the outcomes one might expect from EMF/RFR could be similar.

Autonomic dysregulation

Although there are a fair number of negative studies regarding the impact of EMF/RFR exposure on the autonomic nervous system, increased HRV and autonomic disturbances have been documented (Andrzejak et al. 2008; Szmigielski et al. 1998; Bortkiewicz et al. 2006; Graham et al. 2000; Saunders and Jefferys 2007). Buchner and Eger (2010), in a study in rural Germany of the health impacts of exposures from a new base station yielding novel exposure to EMF/RFR, saw a significant elevation of the stress hormones adrenaline and noradrenaline during the first six months with a concomitant drop in dopamine, with a failure to restore the prior levels after a year and a half. These impacts were felt by the young, the old and the chronically ill, but not by healthy adults (Buchner and Eger 2011).

Effects on the neonate are also evident. Bellieni et al (2008) found that heart rate variability is adversely affected in infants hospitalized in isolettes or incubators where ELF-EMF levels are in the 0.8 to 0.9 μT range (8 to 9 mG). Infants suffer adverse changes in heart rate variability, similar to adults (Bellieni et al. 2008). This electromagnetic stress may have lifelong developmental impacts, based on a study showing that in utero beta 2 agonist exposure can potentially induce a permanent shift in the balance of sympathetic-to-parasympathetic tone (Witter et al. 2009).

Meanwhile clinical observation and a growing body of literature support a major role for stress in ASDs (Anderson and Colombo 2009; Anderson, Colombo, and Unruh 2012; Daluwatte et al. 2012; Ming et al. 2011), with variability amongst individuals in the severity of the stress response but a tendency to have high tonic sympathetic arousal at

baseline (Hirstein, Iversen, and Ramachandran 2001; Toichi and Kamio 2003; Ming, Julu, et al. 2005; Mathewson et al. 2011; Cheshire 2012; Chang et al. 2012).

The impact of EMF/RFR exposure can also be greatly influenced by the stress system status of the individual being exposed. Tore et al sympathecotomized some of his rats before exposure to GSM, to simulate cell phone exposure (Tore et al. 2002; Tore et al. 2001). Salford et al. (2012) reviewed the results:

> *"Comparing the animals, which had been subjected to ganglionectomy, to the other animals, Töre et al. made an interesting observation: as expected, albumin extravasation was more prominent in the sympathectomised sham-exposed rats as compared to normal exposed rats. This was due to the fact that the sympathectomised rats were in a chronic inflammation-prone state with hyper-development of pro-inflammatory structures, such as the parasympathetic and sensory inputs as well as mast cells, and changes in the structure of the blood vessels. Such an inflammation-prone state has a well-known effect on the BBB leakage. However, when comparing sham-exposed sympathectomised rats to GSM-exposed sympathectomised rats, a remarkable increase in albumin leakage was present in the GSM exposed sympathectomised rats compared to the sham rats.* ***In the GSM-exposed sympathectomised rats, both brain areas and the dura mater showed levels of albumin leakage resembling those observed in positive controls after osmotic shock****.[emphasis added] Indeed, more attention should be paid to this finding, since it implicates that the sensitivity to EMF-induced BBB permeability depends not only on power densities and exposure modulations, but also on the initial state of health of the exposed subject."* (Salford, Nittby, and Persson 2012)

This dramatically greater impact on an autonomically and immunologically vulnerable set of animals raises concerns since the vulnerabilities of these animals bear some resemblance to the pathophysiological challenges of individuals with ASDs.

The interconnection between stress and brain connectivity (or coherence) in ASDs is brought out by Narayanan et al. (2010) n a pilot study testing the impact of the beta blocker propranolol on brain functional connectivity measured using functional MRI(Narayanan et al. 2010). A fairly immediate increase in functional connectivity was noted from propranolol – but not from nadolol which has the same vascular effects but does not cross the BBB. Propranolol decreases the burden of norepinephrine, thereby reducing the impact of stress systems on brain processing, and the authors interpreted these effects as creating an improvement of the brain's signal-to-noise ratio{Hasselmo, 1997 #2594}, allowing it to utilize and coordinate more remote parts of its networks. This suggests that stressors such as EMF/RFR, by adding non-biologically meaningful noise to the system, might have the opposite effects, degrading coherent integration.

C. DE-TUNING OF THE BRAIN AND ORGANISM

1. Electromagnetic signaling, oscillation and synchrony are fundamental, and vulnerable

While electrophysiological activity is an intrinsic property of the nervous system, electromagnetic signaling are vital parts of every cell and of molecular structure.

> *"All life on earth has evolved in a sea of natural low-frequency electromagnetic (EM) fields. They originate in terrestrial and extraterrestrial sources. The ever-growing use of electric power over the last century has sharply modified this natural environment in urban environments. Exposure to power-frequency fields far stronger than the natural environment is now universal in civilized society."* (Adey 1994)

Adey published some of the earliest scientific studies on the "cooperativity" actions of cells in communication. Studies showing us that the flux of calcium in brain tissue and immune cells is sensitive to ELF-modulated radiofrequency fields is actually telling us that some of the most fundamental properties of cells and thus of our existence can be modulated by EMF/RFR.

> *"…in first detection of environmental EM fields in tissues, there appears to be a general consensus that the site of field action is at cell membranes. Strands of protein are strategically located on the surface of cells in tissue, where they act as detectors of electrical and chemical messages arriving at cell surfaces, transducing them and transmitting them to the cell interior. The structural basis for this transductive coupling by these protein strands is well known. Through them, cell membranes perform a triple role, in* ***signal detection, signal amplification, and signal transduction to the cell interior****."* (Adey 1994)
> Communication between cells through gap junctions, which is a means of "metabolic cooperation," is also vulnerable to disruption, as discussed earlier.

Oscillation is also a universal phenomenon, and biological systems of the heart, brain and gut are dependent on the cooperative actions of cells that function according to principles of non-linear, coupled biological oscillations for their synchrony, and are dependent on exquisitely timed cues from the environment at vanishingly small levels (Buzsaki 2006; Strogatz 2003). The key to synchronization is the joint actions of cells that co-operate electrically - linking populations of biological oscillators that couple together in large arrays and synchronize spontaneously according to the mathematics described for Josephson junctions (Brian Josephson, the 1993 Nobel prize winner for this concept). This concept has been professionally presented in journal articles and also popularized in a book by Prof. Steven Strogatz, a mathematician at Cornell University who has written

about ‘sync’ as a fundamental organizing principle for biological systems (Strogatz 2001) (Strogatz 2003).

> *“Organisms are biochemically dynamic. They are continuously subjected to time-varying conditions in the form of both extrinsic driving from the environment and intrinsic rhythms generated by specialized cellular clocks within the organism itself. Relevant examples of the latter are the cardiac pacemaker located at the sinoatrial node in mammalian hearts and the circadian clock residing at the suprachiasmatic nuclei in mammalian brains. These rhythm generators are composed of thousands of clock cells that are intrinsically diverse but nevertheless manage to function in a coherent oscillatory state. This is the case, for instance, of the circadian oscillations exhibited by the suprachiasmatic nuclei, the period of which is known to be determined by the mean period of the individual neurons making up the circadian clock. The mechanisms by which this collective behavior arises remain to be understood.”* (Strogatz 2003)

The brain contains a population of oscillators with distributed natural frequencies, which pull one another into synchrony (the circadian pacemaker cells). Strogatz has addressed the unifying mathematics of biological cycles and external factors disrupt these cycles. This also applies to mitochondria:

> *“Organisation of mitochondrial metabolism is a quintessential example of a complex dissipative system which can display dynamic instabilities. Several findings have indicated that the conditions inducing instabilities are within the physiological range and that mild perturbations could elicit oscillations. Different mathematical models have been put forth in order to explain the genesis of oscillations in energy metabolism. One model considers mitochondria as an organised network of oscillators and indicates that communication between mitochondria involves mitochondrial reactive oxygen species (ROS) production acting as synchronisers of the energy status of the whole population of mitochondria. An alternative model proposes that extramitochondrial pH variations could lead to mitochondrial oscillations.”* (Iotti, Borsari, and Bendahan 2010)

The field of bioelectromagnetics has studied exposure to very low levels of electromagnetic frequencies.

> These exposures can alter critical properties of chemical reactions. *“In a chemical reaction, the bond breaks and each partner reclaims its electron from the bond, moving away to encounter a new partner. It is now an unattached, highly reactive free radical. Reforming a bond requires a meeting between two radicals with opposite electron spins, the union producing a singlet pair. The*

lifetime of free radicals is typically short, in the range of microseconds to nanoseconds. It is in this brief period that imposed magnetic fields may alter the rate and amount of product of a chemical reaction. Since the effect is only on the kinetics of chemical reactions, they are known as magnetokinetic effects (Steiner and Ulrich, 1989). They occur only in nonthermal states of biomolecular systems, defined as an insensitivity to random thermal interactions during the brief period of their existence (Walleczek, 1994). They are a consequence of a coherent quantum-mechanical step which accompanies free radical formation." (Adey 1994)

Not just chemical reactions but synchronous biological oscillations in cells (pacemaker cells) can be disturbed and disrupted by artificial, exogenous environmental signals, whicn can lead to desynchronization of neural activity that regulates critical functions (including metabolism) in the brain, gut and heart and circadian rhythms governing sleep and hormone cycles (Strogatz, 1987). Buzsaki in his book *Rhythms of the Brain* (2006) says "*rhythms can be altered by a wide variety of agents and that these perturbations must seriously alter brain performance."* (Buzsaki 2006)

Disturbance can get increasingly disruptive as more damage occurs and more systems are thrown out of kilter and out of cooperativity. One can think of the kindling model in which repeated induction of seizures leads to longer and more sever seizures and greater behavioral involvement. The combination of disruptive and stimulatory effects of biologically inappropriate EMF/RFR exposures could contribute to disruption of synchronized oscillation and cooperativity at a myriad of levels but particularly in the brain, and this may contribute to the loss of coherence and complexity in the brain in autism, as well as dysregulation of multiple other bodily systems. Strogatz points out that there are many more ways of being desynchronized than being synchronized{Strogatz, 2003 #1969}. It has even been suggested that autism itself could be due to brain desynchronization {Welsh, 2005 #528}.

2. Behavior as an "emergent property"

Although from a pathophysiological point of view one might hypothesize that a brain with greater indications of oxidative stress along with immune activation and mitochondrial dysfunction might generate different oscillatory activity than a brain in which those pathophysiological features were absent, to date almost no attention has been paid to testing this hypothesis in ASD or neurodevelopmental and neuropsychiatric conditions more generally. From this vantage point it would make sense to propose that the compromised whole body health status of at least many with ASDs would make it harder for them to maintain the resilience of their brain cells and brain activities in the face of potentially disruptive exposures. Yet the investigation of how this might occur remains a largely unexplored frontier. But from the point of view of making sense of the

brain impact of environmental challenges – including but not limited to EMF-RFR – this investigation is crucial.

The pathophysiological perspective that guides this review would suggest a move away from considering the behavioral manifestations of ASDs as core 'traits,' *Instead behaviors may be better understood as 'outputs' or emergent properties – what the brain and body produce – when their physiological attributes are altered* in these fashions for whatever reasons – be they genetic, environmental or many combinations of both (Anderson 2009, 2008; Sieb 2004; Smith and Thelen 2003; Custodio et al. 2007; Herbert 2012). Sleep and consciousness have also been considered 'emergent properties' (Krueger et al. 2008; Krueger and Obal 2003). Brain oscillatory activity is critical for organizing behavior, and it arises from cells and subcellular features that are shaped by the environment and can act differently based on their functional status as well as on account of external sensory or psychosocial stimuli.

In particular, a) brain oscillatory activity is intimately connected with underlying cellular, metabolic and immune status, b) EMF/RFR is capable of perpetrating changes at each of these levels, and c) problems at each of these levels can make other problems worse. And as mentioned earlier, EMF/RFR and various toxicants can co-potentiate damage(Juutilainen and Kumlin 2006; Juutilainen, Kumlin, and Naarala 2006; Verschaeve et al. 2006; Ahlbom et al. 2008; Hoyto et al. 2008; Juutilainen 2008; Luukkonen et al. 2009; Markkanen, Juutilainen, and Naarala 2008), amplifying allostatic load.

Put together, all of this implies that the combination of these EMF/RFR impacts may quite plausibly significantly contribute both to how ASDs happen in individuals and to why there are more reported cases of ASDs than ever before (with studies showing that not all of this increase can be written off as artifact (King and Bearman 2009; Hertz-Picciotto and Delwiche 2009)).

The hopeful side of this framing of the problem comes from moving beyond the increasingly anachronistic idea that autism is determined overwhelmingly by genetic code about which we can do little or nothing. An emerging model that explains much more of what we now know frames ASDs as the dynamic, active outcomes of perturbed physiological processes – again, more like a chronic but changeable 'state' than a 'trait.' In the latter model, one is empowered to strongly reduce exposures and to make aggressive constructive environmental changes – particularly in diet and nutrition, given their protective potency discussed above (Herbert and Weintraub 2012). In this way allostatic load can be reduced, physiological damage can be repaired, homeostasis can be restored and resilience and optimal function can be promoted.

PART III: IMPLICATIONS

A. SUMMARY

In the above review, the case has been made that ASDs involve physiological challenges at multiple levels, and that these challenges are paralleled in the physiological impacts of EMF/RFR exposure. Evidence has also been presented to suggest that the many levels of damage and degradation of physiological and functional integrity are profoundly related to each other. Although autism spectrum disorders (ASDs) are defined by problems with behavior, communication, social interaction and sensory processing, under the surface they also involve a range of disturbances of underlying biology that find striking parallels in the physiological impacts of electromagnetic frequency and radiofrequency exposures (EMF/RFR). At the cellular and molecular level many studies of people with ASDs have identified oxidative stress and evidence of free radical damage, evidence of cellular stress proteins, as well as deficiencies of antioxidants such as glutathione. Elevated intracellular calcium in ASDs can be associated with genetic mutations but more often may be downstream of inflammation or chemical exposures. Cell membrane lipids may be peroxidized, mitochondria may function poorly, and immune system disturbances of various kinds are common. Brain oxidative stress and inflammation as well as measures consistent with blood-brain barrier and brain perfusion compromise have been documented. Changes in brain and autonomic nervous system electrophysiology can be measured and seizures are far more common than in the population at large. Sleep disruption and high levels of stress are close to universal. In parallel, all of these phenomena have also been documented to result from or be modulated by EMF/RFR exposure. Moreover, some people with ASDs have de novo mutations (that their parents do not have), and EMF/RFR exposures could contribute to this due to their potential genotoxicity. EMF/RFR exposure during pregnancy may send spurious signals to developing brain cells during pregnancy, altering brain development during critical periods, and may increase oxidative stress and immune reactivity that can increase risk for later developmental impairments, with further disruption later in development increasing risk, physiological dysregulation and severity of outcome.

All of this does not prove that EMF/RFR exposures cause autism, but it does raise concerns that they could contribute by increasing risk, and by making challenging biological problems and symptoms worse in these vulnerable individuals. Placed alongside the dramatic rise in reported cases of ASDs, that parallels the dramatic rise in deployment of wireless technologies, a strong case can be made for aggressively investigating links between ASDs and EMR/RFR, and minimizing exposures for people with autism as well as families preconceptionally, during pregnancy, and around infants and children at home, at school, and in health care centers and hospitals.

These arguments have implications for how we understand what ASDs 'are' and how they work. These implications call upon us to take the environmental contribution very seriously, which involves on the one hand a sobering appreciation of the vast array of exposures that can contribute to risk via perturbed development and physiological degradation, and on the other hand a sense that there are powerful things we can do to improve the situation.

B. EXPOSURES AND THEIR IMPLICATIONS

Several thousand scientific studies over four decades point to serious biological effects and health harm from EMF and RFR as are intensively reviewed in the many detailed sections of this BioInitiative Report. These studies report genotoxicity, single-and double-strand DNA damage, chromatin condensation, loss of DNA repair capacity in human stem cells, reduction in free-radical scavengers (particularly melatonin), abnormal gene transcription, neurotoxicity, carcinogenicity, damage to sperm morphology and function, effects on behavior, and effects on brain development in the fetus of human mothers that use cell phones during pregnancy. Cell phone exposure has been linked to altered fetal brain development and ADHD-like behavior in the offspring of pregnant mice.

1. Exposures have outpaced precautions

There is no question that huge new exposures to EMF/RFRs have occurred over the past few decades. As discussed extensively in other parts of this Bioinitiative 2012 update {Sage, 2012 #2595}, there is much concern that regulations to date have been based on a very limited sense of the pertinent biology, and particularly that limiting concern to thermal impacts is no longer valid since there is a wealth of evidence by now that non-thermal impacts can be biologically very powerful.

Only in the last two decades have exposures to RFR and wireless technologies become so widespread as to affect virtually every living space, and affect every member of societies around the world. Even as some disease patterns like brain tumors from cell phone use have become 'epidemiologially visible', there are no comprehensive and systematic global health surveillance programs that really keep up to report EMF/RFR health trends (Fragopoulou et al. 2010).

> "*The deployment of new technologies is running ahead of any reasonable estimation of possible health impacts and estimates of probabilities, let alone a solid assessment of risk. However, what has been missing with regard to EMF has been an acknowledgement of the risk that is demonstrated by the scientific studies. There is clear evidence of risk, although the magnitude of the risk is uncertain, and the magnitude of doing nothing on the health effects cost to society is similarly uncertain. This situation is very similar to our history of dealing with*

the hazards of smoking decades ago, where the power of the industry to influence governments and even conflicts of interest within the public health community delayed action for more than a generation, with consequent loss of life and enormous extra health care costs to society." (Sage and Carpenter 2009).

2. The population's exposure has increased

Given the range of physiological impacts described in Part 2, the very rapid global deployment of both old and new forms of emerging wireless technologies in the last two decades needs aggressive evaluation from a public health perspective.

In the United States, the deployment of wireless infrastructure (cell tower sites) to support cell phone use has accelerated greatly in the last decades. The Cellular Telephone Institute of America (CTIA) estimated that in 1997 there were only 36,650 cell sites in the US; but increased rapidly to 131,350 in June 2002; 210,350 in June 2007 and 265,561 in June 2012 {Roche, 2012 #2614;Cellular Telephone Industry of America (CTIA), 2012 June #2615}. About 220,500 cell sites existed in 2008 {Reardon, 2007 #2613}{Cellular Telephone Industry of America (CTIA), 2012 June #2615}{Anonymous, 2005 May 20 #2618}. These wireless facilities for cellular phone voice and data transmission produce RFR over broad areas in communities and are an involuntary and unavoidable source of radiofrequency radiation exposure. Other new RFR exposures that didn't exist before are from WI-FI access points (hotspots) that radiate 24/7 in cafes, stores, libraries, classrooms, on buses and trains, and from personal WI-FI enabled devices (iPads, tablets, PDAs, etc).

Not surprisingly, the use of cell phones has a parallel growth trend. In the late 1980s and early 1990's, only a few percent of the US population were cell phone users. By 2008, eighty-four percent (84%) of the population of the US owned cell phones [16]. CTIA reports that wireless subscriber connections in the US increased from 49 million in June 1997 to 135 million in June 2002 to 243 million in June 2007 to 322 million in June 2012 {Roche, 2012 #2614;Cellular Telephone Industry of America (CTIA), 2012 June #2615}. This represents more than a 100% penetration rate in the US consumer market, up from just a few percent in the early 1990's. The number of wireless subscribers in June 1997 was 18%; in June 2002 it was 47%; in June 2007 it was 81% and in June 2012 it is 101%.

The annualized use of cell phones in the US was estimated to be 2.23 trillion minutes in 2008 [16] and 2.296 trillion minutes in 2010 (CITA, 2012). There are 6 billion users of cell phones world- wide in 2011 up from 2.2 billion in 2008 [17] and many million more users of cordless phones.

The number of US homes with *only* wireless cell phones has risen from 10.5% in 2007 to 31.6% in June of 2012 {Roche, 2012 #2614;Cellular Telephone Industry of America

(CTIA), 2012 June #2615}. There are no statistics for June 1997 nor for June 2002, since landline (non-wireless) phone use predominated. The shift to wireless communications, more minutes of use, and reliance on cell and cordless phones rather than corded phones is an extremely revealing measure of new EMF and RFR exposures for both adults and children.

3. Infants, children and childbearing families are highly exposed and vulnerable

With regard to children, the spread of cell towers in communities, often placed on pre-school, church day-care, and school campuses, means that young children may have hundreds of thousands of times higher RF exposures in home and school environments than existed even 20-25 years ago. In addition, the nearly universal switch to cordless and cell phones, and away from corded landline phones, means close and repetitive exposures to both EMF and RFR in the home. Wireless laptops and wireless internet in schools, and home offices and for homework mean even more chronic exposures to RFR, a designated IARC 2B Possible Human Carcinogen {International Agency for Research on Cancer of the World Health Organization, 2011 May #2616}{Baan, 2011 #2598} . The great utility of handheld devices as communication aids and sources of information and satisfaction for people on the autism spectrum may come with a concerning underbelly.

Exposures prior to conception or during pregnancy and infancy are also important to consider. These exposures can come from faulty wiring, proximity to power lines, or high-frequency transients from a proximate transformer on a utility pole, or internal sources of pulsed RFR in the home (examples include an electronic baby monitor in the crib, a wireless router in the next room, a DECT phone that pulses high emissions of RFR on a continuous basis 24/7, or conversion to all compact fluorescent bulbs that produce significant 'dirty electricity' for occupants due to low-kilohertz frequency fields on electrical wiring and in ambient space. Sick and vulnerable infants in neonatal intensive care units are heavily exposed from being surrounded by equipment, with negative metabolic and autonomic consequences documented and other likely consequences needing further investigation (Bellieni et al. 2008; Bellieni, Tei, et al. 2012).

Wireless phones and laptops exposures produce extremely low frequency pulses from the battery of the wireless device {Sage, 2007 #2611}(Sage and Carpenter 2009) and the exposures to pulsed radiofrequency microwave radiation when the wireless device is transmitting or receiving calls and emails.

Especially since EMF/RFR exposures are already classified as IARC 2B Possible Human Carcinogens, we should be actively investigating these sources of damage to DNA that could reasonably result in 'de novo mutations' but also be aware that common

environmental exposures from EMF and RFR might play a role in the higher rates of concordance for autism (ASD) among twins and siblings.

Researchers also should be aware that common environmental exposures from EMF and RFR might play a role in the higher rates of autism (ASD) among twins and siblings, not solely because of maternal use of wireless devices during pregnancy and paternal sperm exposure to wireless devices peri-conception; but also because such oxidative damage to DNA can occur at levels introduced into the world of the fetus, and young developing infant and child via baby surveillance monitoring devices in the crib and wireless devices in the home. The deployment of technologies poses risks to human fertility and reproduction capacity, to the fetus, to children and adults (Sage and Carpenter 2009).

4. ASD Risk and Genomic Damage to Future Generations

Barouki and Grandjean (2012) make a persuasive case that public health interventions are critically needed in early childhood development to prevent adult diseases that appear decades later (Barouki et al. 2012). Although they do not include EMF or RFR but only chemicals, they do say that physiological stressors, which EMF and RFR certainly have been established to be, should be reduced during critical development windows. They say: "*The current pandemic of non-communicable diseases and the increased prevalence of important dysfunctions demand an open interrogation of why current interventions appear insufficient. We now know that disease risk can be induced very early in the life course and that it is modifiable by nutrients and environmental chemical exposures (along with drugs. infections, and other types of stresses)".*

Part II of this chapter documents the detailed scientific basis for considering EMF/RFR exposures to be of significance to the ASDs crisis. Public health interventions are warranted now to protect the genetic heritage of humans, as well as the other stocks of genetic material in wildlife and plants in the face of what appears to be on-going impairment of these genomes. The risk of genomic damage for future generations is sufficiently documented to warrant strong preventative action and new public safety limits that observe EMF/RFR levels shown to cause adverse effects.

5. De-Tuning the Organism

Genetic mutations may lead to cancer and other diseases in the present and future generations, but the exposures that are capable of creating genotoxic impacts also compromise physiological function Even genotoxicity can have not only specific but also non-specific effects due to inefficiencies, misfolded proteins, and cellular debris, as discussed in the section "Implications of Damage" at the end of the first part of Part II, regarding the rescue of a mouse model of Rett syndrome through enabling a probably generic process of microglial phagocytosis, critical to debris removal, rather than through

correcting some specific molecular defect of the synapse (Derecki et al. 2012; Derecki, Cronk, and Kipnis 2012).

In the present setting, where the argument about the pertinence of the cascade of physiological and genotoxic compromises to autism includes the degradative impact on oscillatory synchronization, it is also worth considering that oscillation is a property of living and even physical systems much more generally, and not just of brain oscillatory networks (Strogatz 2003). Under certain circumstances, phase transitions occur and synchronization emerges, often rather quickly rather than gradually – more like a state change than a gradual trend. On the other hand, as mentioned, synchronization can be lost, and there are an enormous number of ways for a system to be de-synchronized, which may relate to the heterogeneity amongst people with ASD that so vexes researchers.

In the setting of autism, a baby gestated or developing as a neonate in a milieu with excessively elevated EMF/RFR exposures is bound to have interference with the normal development processes, including the organization of information and experience in the brain. This baby's environment also often nutritional insufficiencies (processed denatured pesticide-laden food low in antioxidants, minerals and essential fatty acids essential to cellular protection). The baby's gestational period may have been complicated by the mother's own health issues such as conditions like obesity and diabetes {Krakowiak, 2012 #2617} which converge on inflammation, oxidative stress and other common forms of physiological dysregulation associated with or even just eating nutrient-depleted, pesticide-laden processed food. The exquisite 'tuning up' of the brain and body as it develops will integrate and respond to the environmental inputs it receives, and is particularly sensitive to environmental miscues (whether chemical like endocrine disruptors, EMF/RFR, or other hostile environmental conditions whether hostile or nurturing). To the extent that the baby is burdened with more disorganized or hostile cues than nurturing and organizing cues, that baby may lose resiliency and become more physiologically vulnerable –perhaps approaching a tipping point into decompensation.

From a systems point of view, the phenomenon of 'autistic regression' may occur after an accumulation of multisystem signaling interference leading to a tipping point of loss of some vital systems synchronization and increase in randomization. EMF/RFR exposures could plausibly contribute both to this vulnerability and to the decompensation/desynchronization process – as could other stressors such as infection, toxicity, acute stress. The vulnerability, then, is the 'allostatic load' – the total burden of stressors pressing toward disorganization. The tipping point may come in a variety of ways but upon investigation one is likely to find that unless it is a severe stressor it is not triggered simply by a single source of stress in an otherwise blissfully healthy child, but rather is the "straw that breaks the camel's back' laid atop a prior accumulation of 'allostatic load.'

C. CONCLUSIONS AND RECOMMENDATIONS

1. Change our deployment of EMF/RFR

The deployment of RFR from wireless technologies has incredible momentum, and it has made many things easier and many other things possible for the first time. On the other hand this momentum can interfere with setting up the technology in a fashion truly respectful of biological tolerances. Other sections in the Bioinitiative 2012 update will address recommendations and guidelines for increasing the safety profile. This will undoubtedly provoke controversy. The problems will not get settled immediately, and transformation to healthier arrangements will take time.

> *"There is no question that global implementation of the safety standards proposed in the Bioinitiative Report, if implemented abruptly and without careful planning, have the potential to not only be very expensive but also disruptive of life and the economy as we know it. Action must be a balance of risk to cost to benefit. The major risk from maintaining the status quo is an increasing number of cancer cases, especially in young people, as well as neurobehavioral problems at increasing frequencies. The benefits of the status quo are expansion and continued development of communication technologies. But we suspect that the true costs of even existing technologies will only become much more apparent with time. Whether the costs of remedial action are worth the societal benefits is a formula that should reward precautionary behavior*." (Sage and Carpenter 2009)

2. Encourage precautions right now based on present knowledge

In the meantime many people have already started taking precautionary measures, and more will wish to do so. Physicians and health care people should raise the visibility of EMF/RFR as a plausible environmental factor in clinical evaluations and treatment protocols. Reducing or removing EMF and wireless RFR stressors from the environment is a reasonable precautionary action given the overall weight of evidence.

- Children with existing neurological problems that include cognitive, learning, attention, memory, or behavioral problems should as much as possible be provided with wired (not wireless) learning, living and sleeping environments,
- Special education classrooms should aim for 'no wireless' conditions to reduce avoidable stressors that may impede social, academic and behavioral progress.
- All children should reasonably be protected from the physiological stressor of significantly elevated EMF/RFR (wireless in classrooms, or home environments).
- School districts that are now considering all-wireless learning environments should be strongly cautioned that wired environments are likely to provide better learning and teaching environments, and prevent possible adverse health consequences for both students and faculty in the long-term.

- Monitoring of the impacts of wireless technology in learning and care environments should be performed with sophisticated measurement and data analysis techniques that are cognizant of the non-linear impacts of EMF/RFR and of data techniques most appropriate for discerning these impacts.
- There is sufficient scientific evidence to warrant the selection of wired internet, wired classrooms and wired learning devices, rather than making an expensive and potentially health-harming commitment to wireless devices that may have to be substituted out later, and
- Wired classrooms should reasonably be provided to all students who opt-out of wireless environments.

Undoubtedly risks and the above recommendations will be dismissed by those poised to benefit from the sale of these new systems. Many people also feel that new possibilities have opened up to themselves and the world through wireless technologies. But the public needs to know that these risks exist, that transition to wireless should not be presumed safe, and that it is very much worth the effort to minimize exposures that still provide the benefits of technology in learning, but without the threat of health risk and development impairments to learning and behavior in the classroom.

Broader recommendations also apply, related to reducing the physiological vulnerability to exposures, reduce allostatic load and build physiological resiliency through high quality nutrition, reducing exposure to toxicants and infectious agents, and reducing stress(Herbert and Weintraub 2012), all of which can be implemented safely based upon presently available knowledge.

3. Build an environmentally physiologically centered research program in ASDs as a platform for investigating the EMR/RFR-ASD linkage

This review has been structured around the physiological parallels between ASDs and the impacts of EMF/RFR. What is missing from the autism research agenda is some cross-study of these two bodies of research evidence. To do this we will need both a recognition of the importance of these risks, and a collaborative multi-site research program centered around a "middle-out" physiological approach that can incorporate the the gene-brain-behavior agenda that has dominated ASD research into a broader framework (Herbert 2013). While the middle-out approach is an emerging framework in systems biology that can incorporate complexity and nonlinear, multileveled modeling (Cristofolini et al. 2008; de Graaf et al. 2009; Majumder and Mukherjee 2011; Vinga et al. 2010; Walker and Southgate 2009), the gene-brain-behavior approach has been based on an expectation of linear mapping across the levels on which it focuses, but instead the systems involved appear to be much more complex, and the physiological levels largely

left out of this linear approach are critically important to helping people with ASDs because they will help not only with understanding how environment impacts function but also with identifying leverage points.

4. Take the evidence as a call to action

Both EMF and RFR exposures are already classified as IARC 2B Possible Human Carcinogens. The substantial scientific literature on EMF and RFR effects on DNA, on immune and blood-brain barrier disruption, on stress proteins, on circadian rhythms and hormone disregulation, and on cognition, sleep, disruption of neural control and altered brainwave activity all argue for reduction of exposures now, and better coordinated research in these areas.

All relevant environmental conditions should be given weight in defining and implementing prudent, precautionary actions to protect public health, including EMF and RFR. Evidence is sufficient to add EMF/RFR prominently to the list of exposures that can degrade the human genome, and impair normal development, health and quality of our physiology. With the rising numbers people with ASDs and other childhood health and developmental disorders, we cannot afford to ignore this component of risk to our children and vulnerable populations. When the risk factors are largely avoidable or preventable, ignoring clear evidence of large-scale health risks to global populations poses unnecessary and unacceptable risks. Taking this evidence as a call to action will be challenging and disruptive in the short term, but constructive in the longer term as we learn to use EMF/RFR in healthier ways.

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Autism and EMF? Plausibility of a pathophysiological link – Part I

Martha R. Herbert [a,*], Cindy Sage [b]

[a] *TRANSCEND Research Program Neurology, Massachusetts General Hospital, Harvard Medical School, Boston, MA 02129, USA*
[b] *Sage Associates, Santa Barbara, CA, USA*

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Abstract

Although autism spectrum conditions (ASCs) are defined behaviorally, they also involve multileveled disturbances of underlying biology that find striking parallels in the physiological impacts of electromagnetic frequency and radiofrequency exposures (EMF/RFR). Part I of this paper will review the critical contributions pathophysiology may make to the etiology, pathogenesis and ongoing generation of core features of ASCs. We will review pathophysiological damage to core cellular processes that are associated both with ASCs and with biological effects of EMF/RFR exposures that contribute to chronically disrupted homeostasis. Many studies of people with ASCs have identified oxidative stress and evidence of free radical damage, cellular stress proteins, and deficiencies of antioxidants such as glutathione. Elevated intracellular calcium in ASCs may be due to genetics or may be downstream of inflammation or environmental exposures. Cell membrane lipids may be peroxidized, mitochondria may be dysfunctional, and various kinds of immune system disturbances are common. Brain oxidative stress and inflammation as well as measures consistent with blood–brain barrier and brain perfusion compromise have been documented. Part II of this paper will review how behaviors in ASCs may emerge from alterations of electrophysiological oscillatory synchronization, how EMF/RFR could contribute to these by de-tuning the organism, and policy implications of these vulnerabilities. Changes in brain and autonomic nervous system electrophysiological function and sensory processing predominate, seizures are common, and sleep disruption is close to universal. All of these phenomena also occur with EMF/RFR exposure that can add to system overload ('allostatic load') in ASCs by increasing risk, and worsening challenging biological problems and symptoms; conversely, reducing exposure might ameliorate symptoms of ASCs by reducing obstruction of physiological repair. Various vital but vulnerable mechanisms such as calcium channels may be disrupted by environmental agents, various genes associated with autism or the interaction of both. With dramatic increases in reported ASCs that are coincident in time with the deployment of wireless technologies, we need aggressive investigation of potential ASC – EMF/RFR links. The evidence is sufficient to warrant new public exposure standards benchmarked to low-intensity (non-thermal) exposure levels now known to be biologically disruptive, and strong, interim precautionary practices are advocated.
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1. Introduction

The premise of this review is that although scant attention has been paid to possible links between electromagnetic fields and radiofrequency radiation exposures (EMF/RFR) and Autism Spectrum Conditions (ASCs), such links probably exist. The rationale for this premise is that the physiological impacts of EMF/RFR and a host of increasingly well-documented pathophysiological phenomena in ASCs have remarkable similarities, spanning from cellular and oxidative stress to malfunctioning membranes, channels and barriers to genotoxicity, mitochondrial dysfunction, immune abnormalities, inflammatory issues, neuropathological disruption and electrophysiological dysregulation – in short, multi-scale contributors to de-tuning the organism. Additional support may be found in the parallels between the rise in reported cases of ASCs and the remarkable increases in EMF/RFR exposures over the past few decades

Reviewing these similarities does not prove that these parallels imply causality. Moreover, the physiological processes affected by EMF/RFR are also impacted by other environmental factors, and are known to be present in myriad other chronic illnesses. A set of in-depth reviews on the

* Corresponding author.
E-mail address: drmarthaherbert@gmail.com (M.R. Herbert).

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science and public health policy implications of EMF/RFR has been published in a special issue of Pathophysiology 16 (2,3) 2009. This two-volume special issue of Pathophysiology offers a broad perspective on the nature of health impacts of man-made EMFs, documenting biological effects and health impacts of EMFs including genotoxicity, neurotoxicity, reproductive and developmental effects, physiological stress, blood–brain barrier effects, immune system effects, various cancers including breast cancer, glioma and acoustic neuroma, Alzheimer's disease; and the science as a guide to public health policy implications for EMF diseases [1]. Many of these reviews have been updated in the BioInitiative 2012 Report [2], with 1800 new papers added. Further reinforcement is published in seminal research reviews including the two-volume Non-Thermal effects and Mechanisms of Interaction between Electromagnetic Fields and Living Matter, Giuliani L and Soffritti, M (Eds.), ICEMS, Ramazzini Institute, Bologna, Italy (2010) [3]; the World Health Organization INTERPHONE Final Report (2010) [4]; and the WHO International Agency for Research on Cancer RFR Monograph [5] designating RFR as a Group 2B Possible Human Carcinogen. The National Academy of Sciences Committee on Identification of Research Needs Relating to Potential Biological or Adverse Health Effects of Wireless Communication Devices (2008) [6] called for health research on wireless effects on children and adolescents and pregnant women; wireless personal computers and base station antennas; multiple element base station antennas under highest radiated power conditions; hand-held cell phones; and better dosimetric absorbed power calculations using realistic anatomic models for both men, women and children of different height and ages. Yet EMF/RFR does not need to be a unique contributor to ASCs to add significantly to system overload ('allostatic load') and dysfunction [7]. Even so these pathophysiological overlaps do suggest that the potential for an EMF/RFR-ASC connection should be taken seriously, and that their biological fragility may make many with ASCs more likely to experience adverse EMF/RFR impacts. This is a sufficient basis to recommend that precautionary measures should be implemented, that further research should be prioritized, and that policy level interventions based on existing and emerging data should be designed and pursued. Moreover, pursuing this link could help us understand ASCs better and find more ways to improve the lives of people with ASCs and of so many others.

This paper is divided into two parts. Part I (http://dx.doi.org/10.1016/j.pathophys.2013.08.001) describes the pathophysiology and dynamism of common behavioral manifestations in autism, and pathophysiological damage to core cellular processes that is associated both with ASCs and with impacts of EMF/RFR. Part II (http://dx.doi.org/10.1016/j.pathophys.2013.08.002) reviews how behaviors in ASCs may emerge from alterations of electrophysiological oscillatory synchronization and how EMF/RFR could contribute to these by de-tuning the organism. Part II also discusses public health implications, and proposes recommendations for harm prevention and health promotion.

2. Physiological pathogenesis and mechanisms of autism spectrum conditions

2.1. How are biology and behavior related?

Appreciating the plausibility of a link between ASCs and EMF/RFR requires considering the relationship between ASC's behavioral and biological features. ASCs were first labeled as 'autism' in 1943 by Leo Kanner, a child psychiatrist who extracted several key behavioral features, related to communication and social interaction challenges and a tendency toward restricted interests and repetitive behaviors [8]. There has been some modification of the characterization of these behavioral features, but ASCs are still defined behaviorally, although sensory issues such as hypo- or hyper-reactivity have recently been included in the diagnostic criteria (Diagnostic and Statistical Manual of Mental Disorders or DSM-V) [9,10].

2.1.1. Transduction is fundamental but poorly understood

To evaluate how an environmental factor such as EMF/RFR could lead to autism and/or influence its severity or incidence, we examine how effects of EMF/RFR exposure may be transduced into changes in nervous system electrical activity, and how these in turn generate the set of behaviors we have categorized as 'autism.'[11] This means not taking behaviors as given, or as purely determined by genetics, but exploring the full range of biology that generates these features and challenges.

2.1.2. More than brain

Although 'autism' has long been considered to be a psychiatric or neurological brain-based disorder [12,13], people diagnosed with ASCs often have many biological features including systemic pathophysiological disturbances (such as oxidative stress, mitochondrial dysfunction and metabolic and immune abnormalities) [14–17] as well as symptomatic medical comorbidities (such as gastrointestinal distress, recurrent infections, epilepsy, autonomic dysregulation and sleep disruption) [18–26] in addition to the core defining behaviors [27]. Because of variability among individuals, the relevance of many of these biological features has been dismissed as secondary and not intrinsically related to the 'autism.'

2.1.3. Heterogeneity: more genetic and environmental than physiological

Presently large numbers of genes and environmental contributors to ASCs are under consideration. Over 800 genes have been associated with ASCs, and over 100 different rare genetic syndromes are frequently accompanied by ASCs, but

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no clear unifying mechanism has been identified [28–33]. Similarly, a large number of potential environmental contributors are under investigation ranging from toxicants and Vitamin D deficiency or failure to take prenatal vitamins to air pollution and stress or infection in pregnancy [34–41].

By contrast, a smaller set of disturbances are showing up physiologically as common across substantial numbers of people with ASCs – as well as in myriad other chronic conditions whose prevalence also appears to be increasing [42,43]. These include oxidative stress, inflammation, and mitochondrial dysfunction. EMF/RFR exposure is associated with many of the same biological effects and chronic health conditions [1]. This environmentally vulnerable physiology [44], which may serve as final common pathways triggered by diverse genetic and environmental contributors, will be discussed in Section 3 of Part I as well as in Part II; it may or may not need to rest on underlying genetic vulnerability.

2.1.4. EMF/RFR research may help us understand how ASCs 'work'

Some correlations between biological and behavioral features have been identified – e.g., a higher level of immune abnormalities correlates with more aberrant behaviors [26,45–50]. In order to move beyond correlations to identifying *mechanisms* by which the *transduction of pathophysiology into behavior* might actually occur, an important component is studying the relationship between systemic pathophysiology and nervous system electrophysiology.

The brain is simultaneously a tissue-based physical organ that can be compromised by cellular pathophysiology as well as altered developmental processes and an information processing system that operates through networks of synchronized electrical oscillations (brain waves) – and EMF/RFR impacts may occur directly at both of these levels. To date the emphasis in ASC research has largely been on 'structure-function' relationships that have been anatomy-centered. Thus, exploring how EMF/RFR impacts ASCs may answer questions of how pathophysiological and electrophysiological information-processing interacts.

2.2. Time courses of mechanisms

Researchers have mainly looked for causes of autism in mechanisms that occur early and create permanent change or damage. This approach is logical if one assumes that genetic influences are overwhelmingly predominant, and 'autism' is a fixed lifelong trait. However evidence is emerging that ASCs may be more state-like and variable than trait-like and fixed.

2.2.1. Plasticity

A remarkable shift is occurring in conceptual thinking about ASCs and brain plasticity [51]. There are growing numbers of reports of improvement and loss of diagnosis, reversal of neurological symptoms in a growing number of mouse models of genetic syndromes that in humans prominently feature autism [52–62], short-term pharmaceutically-induced improvement in brain connectivity [63], and transient reversal or abeyance of symptomatology under various circumstances (including fever, fluid-only diet, and certain antibiotic treatments [50,64]). Reversals undermine the idea that ASCs derive from an intrinsically 'broken brain', and short time frames of marked improvement cannot be accounted for by remodeling of the brain's anatomical substrate [65]. 'Brain waves' and their synchronization, on the other hand, could easily vary over short time periods.

Also, evidence of average to superior intelligence in most people with autism [66,67], as well as of domains of perceptual superiority [68–76], call into question the assumption that ASCs are intrinsically associated with cognitive deficits.

2.2.2. Mechanisms that operate actively throughout the life-course

EMF/RFR effects can occur within minutes (Blank, 2009) and may, in part, explain clinical reports of 'intermittent autism' – for example, some children with mitochondrial disease who have ups and downs of their bioenergetics status 'have autism' on their bad days but don't display autistic features on their good days [77]. These children with their vulnerable, barely compensated mitochondria could very well be teetering right at the brink of a minimally adequate interface of metabolic and electrophysiological dysfunction. Everyday exposures to allergens, infection, pesticide on the school playground, as well as EMF/RFR interference with electrophysiology might reasonably contribute to the bad days. Stabilizing more optimal nervous system performance [78] including through environmental control of excessive EMF/RFR exposure could perhaps achieve more 'good days'.

2.2.3. Pathophysiology and allostatic load

The model of 'allostatic load' – the sum total of stressors and burdens [79]– may be central to understanding how the many risk factors interact to create autism – and to create a spectrum of levels of severity across so many of ASD's associated features. This accumulation increases chronic stress, and a growing number of papers document indicators of chronic stress in individuals with ASCs (as will be discussed in Part II). The 'allostatic load' concept dovetails well with a model of progressive exacerbation of pathophysiological disturbances that occurs in the pathogenesis of many chronic diseases [43]. It is also critical to understand that many different environmental factors converge upon a much smaller number of environmentally vulnerable physiological mechanisms [44], so that large numbers of small exposures may have effects from small numbers of large exposures.

EMF/RFR exposures have demonstrated biological effects at just about every level at which biology and physiology have been shown to be disrupted in ASCs. Further EMF/RFR has been shown to potentiate the impact of various toxicants when both exposures occur together [80]; this may be additive or more than additive. This suggests that EMF/RFR may synergize with other contributors and make things worse. A cascade of exposures interacting with vulnerabilities in

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an individual can potentially lead to a tipping point for that person, such as the phenomenon of autistic regression experienced by a substantial subset of people with ASCs.

Just a few decades ago, EMF/RFR exposures were not present in the environment at today's levels. Levels have increased several thousand-fold or more in the past two decades from wireless technology alone; with unplanned side effects from pulsed RFR that is a newly classified Group 2B possible human carcinogen [5]. Nearly six billion people globally own wireless phones. Many millions are exposed to wireless exposures from use of wireless devices and wireless antenna facilities [81]. For this as well as for physiological reasons, 'allostatic loading' as a viable concept for the study of ASCs should reasonably address EMF/RFR as one of the exposures of relevance to the overall stress load, since it is now a chronic and unremitting exposure in daily life at environmentally relevant levels shown to cause bioeffects from preconception and pregnancy through infancy, childhood and the whole life-course.

3. Parallels in pathophysiology

This section will review parallels in pathophysiology between ASCs and impacts of EMF/RFR. It will begin with a review of mechanisms of direct impact and damage at the level of molecules, cells, tissues and genes. It will then move on to consider how these levels of damage lead to degradation of the integrity of functional systems including mitochondrial bioenergetics, melatonin metabolism, immune function and nervous system physiology. The review of parallels concludes with electromagnetic signaling and synchronized oscillation from membranes to nervous system. It will discuss how the ensemble of pathophysiological disturbances, which are themselves final common pathways that can be caused or worsened by many stressors, combine to converge upon electrophysiology. This leads to the implication that 'aberrant' neural systems and somatic function and behaviors might be better understood as consequences or 'outputs' of disturbed underlying physiology to which EMF/RFR is a plausible contributor.

3.1. Damage: means and domains

ASCs have been conceptualized as 'neurodevelopmental' which has focused attention on how genes and environment could alter brain development. This leads to the unstated presumption that virtually everything important about the brain in ASCs has to do with differences in the way it was formed, and that all "malfunction" derives from this "malformation." In genetics this has led to a hunt for neurodevelopmental genes. There is no question that environmental impacts can alter brain development, and impact brain function across the lifespan.

However the influence of the environment on neurodevelopmental conditions such as ASCs does not stop there. Evidence is accumulating showing that increased expression of genes associated with physiological dysregulation, as well as *single-nucleotide polymorphisms* (SNPs) associated with these issues, may be if anything more prominent than alterations of 'neurodevelopmental' genes [82]. In a study of gene expression in ASCs, Down syndrome and Rett syndrome, these authors state, "*(O)ur results surprisingly converge upon immune, and not neurodevelopmental genes, as the most consistently shared abnormality in genome-wide expression patterns. A dysregulated immune response, accompanied by enhanced oxidative stress and abnormal mitochondrial metabolism seemingly represents the common molecular underpinning of these neurodevelopmental disorders.*" Others have also found pathophysiology-related genes as figuring most prominently in alterations of gene expression in ASC [83–86]. SNPs associated with methylation abnormalities, impaired glutathione synthesis and mitochondrial dysfunction also have been identified as significant risk factors.

Genetics may create risk, but the actual nervous system and health consequences probably come from dysfunction at the physiological level. As mentioned, evidence for pathophysiological dysfunction in ASCs increasingly abounds. In particular, a growing body of evidence widely reported in both the EMF/RFR and ASC literature documents immune aberrations, low total and reduced glutathione levels, lower activity of the anti-oxidative stress system and mitochondrial dysfunction. These phenomena may be both genetically and environmentally modulated. As will be discussed further below, they are certainly pertinent to the neurodevelopment of the brain, which has been by far the dominant focus autism research, but it does not stop there as they can significantly modulate brain function in real time, as well as shape the function of the entire organism, including the autonomic system, the cardiovascular, endocrine, immune, gastrointestinal and reproductive systems and more. These systemic impacts may in turn feed back into the nervous system, modulating how it functions.

3.1.1. Cellular stress

3.1.1.1. Oxidative stress. Autism (ASC) research indicates that oxidative stress may be a common attribute amongst many individuals with autism. In the past decade the literature on this has moved from a trickle to a flood. Studies document reduced antioxidant capacity, increased indicators of oxidative stress and free radical damage, alterations in nutritional status consistent with oxidative stress, altered lipid profiles, and pertinent changes not only in blood but also in brain tissue. Associations of ASCs with environmental exposures such as air pollution and pesticides are indirectly supportive as well, since such exposures are linked in other literature to oxidative stress [43,87–101].

Reactive oxygen species are produced as a normal consequence of mitochondrial oxidative metabolism as well as other reactions, but when their number exceeds the cell's antioxidant capacity a situation of oxidative stress develops. It

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is certainly the case that oxidative stress can be a consequence of exposures to chemical toxicants, or of the interactive impacts of toxicants, nutritional insufficiencies and genetic vulnerabilities. This set of risk factors has received considerable attention for the potential roles each component and various possible combinations could play in causing or exacerbating autism.

Less often mentioned in the ASC pathophysiology literature is that it is also well established that EMF/RFR exposures can be associated with oxidative damage. Published scientific papers that demonstrate the depth of EMF and RFR evidence reporting oxidative damage in human and animal models are profiled by Lai and colleagues [102–104]. These cellular effects can occur at low-intensity, legal levels of exposure that are now 'common environmental levels' for pregnant women, the fetus, the infant, the very young child, and the growing child as well as for adults. Electromagnetic fields (EMF) can enhance free radical activity in cells [105,106] particularly via the Fenton reaction, and prolonging the exposure causes a larger increase, indicating a cumulative effect. The Fenton reaction is a catalytic process of iron to convert hydrogen peroxides, a product of oxidative respiration in the mitochondria, into hydroxyl free radical, which is a very potent and toxic free radical [103,104]. Free radicals damage and kill organelles and cells by damaging macromolecules, such as DNA, protein and membrane components.

Further indications of a link to oxidative stress are findings that EMF and RFR at very low intensities can modulate glutamate, glutathione and GABA, and affect mitochondrial metabolism. Alterations in all these substances and processes have been documented in ASCs [25,86,89,90,92,107–127]. On the EMF/RFR side, Campisi et al. (2010) report that increased glutamate levels from 900 MHz cell phone frequency radiation on primary rat neocortical astroglial cell cultures induced a significant increase in ROS levels and DNA fragmentation after only 20 min with pulsed RFR at non-thermal levels [128].

Fragopoulou et al. (2012) conducted proteomics analysis of proteins involved in brain regulation in mice as a consequence of prolonged exposure to EMF [129]. They identified altered expression of 143 proteins, ranging from as low as 0.003-fold downregulation up to 114-fold overexpression with affected proteins including neural function-related proteins including Glial Fibrillary Acidic Protein (GFAP), alpha-synuclein, Glia Maturation Factor beta (GMF), apolipoprotein E (apoE), heat shock proteins, and cytoskeletal proteins (i.e., neurofilaments and tropomodulin), as well as proteins of brain metabolism such as aspartate aminotransferase and glutamate dehydrogenase. The authors pointed out that oxidative stress was consistent with some of these changes.

Aberrations in glutathione metabolism and deficiencies in reserves of reduced glutathione are increasingly associated with ASCs, both systemically and in the brain. The parallel with EMF/RFR impacts here is strong, since glutathione reduction associated with EMF/RFR is reported in at least twenty three relevant research studies in both human and animal studies since 1998, including the following citations [130–144]. It is increasingly appreciated that glutathione is a final common pathway, a critical piece of environmentally vulnerable physiology, as glutathione reserves are compromised by an enormous number of environmental stressors, so that the cumulative impact upon glutathione may be far greater than could be predicted by the magnitude of any specific exposure [145], which supports an 'allostatic loading' model.

Also of note are studies showing that the effects of EMF/RFR can be reduced by supplementation with antioxidants and radical scavengers. As an example, Vitamins E and C reduced adverse impacts on rat endometrium from 900 MHz EMR exposure [137]. Gingko biloba has also prevented mobile phone-induced increases in malondialdehyde and nitric oxide levels in brain tissue as well as decreases in brain superoxide dismutase and glutathione peroxidase activities and increases in brain xanthine oxidase and adenosine deaminase activities, and treated rats were spared the histopathological cell injury found in the untreated rats [146]. Substantial further literature on antioxidants and radical scavengers is reviewed in Belyaev's contribution to the Bioinitiative 2012 Report [147].

3.1.1.2. Stress protein (heat shock protein) responses. Another well-documented effect of exposure to low-intensity extremely low frequency and RFR is the creation of stress proteins (heat shock proteins) indicating that a cell is being placed under physiological stress [148–154]. Heat shock proteins are in a family of inducible proteins that are initiated when any increased need for protection from stray electrons occurs [155,156]. The HSP response is generally associated with heat shock, exposure to toxic chemicals and heavy metals, and other environmental insults. HSP is a signal of cells in distress. Plants, animals and bacteria all produce stress proteins to survive environmental stressors like high temperatures, lack of oxygen, heavy metal poisoning, and oxidative stress. It should also be noted that the generation of HSP stress proteins can have constructive medical applications, such as protection from reperfusion of the heart following ischemic injury [157]. Another concomitant impact of cellular stress can be protein misfolding, which has been documented in association with exposure to EMF/RFR [158,159].

Although a number of papers have demonstrated increases in HSPs in people with ASCs [160–164], it has been investigated far less often than oxidative stress. Part of the research needed to study possible influences of EMF/RFR on ASCs would be more careful study of HSPs in ASCs.

3.1.2. Membranes and channels

3.1.2.1. Cell membranes and lipid peroxidation. Cell and organelle membranes play roles in partitioning cells from the extracellular milieu as well as in sustaining boundaries and regulating flow of materials between cellular compartments needing different metabolic parameters for their activities.

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They also play critical roles in maintaining electrical differences and the flow of electricity.

Adey (2002) summarized studies that report cell membranes as the site of initial field transductive coupling.

"*Collective evidence points to cell membrane receptors as the probable site of first tissue interactions with both ELF and microwave fields for many neurotransmitters* [165], *hormones* [166,167], *growth-regulating enzyme expression* [168–171], *and cancer-promoting chemicals* [172,173]. *In none of these studies does tissue heating appear involved causally in the responses.*" [174]

Membranes are well-known targets of oxidative stress. Membrane damage is a major route through which free radical damage proliferates through the cellular system. Lipid peroxidation of membranes most often affects polyunsaturated fatty acids such as EPA and DHA which are the most abundant and vulnerable lipids in the brain where the damage they sustain can have serious impacts – DHA is 40% of PUFAs (brain polyunsaturated fatty acids). Lipid peroxidation of membranes has been identified as an effect of EMF/RFR in multiple studies [175,176]. A variety of other mechanisms for membrane alteration related to EMF/RFR have been intimated in the literature. Physicochemical properties of membranes such as phase transition of phosphatidylcholine can be shifted by non-thermal effects of microwave radiation [177]. Membrane potential and currents may also be impacted by pulsed radiofrequency fields [178]. This has been observed graphically in altered cellular movement in Paramecium caudatum, with these cells becoming broader, with a broader-appearing cytopharynx, with their pulse vesicles having difficult in expelling their content outside the cell, and with less efficient movement of cilia [179] which the authors suggested might be due to targeting of the cellular membrane. The impacts on this unicellular organism may help us imagine what the impact of EMF/RFR might be on cells with some structural similarities, such as columnar epithelial cells and ciliated cells in mucosal surfaces in the respiratory system, digestive tract, uterus and fallopian tubes and central spinal cord.

Indications of lipid peroxidation of membranes has been documented in ASCs, including malonaldehyde and isoprostanes, as well as alteration of membrane phospholipids and prostaglandins [98,100,115,162,180–184]. In one study the isoprostane levels showed a biomodal distribution with the majority of ASC subjects showing moderate increase but a smaller group showing dramatic increases [183]. Thromboxane, reflecting platelet activation, was also elevated in one study [98]. Given that this phenomenon has been identified in many people with ASCs, it is plausible that such individuals will likely be more vulnerable to having such cellular injuries caused, worsened or both by EMF/RFR exposures.

3.1.2.2. Calcium channels. EMF/RFR exposures have been shown to alter or disturb calcium signaling [185] through a variety of mechanisms, including membrane leakage [186], alteration of calcium-binding proteins and GFAP reactivity [187,188], and altered ultrastructural distribution of calcium and calcium-activated ATPases after exposure [189]. Adey (2002) provided an overview of key studies on calcium efflux and the importance of calcium in cell signaling. "*Early studies described calcium efflux from brain tissue in response to ELF exposures* [190,191], *and to ELF-modulated RF fields* [190–193]. *Calcium efflux from isolated brain subcellular particles (synaptosomes) with dimensions under 1.0 μm also exhibit an ELF modulation frequency-dependence in calcium efflux, responding to 16 Hz sinusoidal modulation, but not to 50 Hz modulation, nor to an unmodulated RF carrier* [194]. *In the same and different cell culture lines, the growth regulating and stress responsive enzyme ornithine decarboxylase (ODC) responds to ELF fields* [170,195] *and to ELF-modulated RF fields.*" [168,170,171,196].

Dutta et al. (1992) reported:

"*Radio-frequency electromagnetic radiation (RFR) at 915 and 147 MHz, when sinusoidally amplitude modulated (AM) at 16 Hz, has been shown to enhance release of calcium ions from neuroblastoma cells in culture. The dose-response relation is unusual, consisting of two power-density "windows" in which enhanced efflux occurs, separated by power-density regions in which no effect is observed. Thus RFR affects both calcium-ion release and AChE activity in nervous system-derived cells in culture in a common dose-dependent manner.*" [197]

Alterations in calcium signaling impacts are of central importance in ASC pathophysiology, and have been documented to occur with some EMF/RFR exposures. Calcium channels play an important role in regulating neuronal excitability. Disturbance during development may be contributory to the development of ASCs, and is often associated with vulnerability to seizures. Gene alterations associated with a number of voltage-gated calcium channels have been identified in ASCs [198–202]. However, based on an examination of patient laboratory and phenotype data it has been argued that aberrant calcium signaling could be downstream: Palmieri and Persico (2010) suggest that "*an abnormal neuroimmune response as a relevant player in elevating intracellular Ca^{2+} levels, deranging neurodevelopment, driving oxidative stress, and ultimately affecting synaptic function and neural connectivity especially in long-range neuronal pathways physiologically responsible for integrated information processing*" [203]. Peng and Jou (2010) have in turn shown how increased intracellular calcium can cause oxidative stress, and a vicious circle: "*. . . mitochondrial ROS [reactive oxygen species]rise can modulate Ca^{2+} dynamics and augment Ca^{2+} surge. The reciprocal interactions between Ca^{2+} induced ROS increase and ROS modulated Ca^{2+} upsurge may cause a feedforward, self-amplified loop creating cellular damage far beyond direct Ca^{2+} induced damage*" [204].

Environmental as well as genetic routes to calcium signaling dysfunction have been identified [205] including chemicals such as the polyaromatic hydrocarbons.

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PCB-95 in particular modulates the calcium-dependent signaling pathway responsible for activity-dependent dendritic growth [206,207]. In fact, once a genetic mutation has been associated with altering a critical signaling pathway and conferring risk for autism, chemicals or other environmental agents can be identified that target the same pathways and also confer ASC risk. Stamou et al. (2012) have reviewed this strategy of identifying multiple mechanisms converging on common signaling pathways regarding Ca(2+)-dependent mechanisms as well as extracellular signal-regulated kinases (ERK)/phosphatidylinositol-3-kinases (PI3K) and neuroligin-neurexin-SHANK [208]. From this point of view, there may be no particular reason to privilege genetic mutations in their contribution to a disturbance of calcium signaling, since whether this function becomes derailed due to a genetic mutation, from a chemical toxin or from EMF/RFR perturbation of calcium signaling, the functional effect is comparable.

3.1.3. Junctions and barriers

The damage discussed so far has been at the molecular and subcellular level. However impacts from this level reverberate up to larger scales in the system. Where membranes create boundaries between cells and subcellular compartments, barriers do this at a larger scale. Cells become capable of forming barriers between each other through tight junctions which block substances and cells from 'slipping through the cracks,' so to speak, between the cells. Conversely, gap junctions are subcellular structures providing openings that allow physical passage of materials between cells otherwise separated by membranes.

Such connections between cells can also be altered by electromagnetic fields and radiofrequency exposures, at least under certain circumstances. High frequency magnetic fields have been observed to be associated with a sharp decrease in intercellular gap junction-like structures, in spite of increased gene expression for pertinent proteins [209]. Changes in tight junctions have been observed upon exposure to microwave and x-ray irradiation [210].

A number of papers in the ASC research field document problems pertinent to junctions. Connexin abnormalities have been documented in neuropathological studies [211] and MacFabe and colleagues identified lipid alterations associated with oxidative stress, membrane fluidity and the modulation of gap junction coupling [212]. Decrease in platelet endothelial cell adhesion molecule-1 were reduced and this reduction correlated with repetitive behavior and abnormal brain growth; adhesion molecules modulate permeability and signaling at the blood–brain barrier as well as leukocyte infiltration into the central nervous system [213].

EMF and RFR might also compromise biologically important barrier structures that separate blood flow from organs like the brain [214]. This raises important questions regarding whether other 'barriers' that keep blood flow separate from the gut (gut-blood barrier), or the placenta (blood–placenta barrier) or the eye (ocular-blood barrier) may also be rendered pathologically leaky, and allow albumin, toxins, pro-inflammatory cytokines and infectious agents to cross these barriers, which may invoke immune responses in the intestines, and may impact the developing fetus [215]. While there are a fair number of negative studies, there are also many studies showing and association between EMF/RFR and pathological leakage of the blood–brain barrier (BBB), as well as evidence in animal studies of damage to brain cells and damage to or death of neurons. Such leakage has been shown to be potentiated by physiological factors such as diabetes and insulin (Gulturk et al., 2010) and has also potentiated viral lethality in a dose-dependent fashion (Lange et al., 1991). Many of the positive findings were associated with non-thermal exposures comparable to normal cell phone radiation exposure [216–222]. There are scattered reports of increased permeability across other membranes and barriers, such as the blood–testicle barrier in mice (Wang, 2008; Wang et al., 2010 and the rat liver canalicular membrane [223]). A 1992 study by Kues et al. reported that "*studies in our laboratory have established that pulsed microwaves at 2.45 GHz and 10 mW/cm^2 are associated with production of corneal endothelial lesions and with disruption of the blood–aqueous barrier in the non-human primate eye*" [224]. A recent study showing impact of high-frequency electromagnetic fields on trophoblastic connexins [209] may indicate the vulnerability of the placenta and placental barrier function to electromagnetic fields. A thorough review and methodological discussion of literature regarding EMF/RFR impacts on the BBB is provided by Salford in Section 10 of the BioInitiative 2012 Report [214].

BBB integrity can be compromised by oxidative stress which can lead to increased permeability [225], and the resultant extravasation of albumin into brain parenchyma can be excitotoxic and neurotoxic [226,227]. The interaction of these factors may contribute to a feed-forward vicious cycle that can result in progressive synaptic and neuronal dysfunction as seen in various neurodegenerative diseases [228].

The evidence suggesting possible existence of barrier function compromise in people with ASCs is largely indirect. The existence of brain neuroinflammation in ASCs has been documented in a growing number of studies [160,229,230], and this is known to be associated with BBB permeability [231–233]. In a review of clinical MRI findings in ASCs 19/59 showed white matter signal abnormalities [234], which in other settings have been associated with cerebral hypoperfusion, though not necessarily in the same locations as the hyperintensities [235,236]. Blood flow abnormalities, predominantly hypoperfusion, documented in a few dozen PET and SPECT studies, could also be caused by and/or associated with physiological phenomena associated with vascular permeability as will be revisited below. Increased intestinal permeability has been documented (although its absence has also been documented) [237–243] and discussed in the context of food exposures, particularly gluten [244–250]. The reactivity to large numbers of different foods, clinically observed in many children with autism, has been framed by

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some as a manifestation of indiscriminate exposure of the immune system and the brain to food proteins on account of intestinal permeability as well as BBB permeability [251]. This reactivity could in turn feed in to aberrant immune responsivity which in turn could further amplify barrier vulnerability [248].

A number of studies have made an association between an increased risk of having a child with autism and maternal infection during pregnancy. This phenomenon looks like it is a result of the maternal immune system response rather than being due to an impact deriving from a specific infectious agent; but the potential for an accompanying compromise of the placental barrier is also conceivable in this setting. Under these circumstances the fetal risk of exposure to maternal blood toxins, cytokines and stress proteins in utero could potentially be increased if placenta barrier (BPB) function were impaired. The integrity, or compromise thereto, of the maternal-fetal interface via the placenta is an important modulator of brain development [252].

3.1.4. Genetic alterations and reproductive impacts

The overwhelming emphasis in recent decades in autism research has been on genetics, and on finding linkages between genes, brain and behavior, in part because of the high heritability of autism that was calculated from the concordance rates of monozygotic (identical) vs. dizygotic (fraternal) twins found in by a series of small twin studies performed some decades ago. In recent years the genetic premises of this seemingly obvious framing of autism as overwhelmingly genetic have been undermined at several levels [253]. First, the number of reported cases is increasing, making it more difficult to maintain that ASCs are purely genetic because these increases can only be partly explained away by greater awareness or other data artifacts [254,255]. Second, the complexity of the ways we understand how genes might relate to autism has grown, from an expectation a decade ago that a small number of genes (even less than a dozen) would explain everything to an identification of close to a thousand genes associated with autism with common threads linking only a small subset [256,257], as well as 'de novo' mutations present in ASC children but not their parents and even 'boutique' mutations not shared beyond an individual family. Moreover, a recent twin study that was much larger than any of the prior such studies identified a modest genetic role but a substantial environmental role [258]. Indeed even concordance between identical twins appears to be influenced by whether the twins shared a placenta [259]. All of this calls into question the idea that genetics can be presumed to be the 'cause' of autism simply based upon heritability calculations, and upgrades the importance of looking not only at the environment and environmentally vulnerable physiology, but also at acquired mutations.

3.1.4.1. Genotoxicity. Genotoxicity has been proposed as a mechanism for the generation of 'de novo' mutations (found in children but not their parents) being found in ASCs [260]. Reviews and published scientific papers on genotoxicity and EMF report that both ELF-EMF and RFR exposures are genotoxic – i.e., damaging to DNA – under certain conditions of exposure, including under conditions of intermittent and/or chronic ELF and RFR exposure that are of low-intensity and below current world safety standards [104,105,261–266]. Types of genetic damage reported have included DNA fragmentation and single- and double-strand DNA breaks, micronucleation and chromosome aberrations, all of which indicate genetic instability [102,103].

Researchers have recently identified large numbers of de novo mutations, more likely to be transmitted by fathers than by mothers to their children [267–269]. This is consistent with the EMF/RFR literature that repeatedly documents DNA damage to sperm from cell phone radiation (see Section 3.1.4.1.2). The Eichler team at the University of Washington found that 39% of the 126 most severe or disruptive mutations map to a network associated with chromatin remodeling that has already been ranked as significant amongst autism candidate genes [268]. Although the relationship between the prominence of chromatin-related gene mutations and the impacts of EMF/RFR on chromatin condensation has not been clarified, the parallels support further investigation.

3.1.4.1.1. Contributors to genotoxicity.

- Oxidative stress and free radical damage to DNA

  Oxidative stress and excessive free radical production are very well known to be potentially genotoxic. They can be a consequence of myriad environmental factors, including but by no means limited to EMF/RFR. The DNA damage that can result could very well be one cause of 'de novo' mutations which to date have been found in only a small percentage of individuals with ASCs. Although there is not a consensus at this time about the rates or causes of de novo mutations in ASCs, environmentally triggered oxidative stress and free radical damage that we know are present in large numbers of people with ASCs can be genotoxic, and this warrants a serious investigation of the potential contribution of EMF and RFR to de novo mutations in ASC. Further, the huge increases in exposure to EMF/RFR in daily life due to electrification and the global saturation of RFR from wireless technologies [81] reinforce this need.
- Challenge to DNA repair mechanisms

  When the rate of damage to DNA exceeds the rate at which DNA can be repaired, there is the possibility of retaining mutations and initiating pathology. Failure to trigger DNA damage repair mechanisms, or incomplete or failed repair, may be a consequence of a variety of commonplace stressors, including EMF/RFR exposure. A decrease in DNA repair efficiency has been reported to result from exposure to low-intensity RFR in human stem cells, and other cells. Mobile phone frequency GSM exposure at the frequency of 915 MHz consistently inhibited DNA repair foci in lymphocytes [270–272]. Belyaev, Markova and colleagues (2005), and Markova et al. (2009)

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reported that very low-intensity microwave radiation from mobile phones inhibits DNA repair processes in human stem cells. A significant reduction in 53BP1 (tumor suppressor p53 binding protein 1) foci was found in cells exposed to microwave radiofrequency radiation within one hour of exposure. Fibroblast cells were impacted in this fashion but adapted over time, whereas stem cells were similarly affected (inhibited 53BP1 foci) but did not adapt to microwave radiation during chronic exposure [270,271]. Additional challenges to DNA repair mechanisms include not only toxicants and other damaging inputs but also nutritional insufficiencies of substances important to the proper functioning of DNA repair mechanisms, including Vitamin D, essential fatty acids, and minerals such as selenium and molybdenum [273]. The high possibility that various such contributors may combine supports an 'allostatic load' model of environmental injury and genotoxicity.

- Chromatin condensation

The work of Markova, Belyaev and others has repeatedly shown that RFR exposure can cause chromatin condensation, which is a hallmark of DNA damage. Belyaev (1997) reported that super-low intensity RFR resulted in changes in genes, and chromatin condensation of DNA at intensities comparable to exposures from cell towers (typically at RFR levels of 0.1 to one microwatt per centimeter squared ($\mu W/cm^2$)) [274]. Significant microwave (MW)-induced changes in chromatin conformation were observed when rat thymocytes were analyzed between 30–60 min after exposure to MW [275].

In recent studies, human lymphocytes from peripheral blood of healthy and hypersensitive to EMF persons were exposed to non-thermal microwave radiation (NT MW) from the GSM mobile phones [270,271]. NT MW induced changes in chromatin conformation similar to those induced by heat shock, which remained up to 24 h after exposure. The same group has reported that contrary to human fibroblast cells, which were able to adapt during chronic exposure to GSM/UMTS low intensity RFR exposure, human stem cells did not adapt [272].

3.1.4.1.2. Gonadal and germline impacts. De novo mutations have been shown to be more of a problem related to paternal age [268,276–279], and this may be related to the impact of environmental factors such as EMF/RFR on the stem cell genome, particularly in sperm which have no DNA repair capacity. Vulnerability of testes and ova, and of sperm and egg cells, relates to the tissue milieu in which damage to the germline can take place, as well as on the greater vulnerability of stem cells. Several international laboratories have replicated studies showing adverse effects on sperm quality, motility and pathology in men who use and particularly those who wear a cell phone, PDA or pager on their belt or in a pocket [106,280–284]. Other studies conclude that usage of cell phones, exposure to cell phone radiation, or storage of a mobile phone close to the testes of human males affect sperm counts, motility, viability and structure [175,284,285]. Animal studies have demonstrated oxidative and DNA damage, pathological changes in the testes of animals, decreased sperm mobility and viability, and other measures of deleterious damage to the male germ line [134,286–290]. Of note, altered fatty acids consistent with oxidative stress have been found in sperm cells in male infertility [291,292].

There are fewer animal studies that have studied effects of cell phone radiation on female fertility parameters. Panagopoulous et al. (2012) report decreased ovarian development and size of ovaries, and premature cell death of ovarian follicles and nurse cells in *Drosophila melanogaster* [293]. Gul et al. (2009) report rats exposed to stand-by level RFR (phones on but not transmitting calls) caused decrease in the number of ovarian follicles in pups born to these exposed dams [294]. Magras and Xenos (1997) reported irreversible infertility in mice after five (5) generations of exposure to RFR at cell phone tower exposure levels of less than 1.0 $\mu W/cm^2$ [295].

3.1.4.1.3. Implications of genotoxicity. The issue of genotoxicity puts the contribution of genetic variation into a different light – as something that needs to be accounted for, not necessarily assumed as the starting point. In this regard it has been speculated that the apparent higher rates of autism in Silicon Valley, discussed in the past as related to 'geek genes'[296], might be conditioned by higher levels of exposure to EMF/RFR. The relationship between the greater vulnerability of male sperm than of female eggs to adverse effects of EMF/RFR exposure and the marked (4:1) predominance of paternal origin of de novo point mutations (4:1 bias), also deserves further careful attention [268].

3.1.5. Implications of damage

We have reviewed parallels between ASC and EMF/RFR in molecular, cellular and tissue damage, including cellular stress (oxidative stress, the heat shock response and protein misfolding), injury of membranes, aberrant calcium signaling, and compromise of cell junctions and barriers. The genotoxicity of EMF/RFR was reviewed in relation to issues of environmental contributions to autism and of the phenomenon of de novo mutations. The compromise of the tissue substrate appears to have many commonalities in ASCs and in EMF/RFR exposures. Also notable was the possibility of attenuating some of the damage through increasing antioxidant status.

Regarding Rett syndrome, a genetic syndrome often associated with autistic behaviors, these commonalities come to mind in considering the implications of a recent study documenting arrest of symptomatology in a mouse model of Rett syndrome through a bone marrow transplant of wild-type microglia [297,298]. The introduction of these competent microglia cells did not directly target the neuronal defect associated with the MECP2 gene mutation; instead the benefits of the transplant were due to overcoming the inhibition of phagocytosis caused by the MECP2 mutation that was absent in the wild-type microglia. Phagocytosis involves removing debris. This suggests that while research has focused on how

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specific molecular defects, particularly in the synapse, may contribute to Rett pathophysiology, there may also be an important contribution from cellular debris, misfolded proteins and other disordered cellular structure and function. Such disorder could be accumulating in cells under the conditions of pathophysiological disarray reviewed above. Based on this study as well as on the levels of damage just reviewed, cellular problems that are pertinent to ASCs most likely go beyond any specific defect introduced by a mutation. Additionally it is conceivable that many of the mutations may be not part of normal background variation but instead collateral damage from the same environmental factors that are also driving the damage to the physiology.

3.1.6. Summary of Part I and preview of Part II

The data reviewed above in Part I of this two part paper documents a series of parallels between the pathophysiological and genotoxic impacts of EMF/RFR and the pathophysiological underpinnings of ASCs. DNA damage, immune and blood–brain barrier disruption, cellular and oxidative stress, calcium channel, disturbed circadian rhythms, hormone dysregulation, and degraded cognition, sleep, autonomic regulation and brainwave activity all have commonalities between ASCs and EMF/RFR, and the disruption of disruption fertility and reproduction associated with EMF/RFR may also be related to the increasing incidence of ASCs. All of this argues for reduction of exposures now, and better coordinated research in these areas.

These pathophysiological parallels are laid out after identifying the dynamic features of ASCs that could plausibly arise out of such pathophysiological dysregulation. The importance of transduction between levels was also discussed in Part I, and will be elucidated in much more detail in Part II where more detail will be given about the possible interfaces between the cellular and molecular pathophysiology reviewed above and the higher-level disruption of physiological systems, brain tissue and nervous system electrophysiology.

The emergence of ever larger amounts of data is transforming our understanding of ASCs from static encephalopathies based on genetically caused brain damage to dynamic encephalopathies where challenging behaviors emanate from physiologically disrupted systems. In parallel, the emergence of ever larger bodies of evidence supporting a large array of non-thermal but profound pathophysiological impacts of EMF/RFR is transforming our understanding of the nature of EMF/RFR impacts on the organism.

At present our policies toward ASCs are based on outdated assumptions about autism being a genetic, behavioral condition, whereas our medical, educational and public health policies related to treatment and prevention could be much more effective if we took whole-body, gene-environment considerations into account, because there are many lifestyle and environmental modifications that could reduce morbidity and probably incidence of ASCs as well.

At present our EMF/RFR standards are based on outdated purely thermal considerations, whereas the evidence is now overwhelming that limiting regulations in this way does not address the much broader array of risks and harm now known to be created by EMF/RFR.

In particular, the now well-documented genotoxic impacts of EMF/RFR, placed in parallel with the huge rise in reported cases of ASCs as well as with the de novo mutations associated with some cases of ASCs (as well as other conditions), make it urgent for us to place the issue of acquired as well as inherited genetic damage on the front burner for scientific investigation and policy remediation.

With the rising numbers people with ASCs and other childhood health and developmental disorders, and with the challenges to our prior assumptions posed ever more strongly by emerging evidence, we need to look for and act upon risk factors that are largely avoidable or preventable. We would argue that the evidence is sufficient to warrant new public exposure standards benchmarked to low-intensity (non-thermal) exposure levels causing biological disruption and strong, interim precautionary practices are advocated. Further evidence to support the pathophysiological support for parallels between ASCs and EMF/RFR impacts and for taking action will be offered in Part II.

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Autism and EMF? Plausibility of a pathophysiological link part II

Martha R. Herbert [a,*], Cindy Sage [b]

[a] *Massachussetts General Hospital Harvard Medical School Boston, TRANSCEND Research Program Neurology, Boston, MA, USA*
[b] *Sage Associates, Santa Barbara, CA, USA*

Abstract

Autism spectrum conditions (ASCs) are defined behaviorally, but they also involve multileveled disturbances of underlying biology that find striking parallels in the physiological impacts of electromagnetic frequency and radiofrequency radiation exposures (EMF/RFR). Part I (Vol 776) of this paper reviewed the critical contributions pathophysiology may make to the etiology, pathogenesis and ongoing generation of behaviors currently defined as being core features of ASCs. We reviewed pathophysiological damage to core cellular processes that are associated both with ASCs and with biological effects of EMF/RFR exposures that contribute to chronically disrupted homeostasis. Many studies of people with ASCs have identified oxidative stress and evidence of free radical damage, cellular stress proteins, and deficiencies of antioxidants such as glutathione. Elevated intracellular calcium in ASCs may be due to genetics or may be downstream of inflammation or environmental exposures. Cell membrane lipids may be peroxidized, mitochondria may be dysfunctional, and various kinds of immune system disturbances are common. Brain oxidative stress and inflammation as well as measures consistent with blood–brain barrier and brain perfusion compromise have been documented. Part II of this paper documents how behaviors in ASCs may emerge from alterations of electrophysiological oscillatory synchronization, how EMF/RFR could contribute to these by de-tuning the organism, and policy implications of these vulnerabilities. It details evidence for mitochondrial dysfunction, immune system dysregulation, neuroinflammation and brain blood flow alterations, altered electrophysiology, disruption of electromagnetic signaling, synchrony, and sensory processing, de-tuning of the brain and organism, with autistic behaviors as emergent properties emanating from this pathophysiology. Changes in brain and autonomic nervous system electrophysiological function and sensory processing predominate, seizures are common, and sleep disruption is close to universal. All of these phenomena also occur with EMF/RFR exposure that can add to system overload ('allostatic load') in ASCs by increasing risk, and can worsen challenging biological problems and symptoms; conversely, reducing exposure might ameliorate symptoms of ASCs by reducing obstruction of physiological repair. Various vital but vulnerable mechanisms such as calcium channels may be disrupted by environmental agents, various genes associated with autism or the interaction of both. With dramatic increases in reported ASCs that are coincident in time with the deployment of wireless technologies, we need aggressive investigation of potential ASC—EMF/RFR links. The evidence is sufficient to warrant new public exposure standards benchmarked to low-intensity (non-thermal) exposure levels now known to be biologically disruptive, and strong, interim precautionary practices are advocated.
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Keywords: Autism; EMF/RFR; Cellular stress; Oxidative stress; Mitochondrial dysfunction; Oscillatory synchronization; Environment; Radiofrequency; Wireless; Children; Fetus; Microwave

1. Recap of part I and summary of part II

Part I of this two-part article previously documented a series of parallels between the pathophysiological and genotoxic impacts of EMF/RFR and the pathophysiological, genetic and environmental underpinnings of ASCs. DNA damage, immune and blood–brain barrier disruption, cellular and oxidative stress, calcium channel dysfunction, disturbed circadian rhythms, hormone dysregulation, and degraded cognition, sleep, autonomic regulation and brainwave activity—all are associated with both ASCs and EMF/RFR; and the disruption of fertility and reproduction associated with EMF/RFR may also be related to the increasing incidence of ASCs. All of this argues for reduction of exposures now, and better coordinated research in these areas. These

* Corresponding author.
E-mail address: drmarthaherbert@gmail.com (M.R. Herbert).

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pathophysiological parallels are laid out after identifying the dynamic features of ASCs that could plausibly arise out of such pathophysiological dysregulation. The importance of transduction between levels was also discussed in Part I.

Part II elucidates in much more detail the possible interfaces between the cellular and molecular pathophysiology reviewed above and the higher-level disruption of physiological systems, brain tissue and nervous system electrophysiology. It addresses mitochondrial dysfunction, immune system disregulation, neuroinflammation and brain blood flow alterations, altered electrophysiology, disruption of electromagnetic signaling, synchrony, and sensory processing, de-tuning of the brain and organism, and behavior as an emergent property. The emergence of ever larger amounts of data is transforming our understanding of ASCs from static encephalopathies based on genetically caused brain damage to dynamic encephalopathies where challenging behaviors emanate from physiologically disrupted systems. In parallel, the emergence of ever larger bodies of evidence supporting a large array of non-thermal but profound pathophysiological impacts of EMF/RFR is transforming our understanding of the nature of EMF/RFR impacts on the organism. At present our policies toward ASCs are based on outdated assumptions about autism being a genetic, behavioral condition, whereas our medical, educational and public health policies related to treatment and prevention could be much more effective if we took whole-body, gene-environment considerations into account, because there are many lifestyle and environmental modifications that could reduce morbidity and probably incidence of ASCs as well. Our EMF/RFR standards are also based on an outdated assumption that it is only heating (thermal injury) which can do harm. These thermal safety limits do not address low-intensity (non-thermal) effects. The evidence is now overwhelming that limiting exposures to those causing thermal injury alone does not address the much broader array of risks and harm now clearly evident with chronic exposure to low-intensity (non-thermal) EMF/RFR. In particular, the now well-documented genotoxic impacts of EMF/RFR, placed in parallel with the huge rise in reported cases of ASCs as well as with the de novo mutations associated with some cases of ASCs (as well as other conditions), make it urgent for us to place the issue of acquired as well as inherited genetic damage on the front burner for scientific investigation and policy remediation. With the rising numbers people with ASCs and other childhood health and developmental disorders, and with the challenges to our prior assumptions posed ever more strongly by emerging evidence, we need to look for and act upon risk factors that are largely avoidable or preventable. We argue that the evidence is sufficient to warrant new public exposure standards benchmarked to low-intensity (non-thermal) exposure levels causing biological disruption and strong, interim precautionary practices are advocated. The combined evidence in Parts I and II of this article provide substantial pathophysiological support for parallels between ASCs and EMF/RFR health impacts.

2. Parallels in pathophysiology

2.1. Degradation of the integrity of functional systems

EMF/RFR exposures can yield both psychological and physiological stress leading to chronically interrupted homeostasis. In the setting of molecular, cellular and tissue damage, one would predict that the organization and efficiency of a variety of organelles, organs and functional systems would also be degraded. In this section we will review disturbances from EMF/RFR in systems (including include oxidative and bioenergetics metabolism, immune function and electrophysiological oscillations) that include molecular and cellular components subject to the kinds of damage discussed in the previous section. We will review disturbances that have been associated with EMF/RFR, and consider the parallel disturbances that have been documented in ASCs.

2.1.1. Mitochondrial dysfunction

Mitochondria are broadly vulnerable, in part because the integrity of their membranes is vital to their optimal functioning—including channels and electrical gradients, and their membranes can be damaged by free radicals which can be generated in myriad ways. Moreover, just about every step in their metabolic pathways can be targeted by environmental agents, including toxicants and drugs, as well as mutations [1]. This supports a cumulative 'allostatic load' model for conditions in which mitochondrial dysfunction is an issue, which includes ASCs as well as myriad other chronic conditions.

Mitochondria are commonly discussed in terms of the biochemical pathways and cascades of events by which they metabolize glucose and generate energy. But in parallel with this level of function there also appears to be a dimension of electromagnetic radiation that is part of the activity of these organelles. For example, electromagnetic radiation can be propagated through the mitochondrial reticulum, which along with the mitochondria has a higher refractive index than the surrounding cell and can serve to propagate electromagnetic radiation within the network [2]. It is also the case that "*The physiological domain is characterized by small-amplitude oscillations in mitochondrial membrane potential (delta psi(m)) showing correlated behavior over a wide range of frequencies. . .. Under metabolic stress, when the balance between ROS [reactive oxygen species, or free radicals] generation and ROS scavenging [as by antioxidants] is perturbed, the mitochondrial network throughout the cell locks to one main low-frequency, high-amplitude oscillatory mode. This behavior has major pathological implications because the energy dissipation and cellular redox changes that occur during delta psi(m) depolarization result in suppression of electrical excitability and Ca2 + handling. . .*" [3].

These electromagnetic aspects of mitochondrial physiology and pathophysiology could very well be impacted by EMF/RFR.

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Other types of mitochondrial damage have been documented in at least some of the studies that have examined the impacts of EMF/RFR upon mitochondria. These include reduced or absent mitochondrial cristae [4–6], mitochondrial DNA damage [7], swelling and crystallization [5], alterations and decreases in various lipids suggesting an increase in their use in cellular energetics [8], damage to mitochondrial DNA [7], and altered mobility and lipid peroxidation after exposures [9]. Also noted has been enhancement of brain mitochondrial function in Alzheimer's transgenic mice and normal mice [10]. The existent of positive as well as negative effects gives an indication of the high context dependence of exposure impacts, including physical factors such as frequency, duration, and tissue characteristics [11].

By now there is a large amount of evidence for biochemical and other abnormalities in a large portion of children with autism that are consistent with mitochondrial dysfunction [12–14]. Recently published postmortem brain tissue studies that have added a new dimension of evidence for mitochondrial abnormalities in ASCs will be reviewed in the section on alteration of brain cells below.

Secondary mitochondrial dysfunction (i.e. environmentally triggered rather than rooted directly in genetic mutations) [15–18] could result among other things from the already discussed potential for EMF/RFR to damage channels, membranes and mitochondria themselves as well as from toxicant exposures and immune challenges. In a meta-analysis of studies of children with ASC and mitochondrial disorder, the spectrum of severity varied, and 79% of the cases were identified by laboratory findings without associated genetic abnormalities [16].

2.1.2. Melatonin dysregulation

2.1.2.1. Melatonin, mitochondria, glutathione, oxidative stress. Melatonin is well-known for its role in regulation of circadian rhythms, but it also plays important metabolic and regulatory roles in relation to cellular protection, mitochondrial malfunction and glutathione synthesis [19–21]. It also helps prevent the breakdown of the mitochondrial membrane potential, decrease electron leakage, and thereby reduce the formation of superoxide anions [22]. Pharmacological doses of melatonin not only scavenge reactive oxygen and nitrogen species, but enhance levels of glutathione and the expression and activities of some glutathione-related enzymes [21,23].

2.1.2.2. Melatonin can attenuate or prevent some EMF/RFR effects. Melatonin may have a protective effect in the setting of some EMF/RFR exposures, apparently in relation to these functions just described. EMF/RFR can impact melatonin; one example is exposure to 900 MHz microwave radiation promoted oxidation, which reduced levels of melatonin and increased creatine kinase and caspase-3 in exposed as compared to sham exposed rats [24].

Melatonin can attenuate or prevent oxidative damage from EMF/RFR exposure. In an experiment exposing rats to microwave radiation (MW) from a GSM-900 mobile phone with and without melatonin treatment to study renal impacts [25], the untreated exposed rats showed increases of lipid peroxidation markers as reduction of the activities of superoxide dismutase, catalase and glutathione peroxidase indicating decrement in antioxidant status. However these negative effects were inhibited in the exposed rats treated with melatonin. Melatonin also inhibited the emergence of preneoplastic liver lesions in rats exposed to EMFs [26]. The development of DNA strand breaks was observed in RFR exposed rats; this DNA damage was blocked by melatonin [27]. Exposure of cultured cortical neurons to EMF led to an increase in 8-hydroxyguanine in neuronal mitochondria, a common biomarker of DNA oxidative damage, along with a reduction in the copy number of mitochondrial DNA and the levels of mitochondrial RNA transcripts; but these effects could all be prevented by pretreatment with melatonin [7]. In a study of skin lesion induced by exposure to cell phone radiation, the skin changes in the irradiated group (which included thicker stratum corneum, epidermal atrophy, papillamatosis, basil cell proliferation, increased epidermal granular cell layer and capillary proliferation, impaired collagen tissue distribution and separation of collagen bundles in dermis) were prevented (except for hypergranulosis) by melatonin treatment [28]. Melatonin as well as caffeic acid phenyethyl ester (an antioxidant) both protected against retinal oxidative stress in rates exposed long-term to mobile phone irradiation [29]. Nitric oxide (NO) was increased in nasal and sinus mucosa in rats after EMF exposure, with this NO possibly acting as a defense mechanism suggesting tissue damage; but this was prevented by pretreatment with melatonin [30]. Melatonin treatment significantly prevented the increase in the MDA (malondyaldehyde, a marker of lipid peroxidation) content and XO (xanthine oxidase) activity in rat brain tissue after 40 days of exposure, but it was unable to prevent the decrease of CAT activity and increase of carbonyl group contents [31].

Of note, the melatonin production of infants in isolettes in neonatal intensive care units appears to be impacted by the high ELF-EMF environment, in that when infants were removed from those exposures they showed an increase in melatonin levels [32]. There is an increased prevalence of ASCs in children who were born prematurely [33–43]. There are many potential prematurity-associated factors that could contribute to increased risk for ASCs, but proper melatonin regulation warrants EMF/RFR controls in the newborns' environment.

2.1.2.3. Melatonin and autism. Regarding melatonin status in people with ASCs, a recent meta-analysis summarized the current findings as indicating that "*(1) Physiological levels of melatonin and/or melatonin derivatives are commonly below average in ASC and correlate with autistic behavior, (2) Abnormalities in melatonin-related genes may be a cause of low melatonin levels in ASD, and (3) . . . treatment with melatonin significantly improves sleep duration and sleep onset latency in ASD.*" [44].

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The meta-analysis also showed that polymorphisms in melatonin-related genes in ASC could contribute to lower melatonin concentrations or an altered response to melatonin, but only in a small percentage of individuals, since pertinent genes were found in only a small minority of those screened.

Based on the common presence of both sleep disorders and low melatonin levels, Bourgeron [45] proposed that synaptic and clock genes are important in ASCs, and that future studies should investigate the circadian modulation of synaptic function [45]. A number of melatonin-related genetic variants have been identified as associated with ASCs. Polymorphisms and deletions in the ASMT gene, which encodes the last enzyme of melatonin synthesis, have been found [46–48], and variations have been found as well for melatonin receptor genes [46,47,49]. CYP1A2 polymorphisms have been found in slow melatonin metabolisers, in whom melatonin levels are aberrant and initial response to melatonin for sleep disappeared in a few weeks [50].

2.1.2.4. Autism AND melatonin AND glutathione. Whereas PubMed searches for "autism AND melatonin" and "autism AND glutathione" each coincidentally yielded 72 citations, and "melatonin AND glutathione" yielded 803 citations, the search for "autism AND melatonin AND glutathione" yielded zero citations. This is interesting given the strong connection of melatonin and glutathione metabolically, as discussed above, alongside of the strongly established interest in both glutathione and melatonin in ASC research and increasingly in clinical practice. Hopefully one contribution of an investigation of EMF/RFR links to ASCs will be to help bring attention to this relationship, which may help identify potential environmental and physiological causes for low melatonin in those without pertinent mutations. Of pertinence, tryptophan hydroxylase (TPH2) – the rate limiting enzyme in the synthesis of serotonin, from which melatonin is derived – is extremely vulnerable to oxidation, and tends to misfold when its cysteine residues are oxidized, with the enzyme being converted to a redox-cycling quinoprotein [51–54].

2.1.3. Disturbed immune function

There is by now a broad appreciation of the presence of immune disturbances in ASCs, to the point where there is an emerging discussion of ASCs as neuroimmune disorders [55,56]. Research identifying immune features in ASCs spans from genetics where risk genes have been identified to epigenetics where altered expression of immune genes is being reported as prominent in ASC epigenetics [57–59], and also includes prenatal infectious and immune disturbances as risk factors for autism as well as other neurodevelopmental and neuropsychiatric diseases as well as other conditions such as asthma [60–62]. Immune disturbances in infants and children with ASC are heterogeneous, with some but not all manifesting autoimmunity [63,64]. Anecdotally, recurrent infection is common while on the other hand some get sick less often than their peers. It is common for people with autism to have family members with immune or autoimmune diseases [65]. The immune system is turning out to have an important role in brain development [66–68]. As mentioned, glial activation associated with brain immune response has been identified in a growing number of studies. Whether or not EMF/RFR contributes to these features of ASCs causally, based on the evidence below regarding immune impacts of EMF/RFR exposure [69], it is certainly plausible that such exposures could serve as aggravating factors.

2.1.3.1. Low-intensity exposures. The body's immune defense system is now known to respond to very low-intensity exposures [70]. Chronic exposure to factors that increase allergic and inflammatory responses on a continuing basis is likely to be harmful to health, since the resultant chronic inflammatory responses can lead to cellular, tissue and organ damage over time. Many chronic diseases are related to chronic immune system dysfunction. Disturbance of the immune system by very low-intensity electromagnetic field exposure is discussed as a potential underlying cause for cellular damage and impaired healing (tissue repair), which could lead to disease and physiological impairment [71,72]. Both human and animal studies report that exposures to EMF and RFR at environmental levels associated with new technologies can be associated with large immunohistological changes in mast cells as well as other measures of immune dysfunction and dysregulation. Mast cells not only can degranulate and release irritating chemicals leading to allergic symptoms; they are also widely distributed in the body, including in the brain and the heart, which might relate to some of the symptoms commonly reported in relation to EMF/RFR exposure (such as headache, painful light sensitivity, and cardiac rhythm and palpitation problems).

2.1.3.2. Consequences of immune challenges during pregnancy. As mentioned, infection in pregnancy can also increase the risk of autism and other neurodevelopmental and neuropsychiatric disorders via maternal immune activation (MIA). Viral, bacterial and parasitic infections during pregnancy are thought to contribute to at least 30% of cases of schizophrenia [73]. The connection of maternal infection to autism is supported epidemiologically, including in a Kaiser study where risk was associated with psoriasis and with asthma and allergy in the second trimester [65], and in a large study of autism cases in the Danish Medical registry [74] with infection at any point in pregnancy yielding an adjusted hazard ration of 1.14 (CI: 0.96 – 1.34) and when infection occurred during second trimester the odds ratio was 2.98 (CI: 1.29 – 7.15). In animal models, while there is much variation in study design, mediators of the immune impact include oxidative stress, interleukin-6 and increased placental cytokines [61,68,75]. Garbett et al. [76] commented on several mouse models of the effects of MIA on the fetal brain that "The overall gene expression changes suggest that the response to MIA is a neuroprotective attempt by the

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developing brain to counteract environmental stress, but at a cost of disrupting typical neuronal differentiation and axonal growth." [76]. Maternal fetal brain-reactive autoantibodies have also been identified in some cases [62,77–82].

Although we have evidence of immune impacts of EMF/RFR, the impact of repeated or chronic exposure to EMF and RFR during pregnancy is poorly studied; could this trigger similar immune responses (cytokine production) and stress protein responses, which in turn would have effects on the fetus? Although this has been poorly studied, we do have data that very low cell phone radiation exposures during both human and mouse pregnancies have resulted in altered fetal brain development leading to memory, learning, and attention problems and behavioral problems [83].

2.1.3.3. Potential immune contributions to reactivity and variability in ASCs. Immune changes in ASCs appear to be associated with behavioral change [84–88], but the mechanisms are complex and to date poorly understood [89] and likely will need to be elucidated through systems biology methods that capture multisystem influences on the interactions across behavior, brain and immune regulation [90], including electrophysiology.

Two of the particularly difficult parts of ASCs are the intense reactivity and the variability in assorted symptoms such as tantrums and other difficult behaviors. Children with ASCs who also have gastrointestinal symptoms and marked fluctuation of behavioral symptoms have been shown to exhibit distinct innate immune abnormalities and transcriptional profiles of peripheral blood monocytes [91]. It is worth considering EMF/RFR exposures could be operating through related mechanisms so as to add to 'allostatic loading' in ways that exacerbate behavior. In Johansson 2006 and 2007 a foundation is provided for understanding how chronic EMF/RFR exposure can compromise immune function and sensitize a person to even small exposures in the future [72,92]. Johansson discusses alterations of immune function at environmental levels resulting in loss of memory and concentration, skin redness and inflammation, eczema, headache, and fatigue. Mast cells that degranulate under EMF and RFR exposures and substances secreted by them (histamine, heparin and serotonin) may contribute to features of this sensitivity to electromagnetic fields [92]. Theoharides and colleagues have argued that environmental and stress related triggers might activate mast cells, causing inflammatory compromise and leading to gut–blood–brain barrier compromise, seizures and other ASC ASC symptoms [93,94], and that this cascade of immune response and its consequences might also be triggered in the absence of infection by mitochondrial fragments that can be released from cells in response to stimulation by IgE/anti-IgE or by the proinflammatory peptide substance P [95].

Seitz et al. [96] reviewed an extensive literature on electromagnetic hypersensitivity conditions reported to include sleep quality, dizziness, headache, skin rashes, memory and concentration impairments related to EMF and RFR [96]. Some of these symptoms are common in ASCs, whether or not they are due to EMF/RFR exposure, and the experience of discomfort may be hard to document due to difficulties with self-reporting in many people with ASCs.

Johansson [72] also reports that benchmark indicators of immune system allergic and inflammatory reactions occur under exposure conditions of low-intensity non-ionizing radiation (immune cell alterations, mast cell degranulation histamine-positive mast cells in biopsies and immunoreactive dendritic immune cells) [71,72]. In facial skin samples of electro-hypersensitive persons, the most common finding is a profound increase in mast cells as monitored by various mast cell markers, such as histamine, chymase and tryptase [97]. In ASCs, infant and childhood rashes, eczema and psoriasis are common, and they are common in family members as well [98].

2.1.4. Alteration of and damage to cells in the brain

Brain cells have a variety of ways of reacting to environmental stressors, such as shape changes, metabolic alterations, upregulation or downregulation of neurotransmitters and receptors, other altered functionality, structural damage, production of un-metabolizable misfolded proteins and other cellular debris, and apoptosis; these range along a spectrum from adaptation to damage and cell death. These types of alterations can be looked at in animals under controlled conditions, but in human beings direct cellular examination can only be done on surgical biopsy tissue – which is hardly ever available in people with ASCs – or after death, at which point there has been a whole lifetime of exposures that are generally impossible to tease apart if there were even motivation to do so. This complicates the comparison of brain cell and tissue-related pathophysiology between what is seen in ASCs and what is associated with EMF/RFR exposures.

2.1.4.1. Brain cells. Impact of EMF/RFR on cells in the brain has been documented by some of the studies that have examined brain tissue after exposure, although the interpretation of inconsistencies across studies is complicated by sometimes major differences in impact attributable to differences in frequencies and duration of exposure, as well as to differences in resonance properties of tissues and other poorly understood constraints on cellular response. These studies and methodological considerations have been reviewed in depth in several sections of the 2012 BioInitiative Report [11,99]. A few examples of observations after exposure have included dark neurons (an indicator of neuronal damage), as well as alteration of neuronal firing rate [100], and upregulation of genes related to cell death pathways in both neurons and astrocytes [101]. Astrocytic changes included increased GFAP and increased glial reactivity [102–105], as well as astrocyte-pertinent protein expression changes detected by Fragopoulou et al. [322] as mentioned above. Also observed has been a marked protein downregulation of the nerve growth factor glial maturation factor beta (GMF) which is

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considered as an intracellular signal transduction regulator in astrocytes, which could have significant impact on neuronal-glial interactions as well as brain cell differentiation and tumor development. Diminution of Purkinje cell number and density has also been observed, [106] including in two studies of the impacts of perinatal exposure [107,108]. Promotion of pro-inflammatory responses in EMF-stimulated microglial cells has also been documented [109].

Neuropathology findings in ASCs have been varied and have been interpreted according to various frameworks ranging from a regionalized approach oriented to identifying potential brain relationships to ASC's behavioral features [110] to identifying receptor, neurotransmitter and interneuron abnormalities that could account for an increased excitation/inhibition ratio [111–115]. Studies have documented a range of abnormalities in neurons, including altered cellular packing in the limbic system, reduced dendritic arborization, and reductions in limbic GABAergic systems [116]. Over the past decade a shift has occurred from presuming that all pertinent brain changes occurred prior to birth, to an acknowledgement that ongoing cellular processes appear to be occurring not only after birth but well into adulthood [117]. One of the reasons for this shift was the observation that head size (as well as brain weight and size) was on average larger in children with autism, and the head sizes of children who became diagnosed with autism increased in percentile after birth [118].

2.1.4.2. Neuroinflammation, glial activation and excitotoxicity. Although much attention has been paid in ASC brain literature to specific regions manifesting differences in size and activity in comparison to those without ASCs, there are other observations that are not strictly regional in nature, such as more widely distributed scaling differences (e.g. larger brains, wider brains, increased white matter volume, along with altered functional connectivity and coherence to be discussed below). Recently more studies have appeared identifying pathophysiological abnormalities such as neuroinflammation, mitochondrial dysfunction and glutathione depletion in brain tissue. Neuroinflammation was first identified in a study of postmortem samples from eleven individuals aged 5–44 who had died carrying an ASC diagnosis, in which activated astrocytes and microglial cells as well as abnormal cytokines and chemokines were found. Other research has identified further astrocyte abnormalities such as altered expression of astrocyte markers GFAP abnormalities, with elevation, antibodies, and altered signaling having been documented [119–121]. Increased microglia activation and density as well as increased myeloid dendritic cell frequencies have also been documented [87,122,123], as has abnormal microglial-neuronal interactions [124]. Recently, through use of the PET ligand PK11105, microglial activation was found to be significantly higher in multiple brain regions in young adults with ASCs [125]. Genes associated with glial activation have been documented as upregulated. Garbett et al measured increased transcript levels of many immune genes, as well as changes in transcripts related to cell communication, differentiation, cell cycle regulation and chaperone systems [126]. Voineaugu and colleagues performed transcriptomic analysis of autistic brain and found a neuronal module of co-expressed genes which was enriched with genetically associated variants; an immune-glial module which showed no such enrichment for autism GWAS signals was interpreted as secondary [127], but this seems to involve circular thinking, since it implies that the primary cause must be genetic, which is an assumption deriving from a dominant model, but is not a proven fact.

Neuroinflammation also does not appear to be strictly localized in a function-specific fashion, and it may contribute both to more broadly distributed and more focal features for tissue-based reasons. It may be that brain regions with particular prominence in ASCs may have distinctive cellular characteristics—e.g. the amygdala [128–138], which may have a larger or more reactive population of astrocytes [139] or the basal ganglia which may have greater sensitivity to even subtle hypoxia or perfusion abnormalities. In this case it may be the histology of these areas that makes them vulnerable to environmental irritants, and this may contribute to how environmental factors such as EMF/RFR might trigger or aggravate some of ASC's features. More widely distributed brain tissue pathology be part of what leads to differences in ASCs in brain connectivity. However these types of tissue-function relationships have been poorly investigated. Belyaev has intensively reviewed physical considerations including the contribution of tissue differences to variability in measured EMF/RFR impacts [11].

Various signs of mitochondrial dysfunction and oxidative stress have also been identified in the brain. Findings include downregulation of expression of mitochondrial electron transport genes [140] or deficit of mitochondrial electron transport chain complexes [141], brain region specific glutathione redox imbalance [142], and evidence of oxidative damage and inflammation associated with low glutathione redox status [143]. Oxidative stress markers were measured as increased in cerebellum [144].

Additional support for the presence of tissue pathophysiology-based changes in brains of people with ASCs comes from the various studies documenting reduction in Purkinje cell numbers [117,145–150], possibly due to oxidative stress and an increased excitation/inhibition ratio that could potentially be acquired [150]. Also of note are changes in the glutamatergic and GABAergic systems, which when imbalanced can disturb the excitation/inhibition ratio and contribute to seizure disorders; reductions in GABA receptors as well as in GAD 65 and 67 proteins that catalyse the conversion of glutamate into GABA have been measured [151–153]. A consensus statement on the cerebellum in ASCs stated that, "*Points of consensus include presence of abnormal cerebellar anatomy, abnormal neurotransmitter systems, oxidative stress, cerebellar motor and cognitive deficits, and neuroinflammation in subjects with autism*" [150].

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Some indirect corroboration for these findings has come from neuroimaging, where the initial hypothesis regarding the tissue basis of the larger size of brains in so many people with autism – that it was due to a higher density of neurons and more tightly packed axons – came under question with the emergence of contradictory findings, well reviewed a few years ago by Dager and colleagues [154]. These include reduced rather than increased density of NAA (*n*-acetylaspartate, a marker of neuronal integrity and density that is produced in the mitochondria), reduced rather than increased fractional anisotropy suggesting less tightly packed axonal bundles [155–161] and greater rather than lower diffusivity, all of which may be more consistent with lower density of tissue and tissue metabolites and more fluid, which could be consistent with neuroinflammation and/or oxidative stress. The early postnatal development of such lower fractional anisotropy and increased diffusivity was measured in the process of occurring recently, in the first large prospective longitudinal imaging study of infants, who trended from 6 months to 2 years in the direction of these findings becoming more pronounced—but still with substantial overlap with those infants who did not develop autism [160]. This trend was consistent with prior studies showing increase in head size after birth, and added some information about what was happening in the brain to drive this size increase, although due to its methods it could only indirectly address the possibility that emergence during the first few years of life of tissue pathophysiology disturbances such as neuroinflammation might be contributing to these trends [162].

There is also substantial variability across many different types of brain findings. Of interest is that a number of functional brain imaging and electrophysiology studies have identified greater heterogeneity in response to stimuli between individuals in the ASC group than individuals in the neurotypical control group [163,164]. This may make more sense from the point of view of non-linear response—i.e. a disproportionality between output and input (as well as state and context sensitivity), in a pathophysiologically perturbed brain system. Nonlinearity has also been a significant methodological issue in EMF/RFR research because linear methods of study design and data analysis have often been insensitive to effects, whereas nonlinear methods have been argued to show greater sensitivity [165–175].

It is important to entertain how environmental agents could contribute individually and synergistically to brain changes in ASCs, how different exposures may disturb physiology similarly or differently, and how these changes may develop over progress over time after the earliest periods in brain development. EMF/RFR exposures could be preconceptional, prenatal or postnatal—or all of the above; it is conceivable that this could be the case in ASCs as well.

2.1.4.3. Altered development. There is some evidence for altered brain and organism development in relation to EMF/RFR exposure. Aldad et al. [83] exposed mice in-utero to cellular telephones, with resultant aberrant miniature excitatory postsynaptic currents, and dose-responsive impaired glutamatergic synaptic transmission onto layer V pyramidal neurons of the prefrontal cortex [83]. Lahijani exposed preincubated chicken embryos to 50 Hz EMFs, and made the following morphological observations: "*exencephalic embryos, embryos with asymmetrical faces, crossed beak, shorter upper beak, deformed hind limbs, gastroschesis, anophthalmia, and microphthalmia. H&E and reticulin stainings, TEMS, and SEMs studies indicated EMFs would create hepatocytes with fibrotic bands, severe steatohepatitis, vacuolizations, swollen and extremely electron-dense mitochondria, reduced invisible cristae, crystalized mitochondria with degenerated cristae, myelin-like figures, macrophages engulfing adjacent cells, dentated nuclei, nuclei with irregular envelopes, degenerated hepatocytes, abnormal lipid accumulations, lipid droplets pushing hepatocytes' nuclei to the corner of the cells, abundant cellular infiltrations cellular infiltrations inside sinusoid and around central veins, disrupted reticulin plexus, and release of chromatin into cytosol, with partially regular water layers, and attributed cell damage to elevated free radical induced cell membrane disruptions*" [5].

Although it is of great interest to characterize the changes in development associated with ASCs, it is also difficult to do in human beings because at present diagnosis is not possible until at least 2–3 years after birth. By now there have been a lot of prospective studies of infants at high risk for autism, but the in vivo brain imaging and electrophysiology data from these studies is only starting to be published, and so the for now the main sources of information are still inference backwards from post-mortem or imaging data, and animal models, both of which have clear limitations. Thus it is impossible to seek precise parallels here between what we know about the development of ASCs compared with the impacts of EMF/RFR exposures.

Nevertheless it is of real concern that such exposures have elicited some of the brain tissue changes that have been documented in ASCs, both in early development and subsequently. Already noted above is the question of whether high exposures of neonates to monitoring equipment may affect the melatonin levels of neonates [32]; these exposures also impact heart rate variability [258]. There are no studies yet on infants exposed to baby surveillance monitors or DECT wireless phones. However there are good laboratory testing studies yielding actual measurements of these devices that conclude: "*Maximum incident field exposures at 1 m can significantly exceed those of base stations (typically 0.1–1 V/m). At very close distances the derived or reference exposure limits are violated for baby surveillance monitors and DECT phones. Further, the authors conclude that, based on very strictly controlled laboratory testing of everyday devices like baby monitors and some cordless phones (W)orse case peak spatial SAR values are close to the limit for the public or uncontrolled environments, e.g., IEEE 802.11b and Bluetooth Class I*" [176].

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Even exposure of the fetus to laptop computer wireless emissions through the pregnant mother's use of this device on her lap may involve induction of strong intracorporeal electric current densities from the power supply possibly even more than the device itself [177].

2.1.4.4. Brain blood flow and metabolism. Cerebral perfusion and metabolism abnormalities have been identified in close to two dozen papers studying autistic cohorts. Cerebral perfusion refers to the quantity of blood flow in the brain. Abnormal regulation of cerebral perfusion is found in a range of severe medical conditions including tumors, vascular disease and epilepsy. Cerebral hypoperfusion has also been found in a range of psychiatric disorders [178]. Neurocognitive hypotheses and conclusions, as well as localization of perfusion changes, have been heterogeneous across these papers. Hypoperfusion or diminished metabolism has been identified in frontal regions [179–184], temporal lobes [179,181,183–190], as well as a variety of subcortical regions including basal ganglia [181,188,189], cerebellum [188], limbic structures [184,191] and thalamus [188,189,191]—i.e. in a widely distributed set of brain regions. Possibly because virtually all of these studies were oriented toward testing neuropsychological rather than pathophysiological hypotheses, there were no probes or tests reported to unearth the tissue level alterations that might be underlying these reductions in blood flow in these brains.

While a large number of animal studies have documented blood–brain barrier (BBB) abnormalities from EMF/RFR exposures, only a few PET studies have been performed evaluating EMF exposure effects upon brain glucose metabolism. Volkow et al. performed PET scans both with and without EMF exposure (50 min of GSM-900 with maximum SAR of 0.901 W/kg), and the participants were blinded to the exposure situation [192]. A 7% increase in metabolism in the exposure situation compared to controls was identified regionally on the same side of the head as where the mobile phone was placed. The strength of the E-field from the phones correlated positively with the brain activation, which the authors hypothesized was from an increase in brain neuron excitability. A subsequent smaller study by Kwon et al. demonstrated not increased but decreased brain ^{18}FDG uptake after GSM-900 exposure [193].

Many possible mechanisms could be involved in the metabolic and perfusion abnormalities identified, ranging from altered neuronal activity that was hypothesized in the Volkow et al. [192] ^{8}FDG PET study to narrowing of vascular lumen in the setting of reduced perfusion. Underlying tissue pathophysiology-based phenomena could influence the measurable metabolism and perfusion abnormalities, via mechanisms such as excitotoxicity, cell stress response, constriction of capillary lumen by activated astrocytes, volume effects of vascular extravasation, subtle alterations in blood viscosity due to immune or oxidative stress-associated blood chemical changes, with other possibilities as well. Differences in findings between papers could relate at least in part to study design and nonlinearity issues.

2.1.5. Electrophysiology perturbations

At this stage the argument we hit a key pivot point, where we look at how the alterations in molecular, cellular and systems physiological function, which occur in the brain as well as in the body, impact the transduction into the electrical signaling activities of the brain and nervous system. Certainly the cells and tissues whose physiological challenges we have already discussed provide the material substrate for the electrical activity. Although ASC behaviors are influenced by many factors, they must in principle be mediated through nervous system electrophysiology.

If the cells responsible for generating synapses and oscillatory signaling are laboring under cellular and oxidative stress, lipid peroxidation, impaired calcium and other signaling system abnormalities, then mitochondrial metabolism will fall short, all the more so because of the challenges from the immune system which in turn be triggered to a major extent by environment. How well will synaptic signals be generated? How well will immune-activated and thereby distracted glial cells be able to modulate synaptic and network activity? [194–197].

At present we are in the early stages of being able to formulate these questions well enough to address them empirically. We do know that microglial activation can impact excitatory neurotransmission mediated by astrocytes [198]. We do know that the cortical innate immune response increases local neuronal excitability and can lead to seizures [199,200]. We do know that inflammation can play an important role in epilepsy [201]. We know less about lower levels of chronic or acute pathophysiological dysfunction and how they may modulate and alter the brain's electrophysiology.

2.1.5.1. Seizures and epilepsy. EEG signals in ASCs are abnormal on a variety of levels. At the most severe level, EEGs show seizure activity. Although less than 50% of people with ASCs clearly have seizures or epilepsy a much larger number have indications of epileptiform activity, and an even larger percent have subclinical features that can be noted by a clinical epileptologist though not necessarily flagged as of clinical concern. In addition to the association of some severe epilepsy syndromes (e.g. Landau Kleffner, tuberous sclerosis) with autism, the risk of epilepsy is substantially higher in people with ASCs than in the general population, with a large subset of these individuals experiencing seizure onset around puberty, likely in relation to aberrations in the dramatic and brain-impactful hormonal shifts of that phase of life. Epileptic seizures can be both caused by and cause oxidative stress and mitochondrial dysfunction. Seizures can cause extravasation of plasma into brain parenchyma [202–206] which can trigger a vicious circle of tissue damage from albumin and greater irritability, as discussed above. Evidence suggests that if a BBB is already disrupted, there

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will be greater sensitivity to EMF/RFR exposure than if the BBB were intact [207,208], suggesting that such exposures can further exacerbate vicious circles already underway.

The combination of pathophysiological and electrophysiological vulnerabilities has been explored in relation to the impact of EMF/RFR on people with epilepsy EMF/RFR exposures from mobile phone emissions have been shown to modulate brain excitability and to increase interhemispheric functional coupling [209,210]. In a rat model the combination of picrotoxin and microwave exposure at mobile phone-like intensities led to a progressive increase in neuronal activation and glial reactivity, with regional variability in the fall-off of these responses three days after picrotoxin treatment [211], suggesting a potential for interaction between a hyperexcitable brain and EMF/RFR exposure.

One critical issue here is nonlinearity and context and parameter sensitivity of impact. In one study, rat brain slices exposed to EMF/RFR showed reduced synaptic activity and diminution of amplitude of evoked potentials, while whole body exposure to rats led to synaptic facilitation and increased seizure susceptibility in the subsequent analysis of neocortical slices [212]. Another study unexpectedly identified enhanced rat pup post-seizure mortality after perinatal exposure to a specific frequency and intensity of exposure, and concluded that apparently innocuous exposures during early development might lead to vulnerability to stimuli presented later in development [213].

2.1.5.2. Sleep. Sleep involves a profound change in brain electrophysiological activity, and EEG abnormalities including disrupted sleep architecture figure in sleep challenges in ASCs. Sleep symptoms include bedtime resistance, sleep onset delay, sleep duration and night wakings; and sleep architecture can involve significantly less efficient sleep, less total sleep time, prolonged sleep latency, and prolonged REM latency [214,215], with these sleep problems being worse in children with ASCs who regressed than in those who did not regress into their autism [215]. EEG abnormalities have also been associated with EMF/RFR exposure, including disrupted sleep architecture as well as changes in sleep spindles and in the coherence and correlation across sleep stages and power bands during sleep [216,217].

Sleep disturbance symptoms are also common in both situations. Insomnia is commonly reported in people who are chronically exposed to low-level wireless antenna emissions. Mann and Rosch reported an 18% reduction in REM sleep, which is key to memory and learning functions in humans [321]. In ASCs sleep difficulties are highly pervasive and disruptive not only to the affected individual but also to their whole family due to the associated problems such as noise (e.g. screaming at night) and the need for vigilance.

The multileveled interconnections involved in the modulation of sleep exemplify the interconnectedness of the many levels of pathophysiology reviewed here: "*Extracellular ATP associated with neuro- and glio-transmission, acting via purine type 2 receptors, e.g., the P2X7 receptor, has a role in glia release of IL1 and TNF. These substances in turn act on neurons to change their intrinsic membrane properties and sensitivities to neurotransmitters and neuromodulators such as adenosine, glutamate and GABA. These actions change the network input-output properties, i.e., a state shift for the network*" [218]. With disturbance simultaneously at so many of these levels, it is not surprising that sleep dysregulation is nearly universal in ASCs, and common in the setting of EMF/RFR exposures.

2.1.5.3. Quantitative electrophysiology. While clinical reading of EEG studies is done visually, a growing number of studies are examining EEG and MEG data using digital signal processing analysis to find not only epilepsy, but also abnormalities in the power spectrum, i.e. the distribution of power over the different frequencies present, with some studies showing impaired or reduced gamma-and activity [219–221] and others showing reduction of spectral power across all bands [222] while still others showed increased high-frequency oscillations [223]. Abnormalities in coherence and synchronization between various parts of the brain have been found [224–226], comparable to abnormal functional connectivity measured by fMRI [227] but measurable with higher temporal resolution using EEG or MEG [228–232]. Several studies have identified reduced complexity and increased randomness in EEGs of people with ASCs [233,234], as well as an increase in power but a reduction in coherence [229,235]. Some electrophysiological metrics are emerging as potential discriminators between brain signal from individuals with ASCs and those who are neurotypical, such as a wavelet-chaos-neural network methodology applied to EEG signal [236] and reduced cross-frequency coupling [237].

EMF/RFR also has impacts at levels of brain function measurable by these techniques. At various frequencies and durations of exposure it has been noted to impact alpha and beta rhythms [238], to increase randomness [170,239], to alter power, to modulate interhemispheric synchronization [240], to alter electrical activity in brain slices [241] and to alter the patterns of coordination (spectral power coherence) across the major power bands [242]. Bachman et al. [243] showed statistically significant changes in EEG rhythms and dynamics occurred in between 12% and 20% of healthy volunteers [243]. In children, exposures to cell phone radiation have resulted in changes in brain oscillatory activity during some memory tasks [97,102].

2.1.5.4. Sensory processing. Symptomatic level issues with sensory processing are highly prevalent in ASCs and can include hypersensitivity to external stimuli, hyposensitivity to internal sensations and difficulty localizing sensation including pain, and difficulty processing more than one sensory channel at one time [244–246]. There is now electrophysiological evidence of abnormalities at early (brainstem) stages of sensory processing, as well as in later stages of processing that occur in the cortex [247]. Some studies have

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shown lower and some longer latencies of response to an auditory stimulus [247]. Domains of perception where the performance of people with ASCs is superior to that of neurotypical individuals have been identified [248]. "*It is . . . probable that several mechanisms and neuronal abnormalities, most likely at multiple levels (from single neurons through to inter-area connections), all contribute to varying degrees to the abnormal sensory processing observed in ASD. It is also likely that no single mechanism is unique to one sensory modality, which is why we see such a widely distributed range of abnormalities across modalities*" [247].

It is also possible that the mechanisms may not simply be neural—they may also be modulated by glial, metabolic, immune, perfusional and other physiological processes by common underlying cellular abnormalities, and by physical properties as well. Yet there are few studies focusing upon the interface of tissue pathophysiology with electrophysiology.

Kenet et al. demonstrated environmental vulnerability of sensory processing in the brain by the exposure of rat dams to noncoplanar polychlorinated biphenyls (PCBs), during gestation and for three subsequent weeks of nursing [247]. The rat pups showed normal hearing sensitivity and brainstem auditory responses, but their tonotopic development of the primary auditory cortex was grossly distorted [249]. This study may be particularly relevant for EMF/RFR exposures, as Pessah, a co-author on this Kenet et al. [249] paper, was cited earlier as documenting how the noncoplanar PCBs used in this experiment target calcium signaling as do EMF/RFR exposures—i.e. they both converge upon a common particularly critical cellular mechanism [250,251].

2.1.5.5. Autonomic dysregulation. Although there are a fair number of negative studies regarding the impact of EMF/RFR exposure on the autonomic nervous system, increased HRV and autonomic disturbances have been documented [252–256]. Buchner and Eger [257], in a study in rural Germany of the health impacts of exposures from a new base station yielding novel exposure to EMF/RFR, saw a significant elevation of the stress hormones adrenaline and noradrenaline during the first six months with a concomitant drop in dopamine, with a failure to restore the prior levels after a year and a half. These impacts were felt by the young, the old and the chronically ill, but not by healthy adults [257].

Neonate vulnerability was documented by Bellieni et al. [258] who found that heart rate variability is adversely affected in infants hospitalized in isolettes or incubators where ELF-EMF levels are in the 0.8 to 0.9 μT range (8 to 9 mG). Infants suffer adverse changes in heart rate variability, similar to adults [258]. This electromagnetic stress may have lifelong developmental impacts, based on a study showing that in-utero beta 2 agonist exposure can potentially induce a permanent shift in the balance of sympathetic-to-parasympathetic tone [259].

Meanwhile clinical observation and a growing body of literature support a major role for stress in ASCs [260–263], with variability amongst individuals in the severity of the stress response but a tendency to have high tonic sympathetic arousal at baseline [264–269].

The impact of EMF/RFR exposure can also be greatly influenced by the stress system status of the individual being exposed. Tore et al. sympathecotomized some of his rats before exposure to GSM, to simulate cell phone exposure [207,208]. Sympathectomized rats, which were in a chronic inflammation-prone state, had more prominent albumin leakage than sham-exposed rats. However in the sympathecotmized rats who were exposed to GSM, albumin leakage was greatly increased, to levels resembling those observed in positive controls after osmotic shock. Salford et al. [99] suggest that "*. . .more attention should be paid to this finding, since it implicates that the sensitivity to EMF-induced BBB permeability depends not only on power densities and exposure modulations, but also on the initial state of health of the exposed subject*" [99].

The interconnection between stress and brain connectivity (or coherence) in ASCs is brought out by Narayanan et al. in a pilot study testing the impact of the beta blocker propranolol on brain functional connectivity measured using functional MRI [270]. A fairly immediate increase in functional connectivity was noted from propranolol—but not from nadolol which has the same vascular effects but does not cross the BBB. Propranolol decreases the burden of norepinephrine, thereby reducing the impact of stress systems on brain processing, and the authors interpreted these effects as creating an improvement of the brain's signal-to-noise ratio [271], allowing it to utilize and coordinate more remote parts of its networks. This suggests that stressors such as EMF/RFR, by adding biologically non-meaningful noise to the system, might have the opposite effects, degrading coherent integration.

2.2. De-tuning of the brain and organism

2.2.1. Electromagnetic signaling, oscillation and synchrony are fundamental, and vulnerable

While electrophysiological activity is an intrinsic property of the nervous system, electromagnetic signaling is a vital aspect of every cell and of molecular structure.

All life on earth has evolved in a sea of natural low-frequency electromagnetic (EM) fields. They originate in terrestrial and extraterrestrial sources. The ever-growing use of electric power over the last century has sharply modified this natural environment in urban settings. Exposure to power-frequency fields far stronger than the natural environment is now universal in civilized society. [272]

Adey published some of the earliest scientific studies on the "cooperativity" actions of cells in communication. Studies showing us that the flux of calcium in brain tissue and immune cells is sensitive to ELF-modulated radiofrequency fields is actually telling us that some of the most fundamental properties of cells and thus of our existence can be modulated by EMF/RFR. "*. . .in first detection of environmental*

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EM fields in tissues, there appears to be a general consensus that the site of field action is at cell membranes. Strands of protein are strategically located on the surface of cells in tissue, where they act as detectors of electrical and chemical messages arriving at cell surfaces, transducing them and transmitting them to the cell interior. The structural basis for this transductive coupling by these protein strands is well known. Through them, cell membranes perform a triple role, in ***signal detection, signal amplification, and signal transduction to the cell interior***" [272].

Oscillation is also a universal phenomenon, and biological systems of the heart, brain and gut are dependent on the cooperative actions of cells that function according to principles of non-linear, coupled biological oscillations for their synchrony, and are dependent on exquisitely timed cues from the environment at vanishingly small levels [273,274]. The key to synchronization is the joint actions of cells that co-operate electrically - linking populations of biological oscillators that couple together in large arrays and synchronize spontaneously according to the mathematics described for Josephson junctions (Brian Josephson, the 1993 Nobel prize winner for this concept). This concept has been professionally presented in journal articles and also popularized in a book by Prof. Steven Strogatz, a mathematician at Cornell University who has written about 'sync' as a fundamental organizing principle for biological systems [274,275]. "*Organisms are biochemically dynamic. They are continuously subjected to time-varying conditions in the form of both extrinsic driving from the environment and intrinsic rhythms generated by specialized cellular clocks within the organism itself. Relevant examples of the latter are the cardiac pacemaker located at the sinoatrial node in mammalian hearts and the circadian clock residing at the suprachiasmatic nuclei in mammalian brains. These rhythm generators are composed of thousands of clock cells that are intrinsically diverse but nevertheless manage to function in a coherent oscillatory state. This is the case, for instance, of the circadian oscillations exhibited by the suprachiasmatic nuclei, the period of which is known to be determined by the mean period of the individual neurons making up the circadian clock. The mechanisms by which this collective behavior arises remain to be understood*" [274].

The brain contains a population of oscillators with distributed natural frequencies, which pull one another into synchrony (the circadian pacemaker cells). Strogatz has addressed the unifying mathematics of biological cycles and external factors disrupt these cycles. Others have discussed how this also applies to mitochondria: "*Organisation of mitochondrial metabolism is a quintessential example of a complex dissipative system which can display dynamic instabilities. Several findings have indicated that the conditions inducing instabilities are within the physiological range and that mild perturbations could elicit oscillations. Different mathematical models have been put forth in order to explain the genesis of oscillations in energy metabolism. One model considers mitochondria as an organised network of oscillators and indicates that communication between mitochondria involves mitochondrial reactive oxygen species (ROS) production acting as synchronisers of the energy status of the whole population of mitochondria. An alternative model proposes that extramitochondrial pH variations could lead to mitochondrial oscillations*" [276].

Mitochondrial dysfunction is important in ASCs but is usually conceptualized in purely biochemical terms without mentioning any oscillatory dimension to mitochondrial activity; it is conceivable that the interplay between biochemistry and oscillation could figure significantly in the mechanisms of impact of EMF/RFR in ASCs.

The field of bioelectromagnetics has studied exposure to very low levels of electromagnetic frequencies. Exposures can alter the magnetokinetics of the formation of a chemical bond, shifting the rate and amount of product produced [272].

Not just chemical reactions but synchronous biological oscillations in cells (pacemaker cells) can be disturbed and disrupted by artificial, exogenous environmental signals, which can lead to desynchronization of neural activity that regulates critical functions (including metabolism) in the brain, gut and heart and circadian rhythms governing sleep and hormone cycles [277]. Buzsaki in his book *Rhythms of the Brain* says "*rhythms can be altered by a wide variety of agents and that these perturbations must seriously alter brain performance*." [273].

Disturbance can get increasingly disruptive as more damage occurs and more systems are thrown out of kilter and out of cooperativity. One can think of the kindling model in which repeated induction of seizures leads to longer and more severe seizures and greater behavioral involvement. The combination of disruptive and stimulatory effects of biologically inappropriate EMF/RFR exposures could contribute to disruption of synchronized oscillation and cooperativity at a myriad of levels but particularly in the brain, and this may contribute to the loss of coherence and complexity in the brain in autism, as well as dysregulation of multiple other bodily systems. Strogatz points out that there are many more ways of being desynchronized than of being synchronized [274] (which may relate to ASC's great heterogeneity). It has even been suggested that autism itself could be due to brain desynchronization [278].

2.2.2. Behavior as an "emergent property"

From a pathophysiological point of view one might hypothesize that a brain with greater indications of oxidative stress along with immune activation and mitochondrial dysfunction might generate different oscillatory activity than a brain in which those pathophysiological features were absent. From this vantage point it would make sense to propose that the compromised whole body health status of at least many with ASCs would make it harder for them to maintain the resilience of their brain cells and brain activities in the face of potentially disruptive exposures. Yet the investigation of how this might occur remains a largely unexplored frontier. But

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from the point of view of making sense of the brain impact of environmental challenges – including but not limited to EMF-RFR – this investigation is crucial.

The pathophysiological perspective that guides this review would suggest a move away from considering the behavioral manifestations of ASCs as core, intrinsic, 'hard-wired traits.' *Instead behaviors may be better understood as 'outputs' or emergent properties – what the brain and body produce – when their physiological attributes are altered* in these fashions for whatever reasons—be they genetic, environmental or many combinations of both [279–284]. Sleep and consciousness have also been considered 'emergent properties' [285,286]. Brain oscillatory activity is critical for organizing behavior, and it arises from cells and subcellular features that are shaped by the environment and can act differently based on their functional status as well as on account of external sensory or psychosocial stimuli.

In particular, (a) brain oscillatory activity is intimately connected with underlying cellular, metabolic and immune status, (b) EMF/RFR is capable of perpetrating changes at each of these levels, and (c) problems at each of these levels can make other problems worse. And as mentioned earlier, EMF/RFR and various toxicants can co-potentiate damage [287–294], amplifying 'allostatic load'.

Put together, all of this implies that the combination of these EMF/RFR impacts may quite plausibly significantly contribute both to how ASCs happen in individuals and to why there are more reported cases of ASCs than ever before (1200–1500% increase in reported cases over the past 15–20 years, with studies showing that a substantial portion of this increase (45–65%) cannot be written off as artifact and may well represent true increases [295,296]).

The hopeful side of this framing of the problem comes from moving beyond the increasingly anachronistic idea that autism is determined overwhelmingly by genetic code about which we can do little or nothing. An emerging model that explains much more of what we now know frames ASCs as the dynamic, active outcomes of perturbed physiological processes – again, more like a chronic but changeable 'state' than a 'trait.' In the latter model, one is empowered – and motivated – to strongly reduce exposures and to make aggressive constructive environmental changes – particularly in diet and nutrition, given their protective potency discussed above [297]. In this way 'allostatic load' can be reduced, physiological damage can be repaired, homeostasis can be restored and resilience and optimal function can be promoted.

3. Implications

3.1. Exposures and their implications

Several thousand scientific studies over four decades point to serious biological effects and health harm from EMF and RFR [298,299]. These studies report genotoxicity, single-and double-strand DNA damage, chromatin condensation, loss of DNA repair capacity in human stem cells, reduction in free-radical scavengers (particularly melatonin), abnormal gene transcription, neurotoxicity, carcinogenicity, damage to sperm morphology and function, effects on behavior, and effects on brain development in the fetus of human mothers that use cell phones during pregnancy. Cell phone exposure has been linked to altered fetal brain development and ADHD-like behavior in the offspring of pregnant mice [83].

3.1.1. Exposures have outpaced precautions

There is no question that huge new exposures to EMF/RFRs have occurred over the past few decades. As discussed extensively in the BioInitiative 2012 update [299], there is much concern that regulations to date have been based on a very limited sense of the pertinent biology, and particularly that limiting concern to thermal impacts is no longer valid since there is a wealth of evidence by now that non-thermal impacts can be biologically very powerful. Only in the last two decades have exposures to RFR and wireless technologies become so widespread as to affect virtually every living space, and affect every member of societies around the world. Even as some disease patterns like brain tumors from cell phone use have become 'epidemiologically visible', there are no comprehensive and systematic global health surveillance programs that really keep up to report EMF/RFR health trends [300].

The deployment of new technologies is running ahead of any reasonable estimation of possible health impacts and estimates of probabilities, let alone a solid assessment of risk. However, what has been missing with regard to EMF/RFR has been an acknowledgement of the risk that is demonstrated by the scientific studies. There is clear evidence of risk, although the magnitude of the risk is uncertain, and the magnitude of doing nothing on the health effects cost to society is similarly uncertain. This situation is very similar to our history of dealing with the hazards of smoking decades ago, where the power of the industry to influence governments and even conflicts of interest within the public health community delayed action for more than a generation, with consequent loss of life and enormous extra health care costs to society. [301].

3.1.2. The population's exposure has increased

The very rapid global deployment of both old and new forms of emerging wireless technologies in the last two decades needs aggressive evaluation from a public health perspective, given the range of physiological impacts described in Section 2.

In the United States, the deployment of wireless infrastructure (cell tower sites) to support cell phone use has accelerated greatly in the last decades. The Cellular Telephone Institute of America (CTIA) estimated that in 1997 there were only 36,650 cell sites in the US; but increased rapidly to 131,350 in June 2002; 210,350 in June 2007 and 265,561 in June 2012 [302,303]. About 220,500 cell sites existed in 2008 [303–305]. These wireless facilities for cellular phone voice

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and data transmission produce RFR over broad areas in communities and are an involuntary and unavoidable source of whole-body radiofrequency radiation exposure. Other new RFR exposures that did not exist before are from WI-FI access points (hotspots) that radiate 24/7 in cafes, stores, libraries, classrooms, on buses and trains, and from personal WI-FI enabled devices (iPads, tablets, PDAs, etc).

Not surprisingly, the use of cell phones has a parallel growth trend. In the late 1980s and early 1990's, only a few percent of the US population were cell phone users. By 2008, eighty-four percent (84%) of the population of the US owned cell phones. CTIA reports that wireless subscriber connections in the US increased from 49 million in June 1997 to 135 million in June 2002 to 243 million in June 2007 to 322 million in June 2012 [302,303]. This represents more than a 100% penetration rate in the US consumer market, up from just a few percent in the early 1990's. The number of wireless subscribers in June 1997 was 18%; in June 2002 it was 47%; in June 2007 it was 81% and in June 2012 it was 101%.

The annualized use of cell phones in the US was estimated to be 2.23 trillion minutes in 2008 and 2.296 trillion minutes in 2010 [303]. There are 6 billion users of cell phones worldwide in 2011 up from 2.2 billion in 2008 and many million more users of cordless phones.

The number of US homes with *only* wireless cell phones has risen from 10.5% in 2007 to 31.6% in June of 2012 [302,303]. There are no statistics for June 1997 nor for June 2002, since landline (non-wireless) phone use predominated. The shift to wireless communications, more minutes of use, and reliance on cell and cordless phones rather than corded phones is an extremely revealing measure of new EMF and RFR exposures for both adults and children.

The prevalence of autism has risen in parallel from one (1) in 5000 (1975) to 1 in 2500 (1985) to 1 in 500 (1995) to 1 in 250 (∼2001) to 1 in 166 (∼2004) to 1 in 88 (∼2008) to 1 in 50 (∼2013). All reflected birth cohorts born earlier[1,2]. Further research into autism prevalence studies have debunked the initial contention that higher numbers could be explained away by better diagnosis and broadening of diagnostic criteria[3-6].

3.1.3. Infants, children and childbearing families are highly exposed and vulnerable

The spread of cell towers in communities, often placed on pre-school, church day-care, and school campuses, means that young children may have hundreds of thousands of times higher RFR exposures in home and school environments than existed even 20–25 years ago. In addition, the nearly universal switch to cordless and cell phones, and away from corded landline phones, means that people are experiencing close and repetitive exposures to both EMF and RFR in the home [306]. Wireless laptops and wireless internet in schools, and home offices and for homework mean even more chronic exposures to RFR, a designated IARC 2B Possible Human Carcinogen [307,308]. The great utility of handheld devices as communication aids and engaging sources of information and satisfaction for people on the autism spectrum may come with a concerning biologically harmful underbelly.

Exposures prior to conception or during pregnancy and infancy can come from faulty wiring, proximity to power lines, or high-frequency transients from a proximate transformer on a utility pole. Sources of pulsed RFR inside the home include an electronic baby monitor in the crib, a wireless router in the next room, a DECT phone that pulses high emissions of RFR on a continuous basis 24/7, or conversion to all compact fluorescent bulbs that produce significant 'dirty electricity' for occupants due to low-kilohertz frequency fields on electrical wiring and in ambient space. Sick and vulnerable infants in neonatal intensive care units are heavily exposed from being surrounded by equipment, with negative metabolic and autonomic consequences documented [32,258].

Wireless phones and laptops exposures produce extremely low frequency pulses from the battery of the wireless device [301,306,309] and the exposures to pulsed radiofrequency microwave radiation when the wireless device is transmitting or receiving calls and emails.

Especially since EMF/RFR exposures are already classified as IARC 2B Possible Human Carcinogens, we should be actively investigating these sources of damage to DNA that could reasonably result in 'de novo mutations' but also be aware that common environmental exposures from EMF and RFR might play a role in the higher rates of concordance for autism (ASD) among twins and siblings.

Researchers also should be aware that common environmental exposures from EMF and RFR might play a role in the higher rates of autism (ASD) among twins and siblings, not solely because of maternal use of wireless devices during pregnancy and paternal sperm exposure to wireless devices peri-conception; but also because such oxidative damage to DNA can occur at levels introduced into the world of the fetus, and young developing infant and child via baby surveillance monitoring devices in the crib and wireless devices in the home. The deployment of technologies poses risks to human fertility and reproduction capacity, to the fetus, to children and adults [301].

3.1.4. ASC risk and genomic damage to future generations

Barouki and Grandjean make a persuasive case that public health interventions are critically needed in early childhood development to prevent adult diseases that appear decades later [310]. Although they do not include EMF or RFR but only chemicals, they do say that physiological stressors, which EMF and RFR certainly have been established, should be reduced during critical development windows. They say: "*The current pandemic of non-communicable diseases and the increased prevalence of important dysfunctions demand an open interrogation of why current interventions appear insufficient. We now know that disease risk can be induced very early in the life course and that it is modifiable by*

nutrients and environmental chemical exposures (along with drugs. infections, and other types of stresses)" [310].

Public health interventions are warranted now to protect the genetic heritage of humans, as well as the other stocks of genetic material in wildlife and plants in the face of what appears to be on-going impairment of these genomes. The risk of genomic damage for future generations is sufficiently documented to warrant strong preventative action and new public safety limits that observe EMF/RFR levels shown to cause adverse effects.

3.1.5. De-tuning the organism

Genetic mutations may lead to cancer and other diseases in the present and future generations, but the exposures that are capable of creating genotoxic impacts also compromise physiological function. Even genotoxicity can have not only specific but also non-specific effects due to molecular inefficiencies, misfolded proteins, and cellular debris [311,312].

In the setting of autism, a baby gestated or developing as a neonate in a milieu with excessively elevated EMF/RFR exposures is vulnerable to interference with the normal development processes, including the organization of information and experience in the brain. This baby's environment also often includes nutritional insufficiencies (processed denatured pesticide-laden food low in antioxidants, minerals and essential fatty acids essential to cellular protection). The baby's gestational period may have been complicated by the mother's own health issues such as conditions like obesity and diabetes [313] which converge upon on inflammation, oxidative stress and other common forms of physiological dysregulation. The exquisite 'tuning up' of the brain and body as it develops will integrate and respond to the environmental inputs it receives, and is particularly sensitive to environmental miscues (whether chemical like endocrine disruptors, EMF/RFR which can be both chemically and electromagnetically disruptive, or other environmental conditions whether hostile or nurturing). To the extent that the baby is burdened with more disorganized or hostile cues than nurturing and organizing cues, that baby may lose resiliency and become more physiologically vulnerable –perhaps approaching a tipping point into decompensation such as autistic regression or development of other chronic disease processes.

From a systems point of view, the phenomenon of 'autistic regression' can be understood as occurring after an accumulation of multisystem signaling interference leading to a tipping point of loss of some vital systems synchronization and increase in randomization. EMF/RFR exposures could plausibly contribute both to this vulnerability and to the decompensation/desynchronization process – as could other stressors such as infection, toxicity, acute stress. The vulnerability, then, is the 'allostatic load' – the total burden of stressors pressing toward disorganization. The tipping point may come in a variety of ways; but upon investigation one is likely to find that unless a stressor is severe, the trigger most proximally associated with the decompensation is likely to be the 'straw that breaks the camel's back' laid atop a prior accumulation of 'allostatic load.'

3.2. Conclusions and recommendations

The case has been made that ASCs involve physiological challenges at multiple levels, and that these challenges are paralleled in the physiological impacts of EMF/RFR exposure. Evidence has also been presented to suggest that the many levels of damage and degradation of physiological and functional integrity are profoundly related to each other. Although autism spectrum conditions (ASCs) are defined by problems with behavior, communication, social interaction and sensory processing, under the surface they also involve a range of disturbances of underlying biology that find striking parallels in the physiological impacts of electromagnetic frequency and radiofrequency radiation exposures (EMF/RFR). At the cellular and molecular level many studies of people with ASCs have identified oxidative stress and evidence of free radical damage, evidence of cellular stress proteins, as well as deficiencies of antioxidants such as glutathione. Elevated intracellular calcium in ASCs can be associated with genetic mutations but more often may be downstream of inflammation or chemical exposures. Cell membrane lipids may be peroxidized, mitochondria may function poorly, and immune system disturbances of various kinds are common. Brain oxidative stress and inflammation as well as measures consistent with blood–brain barrier and brain perfusion compromise have been documented. Changes in brain and autonomic nervous system electrophysiology can be measured and seizures are far more common than in the population at large. Sleep disruption and high levels of stress are close to universal. In parallel, all of these phenomena have also been documented to result from or be modulated by EMF/RFR exposure. Moreover, some people with ASCs have de novo mutations (that their parents do not have), and EMF/RFR exposures could contribute to this due to their potential genotoxicity. EMF/RFR exposure during pregnancy may send spurious signals to developing brain cells during pregnancy, altering brain development during critical periods, and may increase oxidative stress and immune reactivity that can increase risk for later developmental impairments, with further disruption later in development increasing risk, physiological dysregulation and severity of outcome.

All of this does not prove that EMF/RFR exposures cause autism, but it does raise concerns that they could contribute by increasing risk, and by making challenging biological problems and symptoms worse in these vulnerable individuals. Placed alongside the dramatic rise in reported cases of ASCs [333], that parallels the dramatic rise in deployment of wireless technologies, a strong case can be made for aggressively investigating links between ASCs and EMR/RFR, and minimizing exposures for people with autism as well as families preconceptionally, during pregnancy, and around infants and children at home, at school, and in health care centers and hospitals.

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These arguments have implications for how we understand what ASCs 'are' and how they work, including an appreciation that it may be the physiological disturbance is what actually generates the 'autism' on a moment-to-moment basis—and that these physiological disturbances are profoundly driven by environmental insults. These implications call upon us to take the environmental contribution very seriously, which involves on the one hand a sobering appreciation of the vast array of exposures that can contribute to risk via perturbed development and physiological degradation, and on the other hand a sense that there are powerful things we can do to reduce risk and improve the situation.

3.2.1. Change our deployment of EMF/RFR

The deployment of RFR from wireless technologies has incredible momentum, and it has made many things easier and many other things possible for the first time. On the other hand this momentum can interfere with setting up the technology in a fashion truly respectful of biological tolerances. "*There is no question that global implementation of the safety standards proposed in the Bioinitiative (2007) Report, if implemented abruptly and without careful planning, have the potential to not only be very expensive but also disruptive of life and the economy as we know it. Action must be a balance of risk to cost to benefit. The major risk from maintaining the status quo is an increasing number of cancer cases, especially in young people, as well as neurobehavioral problems at increasing frequencies. The benefits of the status quo are expansion and continued development of communication technologies. But we suspect that the true costs of even existing technologies will only become much more apparent with time. Whether the costs of remedial action are worth the societal benefits is a formula that should reward precautionary behavior*" [301].

3.2.2. Encourage precautions right now based on present knowledge

Physicians and health care workers should raise the visibility of EMF/RFR as a plausible environmental factor in clinical evaluations and treatment protocols. Reducing or removing EMF and wireless RFR stressors from the environment is a reasonable precautionary action given the overall weight of evidence.

- Children with existing neurological problems that include cognitive, learning, attention, memory, or behavioral problems should as much as possible be provided with wired (not wireless) learning, living and sleeping environments,
- Special education classrooms should aim for 'no wireless' conditions to reduce avoidable stressors that may impede social, academic and behavioral progress.
- Adaptations to preserve the attractive design innovations of technologies such as tablet computers in a 'no wireless' environment should be developed.
- All children should reasonably be protected from the physiological stressor of significantly elevated EMF/RFR (wireless in classrooms, or home environments).
- School districts that are now considering all-wireless learning environments should be strongly cautioned that wired environments are likely to provide better learning and teaching environments, and prevent possible adverse health consequences for both students and faculty in the long-term.
- Monitoring of the impacts of wireless technology in learning and care environments should be performed with sophisticated measurement and data analysis techniques that are cognizant of the non-linear impacts of EMF/RFR and of data techniques most appropriate for discerning these impacts.
- There is sufficient scientific evidence to warrant the selection of wired internet, wired classrooms and wired learning devices, rather than making an expensive and potentially health-harming commitment to wireless devices that may have to be substituted out later, and
- Wired classrooms should reasonably be provided to all students who opt-out of wireless environments.

Broader recommendations also apply, related to reducing the physiological vulnerability to exposures, reduce 'allostatic load' and build physiological resiliency through high quality nutrition, reducing exposure to toxicants and infectious agents, and reducing stress [297], all of which can be implemented safely based upon presently available knowledge.

3.2.3. Build an environmentally physiologically centered research program in ASCs as a platform for investigating the EMR/RFR-ASC linkage

This review has been structured around the physiological parallels between ASCs and the impacts of EMF/RFR. What is missing from the autism research agenda is some cross-study of these two bodies of research evidence. To do this we will need both a recognition of the importance of these risks, and a collaborative multi-site research program centered around a "middle-out" physiological approach [314] that can transcend the limits of the gene-brain-behavior agenda that has dominated ASC research, by incorporating this now clearly limited approach into a broader framework [315]. This still dominant gene-brain-behavior approach has been based on an expectation of linear mapping across the levels on which it focuses, but instead the systems involved appear to be much more complex. The middle-out approach is an emerging more inclusive framework in systems biology that can incorporate complexity and nonlinear, multi-scale modeling [316–320]. The physiological levels largely left out in the gene-only approach are critically important to helping people with ASCs because they will help not only with understanding how environment impacts function but also with identifying leverage points.

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3.2.4. Take the evidence as a call to action

Both EMF and RFR exposures are already classified as IARC Group 2B Possible Human Carcinogens. The substantial scientific literature on EMF and RFR effects on DNA, on immune and blood–brain barrier disruption, on stress proteins, on circadian rhythms and hormone disregulation, and on cognition, sleep, disruption of neural control and altered brainwave activity all argue for reduction of exposures now, and better coordinated research in these areas. The evidence is sufficiently documented to warrant strong preventative action and new public safety limits that observe EMF/RFR levels shown to cause adverse effects.

All relevant environmental conditions should be given weight in defining and implementing prudent, precautionary actions to protect public health, including EMF and RFR. Evidence is sufficient to add EMF/RFR prominently to the list of exposures that can degrade the human genome, and impair normal development, health and quality of our physiology. With the rising numbers people with ASCs and other childhood health and developmental disorders, we cannot afford to ignore this component of risk to our children and vulnerable populations. When the risk factors are largely avoidable or preventable, ignoring clear evidence of large-scale health risks to global populations poses unnecessary and unacceptable risks. Taking this evidence as a call to action will be challenging and disruptive in the short term, but constructive in the longer term as we learn to use EMF/RFR in healthier ways.

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Attachment 1

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Public health implications of wireless technologies

Cindy Sage [a,*], David O. Carpenter [b]

[a] *Sage Associates, 1396 Danielson Road, Santa Barbara, CA 93108, USA*
[b] *Institute for Health and the Environment, University at Albany, Rensselaer, NY, USA*

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Abstract

Global exposures to emerging wireless technologies from applications including mobile phones, cordless phones, DECT phones, WI-FI, WLAN, WiMAX, wireless internet, baby monitors, and others may present serious public health consequences. Evidence supporting a public health risk is documented in the BioInitiative Report. New, biologically based public exposure standards for chronic exposure to low-intensity exposures are warranted. Existing safety standards are obsolete because they are based solely on thermal effects from acute exposures. The rapidly expanding development of new wireless technologies and the long latency for the development of such serious diseases as brain cancers means that failure to take immediate action to reduce risks may result in an epidemic of potentially fatal diseases in the future. Regardless of whether or not the associations are causal, the strengths of the associations are sufficiently strong that in the opinion of the authors, taking action to reduce exposures is imperative, especially for the fetus and children. Such action is fully compatible with the precautionary principle, as enunciated by the Rio Declaration, the European Constitution Principle on Health (Section 3.1) and the European Union Treaties Article 174.
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Keywords: Wireless technology; Brain cancer; Radiofrequency; Cell phones; Wireless antenna facilities; Childrens' health

1. Introduction and background

Exposure to electromagnetic fields (EMF) has been linked to a variety of adverse health outcomes that may have significant public health consequences [1–13]. The most serious health endpoints that have been reported to be associated with extremely low frequency (ELF) and/or RF include childhood and adult leukemia, childhood and adult brain tumors, and increased risk of the neurodegenerative diseases, Alzheimer's and amyotrophic lateral sclerosis (ALS). In addition, there are reports of increased risk of breast cancer in both men and women, genotoxic effects (DNA damage and micronucleation), pathological leakage of the blood–brain barrier, altered immune function including increased allergic and inflammatory responses, miscarriage and some cardiovascular effects [1–13]. Insomnia (sleep disruption) is reported in studies of people living in very low-intensity RF environments with WI-FI and cell tower-level exposures [85–93]. Short-term effects on cognition, memory and learning, behavior, reaction time, attention and concentration, and altered brainwave activity (altered EEG) are also reported in the scientific literature [94–107]. Biophysical mechanisms that may account for such effects can be found in various articles and reviews [136–144].

The public health implications of emerging wireless technologies are enormous because there has been a very rapid global deployment of both old and new forms in the last 15 years. In the United States, the deployment of wireless infrastructure has accelerated greatly in the last few years with 220,500 cell sites in 2008 [14–16]. Eighty-four percent of the population of the US own cell phones [16]. Annualized wireless revenues in 2008 will reach $144 billion and US spending on wireless communications will reach $212 billion by 2008. Based on the current 15% annual growth rate enjoyed by the wireless industry, in the next 5 years wireless will become a larger sector of the US economy than both the agriculture and automobile sectors. The annualized use of cell phones in the US is estimated to be 2.23 trillion minutes in 2008 [16]. There are 2.2 billion users of cell phones worldwide in 2008 [17] and many million more users of cordless phones.

Over 75 billion text messages were sent in the United States, compared with 7.2 billion in June 2005, according to

* Corresponding author. Tel.: +1 805 969 0557; fax: +1 805 969 5003.
E-mail address: sage@silcom.com (C. Sage).

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CTIA, the Wireless Association, the leading industry trade group [16]. The consumer research company Nielsen Mobile, which tracked 50,000 individual customer accounts in the second quarter of this year, found that Americans each sent or received 357 text messages a month then, compared with 204 phone calls. That was the second consecutive quarter in which mobile texting significantly surpassed the number of voice calls [17].

The Electronics Industries Alliance (EIA) represents 80% of the $550 billion US electronics industry "that provides two million jobs for American workers." Its members include companies from the consumer electronics and telecommunications industries, among others [17].

There is intense industry competition for market share. Telecom taxes form an immense revenue generator for the government sector. Sale of the airwaves (auctions selling off wireless bandwidth) is a multi-million dollar industry for governments, and multi-billion dollar global advertising budgets are common. Lobbying dollars from the telecom-related industries are estimated to be $300 million annually. The media is nearly silent on health issues, perhaps in part because of global advertising revenues that compromise journalistic independence and discourage balanced coverage of health, equity and economic issues.

2. Evidence supporting a public health risk

Even if there is only a small risk to health from chronic use of and exposure to wireless technologies, there is the potential for a profound public health impact. RF radiation now saturates the airwaves, resulting in exposure to both users and non-users. The effects are both short-term (sleep disruption, hormone disruption, impairment of cognitive function, concentration, attention, behavior, and well-being) and they are almost certainly long-term (generational impacts on health secondary to DNA damage, physiological stress, altered immune function, electrosensitivity, miscarriage risks, effects on sperm quality and motility leading to infertiility, increased rates of cancer, and neurological diseases including Alzheimer's disease and ALS—at least for ELF exposures). (Chapters 5–12 of the BioInitiative Report [1] and papers in this Supplement.)

There is credible scientific evidence that RF exposures cause changes in cell membrane function, metabolism and cellular signal communication, as well as activation of proto-oncogenes and triggering of the production of stress proteins at exposure levels below current regulatory limits. There is also generation of reactive oxygen species, which cause DNA damage, chromosomal aberrations and nerve cell death. A number of different effects on the central nervous system have also been documented, including activation of the endogenous opioid systems, changes in brain function including memory loss, slowed learning, motor dysfunction and performance impairment in children, and increased frequency of headaches, fatigue and sleep disorders. Melatonin secretion is reduced, resulting in altered circadian rhythms and disruption of several physiological functions. (Chapters 5–12 of the BioInitiative Report [1] and papers in this Supplement.)

These effects can reasonably be presumed to result in adverse health effects and disease with chronic and uncontrolled exposures, and children may be particularly vulnerable [1,19]. The young are also largely unable to remove themselves from such environments. Second-hand non-ionizing radiation, like second-hand smoke may be considered of public health concern based on the evidence at hand.

2.1. Malignant brain tumors

At present, the most persuasive evidence for cancer resulting from RF exposure is that there is a significantly increased risk of malignant glioma in individuals that have used a mobile phone for 10 or more years, with the risk being elevated only on the side of the head on which the phone is used regularly (ipsilateral use) [1,3,4,6–8,18]. While the risk for adults after 10 or more years of use is reported to be more than doubled, there is some evidence beginning to appear that indicates that the risk is greater if the individual begins to use a mobile phone at younger ages. Hardell et al. [18] reported higher odds ratios in the 20–29-year-old group than other age ranges after more than 5 years of use of either analog or cordless phones. Recently in a London symposium Hardell reported that after even just 1 or more years of use there is a 5.2-fold elevated risk in children who begin use of mobile phones before the age of 20 years, whereas for all ages the odds ratio was 1.4. Studies from Israel have found that the risk of parotid gland tumors (a salivary gland in the cheek) is increased with heavy cell phone use [7]. The risk of acoustic neuroma (a benign but space-occupying tumor on the auditory nerve) is also significantly increased on the ipsilateral side of the head after 10 or more years of mobile phone use [1,3]. This relationship has also been documented in some of the published reports of the WHO Interphone Study, a decade-long 13-country international assessment of cell phone risks and cancer [6,8].

Kundi reports that "(E)pidemiological evidence compiled in the last 10 years starts to indicate an increased risk, in particular for brain tumors (glioma, meningioma, acoustic neuroma), from mobile phone use. Considering biases that may have been operating in most studies the risk estimates are rather too low, although recall bias could have increased risk estimates. The net result, when considering the different errors and their impact is still an elevated risk" [19].

The latency for most brain tumors is 20 years or more when related to other environmental agents, for example, to X-ray exposure. Yet, for cell phone use the increased risks are occurring much sooner than twenty years, as early as 10 years for brain tumors in adults and with even shorter latencies in children. This suggests that we may currently be significantly underestimating the impact of current levels of

use of RF technology, since we do not know how long the average latency period really is. If it is 20 years, then the risk rate will likely be much higher than an overall doubling of risk for cell phone users if the peak comes later than 10 years. It may also signal very troubling risks for those who start using cell phones, and perhaps all wireless devices, in early childhood. We may not have proof of effect for decades until many hundreds of thousands of new cases of malignant gliomas are set in motion by long-term cell phone use.

The preliminary evidence that mobile phone use at younger ages may lead to greater risk than for older persons is of particular concern. There is a large body of evidence that childhood exposure to environmental agents poses greater risk to health than comparable exposure during adulthood [20,21]. There is reason to expect that children would be more susceptible to the effects of EMF exposure since they are growing, their rate of cellular activity and division is more rapid, and they may be more at risk for DNA damage and subsequent cancers. Growth and development of the central nervous system is still occurring well into the teenage years so that neurological changes may be of great importance to normal development, cognition, learning, and behavior.

A greater vulnerability of children to developing brain cancer from mobile phone use may be the consequence of a combination of patterns of use, stage of development and physical characteristics related to exposure. In addition to the fact that the brain continues to develop through the teen years, many young children and teenagers now spend very large periods of time using mobile phones. The brain is the main target organ of cell phones and cordless phones, with highest exposure to the same side as the phone is used. Further, due to anatomical reasons, the brain of a child is more exposed to RF radiation than the brain of an adult [22,23]. This is caused by the smaller brain size, a thinner pinna of the ear, thinner skin and thinner skull bone permitting deeper penetration into the child's brain. A recent French study showed that children absorb twice the RF from cell phone use as do adults [24].

In addition to concerns about cancer, there is evidence for short-term effects of RF exposure on cognition, memory and learning, behavior, reaction time, attention and concentration, altered brainwave activity (altered EEG) [95–108], and all of these effects argue for extreme caution with regard to exposure of children. The development of children into adults is characterized by faster cell division during growth, the long period needed to fully develop and mature all organ systems, and the need for properly synchronized neural development until early adulthood. Chronic, cumulative RF exposures may alter the normal growth and development of children and adversely affect their development and capacity for normal learning, nervous system development, behavior and judgment [1,97,102].

Prenatal exposure to EMF has been identified as a possible risk factor for childhood leukemia (1). Maternal use of cell phones has been reported to adversely affect fetal brain development, resulting in behavioral problems in those children by the time they reach school age [25]. Their exposure is involuntary in all cases. Children are largely unable to remove themselves from exposures to harmful substances in their environments.

2.2. Plausible biological mechanisms for a relationship between RF exposure and cancer

2.2.1. DNA damage and oxidative stress

Damage to DNA from ELF and from RF cell phone frequencies at very low intensities (far below FCC and ICNIRP safety limits) has been demonstrated in many studies [1,2,26–35]. Both single- and double-strand DNA damage have been reported by various researchers in different laboratories. This is damage to the human genome, and can lead to mutations which can be inherited, or which can cause cancer, or both.

Non-ionizing radiation is assumed to be of too low energy to cause direct DNA damage. However both ELF and RF radiation induce reactive oxygen species, free radicals that react with cellular molecules including DNA. Free-radical production and/or the failure to repair DNA damage (secondary to damage to the enzymes that repair damage) created by such exposures can lead to mutations. Whether it is greater free-radical production, reduction in anti-oxidant protection or reduced repair capacity, the result will be altered DNA, increased risk of cancer, impaired or delayed healing, and premature aging [36–54]. Exposures have also been linked to decreased melatonin production, which is a plausible biological mechanism for decreased cancer surveillance in the body, and increased cancer risk [34,39,44,46,47,49,50,54]. An increased risk of cancers and a decrease in survival has been reported in numerous studies of ELF and RF [55–69].

2.2.2. Stress proteins (heat shock proteins or HSP)

Another well-documented effect of exposure to low-intensity ELF and RF is the creation of stress proteins (heat shock proteins) that signal a cell is being placed under physiological stress) [70–80]. The HSP response is generally associated with heat shock, exposure to toxic chemicals and heavy metals, and other environmental insults. HSP is a signal of cells in distress. Plants, animals and bacteria all produce stress proteins to survive environmental stressors like high temperatures, lack of oxygen, heavy metal poisoning, and oxidative stress.

We can now add ELF and RF exposures to this list of environmental stressors that cause a physiological stress response. Very low-level ELF and RF exposures can cause cells to produce stress proteins, meaning that the cell recognizes ELF and RF exposures as harmful. This is another important way in which scientists have documented that ELF and RF exposures can be harmful, and it happens at levels far below the existing public safety standards. An additional concern is that if the stress goes on too long, the protective effect is diminished. The reduced response with prolonged exposure means the cell is less protected against

damage, and this is why prolonged or chronic exposures may be harmful, even at very low intensities.

2.2.3. RF-induced gene expression changes

Many environment agents cause diseases, including cancer, not by direct damage to DNA but rather by up- or down-regulation of genes that regulate cell growth and function. Usually there are many genes whose expression is changed, and it is difficult to determine the exact changes responsible for the disease. Both ELF and RF exposures have been shown to result in altered gene expression. Olivares-Banuelos et al. [81] found that ELF exposure of chromaffin cells resulted in changed expression of 53 transcripts. Zhao et al. [82] investigated the gene expression profile of rat neurons exposed to 1800 MHz RF fields (2 W/kg) and found 24 up-regulated genes and 10 down-regulated genes after a 24-h exposure. The altered genes were involved in multiple cellular functions including cytoskeleton, signal transduction pathways and metabolism. Kariene et al. [83] exposed human skin to mobile phone radiation, and found by punch biopsy that 8 proteins were significantly altered in expression, consistent with gene induction. Several other studies have found altered gene expression following RF exposure, although none have been found that explain specific disease states [84].

DNA activation at very low ELF and RF levels, as in the stress response, and DNA damage (strand breaks and micronuclei) at higher levels, are molecular precursors to changes that are believed to lead to cancer. These, along with gene induction, provide plausible biological mechanisms linking exposure to cancer.

The biochemical pathways that are activated are the same for ELF and for RF exposures, and are non-thermal (do not require heating or induced electrical currents). This is true for the stress response, DNA damage, generation of reactive oxygen species as well as gene induction. Thus it is not surprising that the major cancers resulting from exposure to ELF and RF are the same, namely leukemia and brain cancer. The safety standards for both ELF and RF, based on protection from heating, are irrelevant and not protective. ELF exposure levels of only 5–10 mG have been shown to activate the stress response genes (http://www.bioinitiative.org, Sections 1 and 7 [1]).

3. Sleep, cognitive function and performance

The relationship of good sleep to cognition, performance and healing is well recognized. Sleep is a profoundly important factor in proper healing, anti-inflammatory benefits, reduction in physical symptoms of such as tendonitis, over-use syndrome, fatigue-induced lethargy, cognition and learning. Incomplete or slowed physiological recovery is common when sleep is impaired. Circadian rhythms that normalize stress hormone production (cortisol, for example) depend on synchronized sleep patterns.

People who are chronically exposed to low-level wireless antenna emissions report symptoms such as problems in sleeping (insomnia), as well as other symptoms that include fatigue, headache, dizziness, grogginess, lack of concentration, memory problems, ringing in the ears (tinnitus), problems with balance and orientation, and difficulty in multi-tasking [85–93,99]. In children, exposures to cell phone radiation have resulted in changes in brain oscillatory activity during some memory tasks [97,102]. Cognitive impairment, loss of mental concentration, distraction, speeded mental function but lowered accuracy, impaired judgment, delayed reaction time, spatial disorientation, dizziness, fatigue, headache, slower motor skills and reduced learning ability in children and adults have all been reported [85–108].

These symptoms are more common among “electrosensitive” individuals, although electrosensitivity has not been documented in double-blind tests of individual identifying themselves as being electrosensitive as compared to controls [109,110]. However people traveling to laboratories for testing are pre-exposed to a multitude of RF and ELF exposures, so they may already be symptomatic prior to actual testing. There is also evidence that RF exposures testing behavioral changes show delayed results; effects are observed after termination of RF exposure. This suggests a persistent change in the nervous system that may be evident only after time has passed, so is not observed during a short testing period.

3.1. Plausible biological mechanisms for neurobehavioral effects

3.1.1. The melatonin hypothesis

While there remains controversy as to the degree that RF and ELF fields alter neurobehavioral function, emerging evidence provides a plausible mechanism for both effects on sleep and cognition. Sleep is controlled by the central circadian oscillator in the suprachiasmatic nucleus, located in the hypothalamus. The activity of this central circadian oscillator is, in turn, controlled by the hormone, melatonin, which is released from the pineal gland [111]. There is considerable evidence that ELF exposure reduces the release of melatonin from the pineal gland—see Section 12 of the Bioinitiative Report [1]. There has been less study of the effects of RF exposure on melatonin release, but investigations have demonstrated a reduced excretion of the urinary metabolite of melatonin among persons using a mobile phone for more than 25 min per day [112]. In a study of women living near to radio and television transmitters, Clark et al. [113] found no effect on urinary melatonin metabolite excretion among pre-menopausal women, but a strong effect in post-menopausal women.

The “melatonin hypothesis” also provides a possible basis for other reported effects of EMFs. Melatonin has important actions on learning and memory, and inhibits electrophysiological components of learning in some but not all areas of the brain [114,115]. Melatonin has properties as a free-radical scavenger and anti-oxidant [116], and consequently,

a reduction in melatonin levels would be expected to increase susceptibility to cancer and cellular damage. Melatonin could also be the key to understanding the relationship between EMF exposure and Alzheimer's disease. Noonan et al. [117] reported that there was an inverse relationship between excretion of the melatonin metabolite and the 1–42 amino acid form of amyloid beta in electric utility workers. This form of amyloid beta has been found to be elevated in Alzheimer's patients.

3.1.2. Blood–brain barrier alterations

Central nervous system effects of EMFs may also be secondary to damage to the blood–brain barrier (BBB). The blood–brain barrier is a critical structure that prevents toxins and other large molecules that are in peripheral blood from having access to the brain matter itself. Salford et al. [118] have reported that a 2-h exposure of rats to GSM-900 radiation with a SAR of 2–200 mW/kg resulted in nerve cell damage. In a follow-up study, Eberhardt et al. report that 2-h exposures to cell phone GSM microwave RF resulted in leakage of albumin across the blood–brain barrier and neuronal death [119]. Neuronal albumin uptake was significantly correlated to occurrence of damaged neurons when measured at 28 days post-exposure. The lowest exposure level was 0.12 mW/kg (0.00012 W/kg) for 2 h. The highest exposure level was 120 mW/kg (0.12 W/kg). The weakest exposure level showed the greatest effect in opening the BBB [118]. Earlier blood–brain studies by Salford and Schirmacher [120,121] report similar effects.

4. What are sources of wireless radiation?

There are many overlapping sources of radiofrequency and microwave emissions in daily life, both from industrial sources (like cell towers) and from personal items [cell and cordless phones, personal digital assistants (PDAs), wireless routers, etc.]. Published data on typical levels found in some cities and from some sources are available at http://www.bioinitiative.org [1,122–124].

Cell phones are the single most important source of radiofrequency radiation to which we are exposed because of the relatively high exposure that results from the phone being held right against the head. Cell phones produce two types of emissions that should be considered. First, the radiofrequency radiation (typically microwave frequency radiation) is present. However, there is also the contribution of the switching battery pack that produces very high levels of extremely low frequency electromagnetic field [125–127].

Cordless telephones have not been widely recognized as similar in emissions to cell phones, but they can and do produce significant RF exposures. Since people tend to use them as substitutes for in-home and in-office corded or traditional telephones, they are often used for long periods of time. As the range of cordless phones has increased (the distance away that you can carry on a conversation is related to the power output of the phone), the more powerful the RF signal will be. Hence, newer cordless phones may in some cases be similar to the power output of cell phones. The cumulative emissions from cell and cordless phones taken together should be recognized when considering the relative risks of wireless communication exposures.

PDAs such as the BlackBerry, Treo and iPhone units are 'souped-up' versions of the original voice communication devices (cell phones). The often produce far higher ELF emissions than do cell phones because they use energy from the battery very intensively for powering color displays and during data transmission functions (email, sending and receiving large files, photos, etc.) [125–127]. ELF emissions have been reported from PDAs at several tens to several hundreds of milligauss. Evidence of significantly elevated ELF fields during normal use of the PDA has public health relevance and has been reported in at least three scientific papers [125,128,129]. In the context of repetitive, chronic exposure to significantly elevated ELF pulses from PDAs worn on the body, relevant health studies point to a possible relationship between ELF exposure and cancer and pregnancy outcomes [130–133].

We include discussion of the ELF literature for two reasons. As mentioned above ELF activates the same biology as RF, it contributes to the total EMF burden of the body. In addition, PDAs and cell phones emit both radiofrequency/microwave radiation (RF) and extremely low frequency ELF from the battery switching of the device (the power source). Studies show that some devices produce excessively high ELF exposures during voice and data transmission. ELF is already classified as a 2B (Possible) Carcinogen by IARC, which means that ELF is indisputably an issue to consider in the wireless technology debate. ELF has been classified as a Group 2B carcinogen for all humans, not just children. The strongest evidence came from epidemiological studies on childhood leukemia, but the designation applies to all humans, both adults and children [1,25].

Wireless headsets that allow for conversations with cell phones at a distance from the head itself reduce the emissions. Depending on the type of wireless device, they may operate (transmit signal) only during conversations or they may be operational continuously. The cumulative dose of wireless headsets has not been well characterized under either form of use. Substantial cumulative RF exposure would be expected if the user wears a wireless headset that transmits a signal continuously during the day. However a critical factor is where the cell phone is placed. If worn on a belt with a headset, the exposure to the brain is reduced but the exposure to the pelvis may be significant.

Cell towers (called "masts" in Europe and Scandinavian countries) are wireless antenna facilities that transmit the cell phone signals within communities. They are another major source of RF exposures for the public. They differ from RF exposures from wireless devices like cell phones in that they produce much lower RF levels (generally 0.05 to 1–2 $\mu W/cm^2$ in the first several hundred feet around them) in comparison to several hundred microwatts per centimeter

squared for a cell phone held at the head. However they create a constant zone of elevated RF for up to 24 h per day. many hours per day, and the exposure is whole body rather than localized at the head. These facilities are the distribution system for wireless voice communications, internet connections and data transmission within communities. They are often erected on free-standing towers. They may be constructed on telephone poles or electrical poles. They may be built into the façade or rooftops of buildings behind wood screening. These are called stealth installations for wireless antenna facilities. Some installations are camouflaged to resemble 'false trees or rocks'. They emit RF to provide cell service to specific "cells" or locations that receive the signal.

Other forms of wireless transmission that are common in areas providing cell service are wireless land area networks (WLAN), (WiMAX) and WIFI networks. Some cities are installing city-wide WIFI service to allow any user on the street to log into the internet (without cables or wire connections). WIFI installations may have a signal reach for a few hundred feet where WiMAX installations may transmit signal more than 10 miles, so produce a stronger RF emission for those in close proximity. Each type has its particular signal strength and intended coverage area, but what they have in common is the production of continuous RF exposure for those within the area. We do not know what the cumulative exposure (dose) might be for people living, working or going to school in continuously elevated RF fields, nor are the possible health implications yet known. However, based on studies of populations near cell sites in general, there is a constellation of generally observed health symptoms that are reported to occur [85–107]. In this regard it is important to note that children living near to AM radio transmitters have been found to elevated risks of leukemia [134,135]. While AM radio RF fields are lower in frequency than that common in mobile phones, this is a total body irradiation with RF. The fact that leukemia, not brain cancer, is apparent in these studies suggests that leukemia is the cancer seen at the lowest levels of both ELF and RF fields under the circumstances of whole-body exposure.

Commercial surveillance systems or security gates pose an additional source of strong RF exposures. They are ubiquitous in department stores, markets and shops at the entry and exit points to discourage shoplifting and theft of goods. Security gates can produce excessively high RF exposures (although transitory) and have been associated with interference with pacemakers in heart patients. The exposure levels may approach thermal public safety limits in intensity, although no one expects a person to stand between the security gate bars for more than 6 min (safety limits for uncontrolled public access are variable depending on the frequency, but are all averaged over a 6-min exposure period).

RFID chips (radiofrequency identification chips) are being widely used to track purchases and for security of pets, and in some cases to keep track of patients with Alzheimer's disease and of children. RFID chips are implanted in fabrics, inserted in many types of commercial goods, and can be implanted under the skin. They create a detectable signal to track the location of people and goods.

5. Problems with existing public health standards (safety limits)

If the existing standards were adequate none of the effects documented above should occur at levels to which people are regularly exposed. The fact that these effects are seen with our current ambient levels of exposure means that our existing public safety standards are obsolete. It also means that new, biologically based public exposure standards for wireless technologies are urgently needed. Whether it is feasible to achieve low enough levels that still work and also protect health against effects of chronic RF exposure – for all age groups – is uncertain. Whether we can protect the public and still allow the kinds of wireless technology uses we see today is unknown.

The nature of electromagnetic field interactions with biological systems has been well studied [136–144]. For purposes of standard-setting processes for both ELF and RF, the hypothesis that tissue damage can result only from heating is the fundamental flaw in the misguided efforts to understand the basic biological mechanisms leading to health effects.

The thermal standard is clearly untenable as a measure of dose when EMF stimuli that differ by many orders of magnitude in energy can stimulate the same biological response. In the ELF range, the same biological changes occur as in the RF, and no change in temperature can even be detected. With DNA interactions the same biological responses are stimulated in ELF and RF ranges even though the frequencies of the stimuli differ by many orders of magnitude. The effects of EMF on DNA to initiate the stress response or to cause molecular damage reflect the same biology in different frequency ranges. For this reason it should be possible to develop a scale based on DNA biology, and use it to define EMF dose in different parts of the EM spectrum. We also see a continuous scale in DNA experiments that focus on molecular damage where single and double strand breaks have long been known to occur in the ionizing range, and recent studies have shown similar effects in both ELF and RF ranges [144].

Existing standard-setting bodies that regulate wireless technologies, assume that there are no bioeffects of concern at exposure levels that do not cause measurable heating. However, it has been established beyond any reasonable doubt that bioeffects and some adverse health effects occur at far lower levels of RF and ELF exposure where no heating (or induced current) occurs; some effects are shown to occur a thousand times or more below the existing public safety limits. New, biologically based public exposure limits are urgently needed. New wireless technologies for cell and cordless phones, other wireless communication and data transmission systems affect living organisms in new ways that our antiquated safety limits have not foreseen, nor protected against.

The exposure of children to electromagnetic fields has not been studied extensively; in fact, the Federal Communications Commission (FCC) standards for exposure to radiofrequency radiation are based on the height, weight and stature of a 6-foot tall man, not scaled to children or adults of smaller stature. They do not take into account the unique susceptibility of growing children to exposures, nor are there studies of particular relevance to children.

In addition there is a problem in the consideration of the level of evidence taken into consideration by these bodies. There have not been adequate animal models shown to have cancer as an endpoint, and a perception that no single mechanism is proven to explain these associations. Thus these committees have tended to ignore or minimize the evidence for direct hazard to humans, and believe there is no proof of cause and effect. These bodies assume from the beginning that only conclusive scientific evidence (absolute proof) will be sufficient to warrant change, and refuse to take action on the basis of a growing body of evidence which provides early but consequential warning of risks.

The Radiofrequency Interagency Working Group of the US governmental agencies involved in RF matters (RFIAWG) issued a Guidelines Statement in June of 1999 that concluded the present RF standard "may not adequately protect the public" [145]. The RFIAWG identified fourteen (14) issues that they believe are needed in the planned revisions of ANSI/IEEE RF exposure guidelines including "to provide a strong and credible rationale to support RF exposure guidelines". In particular, the RFIAWG criticized the existing standards as not taking into account chronic, as opposed to acute exposures, modulated or pulsed radiation (digital or pulsed RF is proposed at this site), time-averaged measurements that may erase the unique characteristics of an intensity-modulated RF radiation that may be responsible for reported biologic effects, and stated the need for a comprehensive review of long-term, low-level exposure studies, neurological-behavioral effects and micronucleus assay studies (showing genetic damage from low-level RF) [145]. This important document from relevant US agencies questions existing standards in the following ways: (a) selection of an adverse effect level for chronic exposures not based on tissue heating and considering modulation effects; (b) recognition of different safety criteria for acute and chronic exposures at non-thermal or low-intensity levels; (c) recognition of deficiencies in using time-averaged measurements of RF that does not differentiate between intensity-modulated RF and continuous wave (CW) exposure, and *therefore may not adequately protect the public*; (d) having standards based on adult males rather than considering children to be the most vulnerable group.

6. Prudent public health responses

Emerging environmental health problems require preventative public health responses even where scientific and medical uncertainties still exist, but where policy decisions today may greatly reduce human disease and societal costs tomorrow.

Policy decisions in public health must address some amount of uncertainty when balancing likely benefits and estimated costs. Although new insight will allow better appreciation of difficult issues, such as those occurring in environmental and occupational health, an expanded perspective may also enlarge the list of problems that need to be managed. Ignoring the problems carries its own costs (as deferring a decision is a decision in itself). With environmental and other public health problems becoming increasingly complex and international in scope, scientific documentation alone rarely justifies simple solutions [146].

Social issues regarding the controversy over public and occupational exposures to ELF and RF center on the resolute adherence to existing ICNIRP and FCC/IEEE standards by many countries, in the face of growing scientific evidence of health risks at far lower levels [10]. The composition of these committees, usually with excessive representation of the physics and engineering communities rather than public health professionals, results in a refusal to adopt biologically based exposure standards. Furthermore, there is widespread belief that governments are ignoring this evidence and there is widespread distrust of and lack of confidence in governments and their health agencies. The basis on which most review bodies and standard-setting agencies have avoided the conclusion that the science is strong enough to warrant new safety limits for ELF and RF is to require a demonstration of absolute proof before taking action. A causal level of evidence, or scientific certainty standard is implicit in nearly all reviews of the ELF and RF science, although this runs counter to good public health protection policies.

There is no question that global implementation of the safety standards proposed in the Bioinitiative Report, if implemented abruptly and without careful planning, have the potential to not only be very expensive but also disruptive of life and the economy as we know it. Action must be a balance of risk to cost to benefit. The major risk from maintaining the status quo is an increasing number of cancer cases, especially in young people, as well as neurobehavioral problems at increasing frequencies. The benefits of the status quo are expansion and continued development of communication technologies. But we suspect that the true costs of even existing technologies will only become much more apparent with time. Whether the costs of remedial action are worth the societal benefits is a formula that should reward precautionary behavior. Prudent corporate policies should be expected to address and avoid future risks and liabilities, otherwise, there is no market incentive to produce safe (and safer) products.

The deployment of new technologies is running ahead of any reasonable estimation of possible health impacts and estimates of probabilities, let alone a solid assessment of risk. However, what has been missing with regard to EMF has been an acknowledgement of the risk that is demonstrated by

the scientific studies. There is clear evidence of risk, although the magnitude of the risk is uncertain, and the magnitude of doing nothing on the health effects cost to society is similarly uncertain. This situation is very similar to our history of dealing with the hazards of smoking decades ago, where the power of the industry to influence governments and even conflicts of interest within the public health community delayed action for more than a generation, with consequent loss of life and enormous extra health care costs to society. New standards are warranted now, based on the totality of scientific evidence; the risks of taking no-action, the large population at risk, costs associated with ignoring the problem in new and upgraded site selection and construction, and the loss of public trust by ignoring the problem.

Direct medical and rehabilitative health costs associated with treatment for diseases that are reasonably related to wireless technologies may be very large. Although there is uncertainty involved in how much disease is related to wireless exposures, the mere scale of the problem with several billion users of cell phones and even larger impacts on bystander populations (from cell site exposures, from other WI-FI and wireless exposures in-home and commercial use, etc.) the associated public health costs will likely be monumental. Furthermore the costs to families with cancers, neurological diseases or learning disabilities in children related in part or in whole to wireless technologies extend beyond medical costs. They may reasonably extend to family disruption and family psychological problems, losses in job productivity and income loss.

The history of governments and their official health agencies to deal with emerging and newly identified risks to health is not good [147–149]. This is particularly true where industry investments in new products and technologies occur without full recognition, disclosure or even knowledge of possible health consequences. Large economic investments in polluting industries often make for perilously slow regulatory action, and the public health consequences may be very great as a result [150,151].

Free markets do not internalize the costs to society of "guessing wrong". Unexpected or hidden health costs of new technologies may not be seen for many years, when the ability to recall or to identify the precise exposures related to disease outcomes is difficult or impossible. The penalty nearly always falls to the individual, the family or the taxpayer and not to the industry that benefits economically—at least in free-market economies. Thus, the profits go to industry but the costs may go to the individual who can suffer both diminished quality of life and health and economic disadvantage. If all disease endpoints that may be reasonably related to chronic exposure to electromagnetic fields are considered even a small attributable fraction for one or more industries, it will have enormous global impact on public health. The public health implications are immense. But they can be reduced by strong government and public health interventions providing information on alternatives to wireless technologies, public education campaigns, health advisories, requirements for redesign of wireless devices, proscription of use of wireless devices by children and teenagers, strong and independent research programs on causes and prevention of EMF-related diseases, and consultation with all stakeholders on issues relating to involuntary exposures (bystander or second-hand radiation exposures from wireless technologies) (Table 1).

Table 1
Public health implications of wireless technologies argue for change in governmental and health agency actions.

Secure US and EU legislative mandates for safer technologies for communication and data transmission, for security and surveillance needs.
Promote wired alternatives for voice and data communication (cable, fiber-optic)
Discourage or ban use of cell phones by children and young teen-agers
Provide permanent (unremovable) labels on cell phones "Not for use by children under the age of 16"
Implement national public education campaigns on health issues (cell phones, cordless phones, PDAs, wireless internet, city-wide WI-FI, WLAN and WiMAX exposures
Promote industry redesign for safer products: support innovation for alternatives and solutions
Slow or stop deployment of wireless technologies to discourage reliance on wireless technologies for communication and security needs
Put the burden of proof on industry to show "new wireless tech" is safe before deployment
Adopt and enforce restricted use areas for sensitive or more vulnerable segments of society including low-EMF environments in public areas and "No Cell" zones in airports, hospitals, schools
Acknowledge FCC and ICNIRP thermal safety standards are obsolete for wireless technologies
Appoint new standard-setting bodies familiar with biological effects to develop new guidelines for public safety limits.
Develop new biologically based standards that address low-intensity, chronic exposures
Require standard of evidence and level of proof = public health
Reject "causal" standard of evidence for taking action on science
Make industry financially liable for "guessing wrong" and ignoring health risks

The scientific information contained in this Supplement argues for thresholds or guidelines that are substantially below current FCC and ICNIRP standards for localized exposures to wireless devices and for whole-body exposure. Uncertainty about how low such standards might have to go to be prudent from a public health standpoint should not prevent reasonable efforts to respond to the information at hand. No lower limit for bioeffects and adverse health effects from RF has been established, so the possible health risks of wireless WLAN and WI-FI systems, for example, will require further research. No assertion of safety at any level of wireless exposure (chronic exposure) can be made at this time. The lower limit for reported human health effects has dropped 100-fold below the safety standard (for mobile phones and PDAs); 1000–10,000-fold for other wireless (cell towers at distance; WI-FI and WLAN devices). The entire basis for safety standards is called into question, and it is not unreasonable to question the safety of RF at any level.

It is likely that for both ELF and RF, as for other carcinogens, there is no threshold of exposure that is without risk, but the magnitude of the risk increases linearly with the level of exposure. Our society will not go back to the pre-electric and pre-wireless age, but the clear evidence of health hazards to the human population from exposure mandates that we develop ways in which to reduce exposure through education, new technologies and the establishment of biomedically based standards.

7. Conclusions and recommended actions

New ELF limits are warranted based on a public health analysis of the overall existing scientific evidence. These limits should reflect environmental levels of ELF that have been demonstrated to increase risk for childhood leukemia, and possibly other cancers and neurological diseases. ELF limits should be set below those exposure levels that have been linked in childhood leukemia studies to increased risk of disease, plus an additional safety factor. It is no longer acceptable to build new power lines and electrical facilities that place people in ELF environments that have been determined to be risky. These levels are in the 2–4 milligauss (mG) range (0.2–0.4 μT), not in the 10 s of mG or 100 s of mG. The existing ICNIRP limit is 1000 mG (100 μT) and 904 mG (90.4 μT) in the US for ELF is outdated and based on faulty assumptions. These limits are can no longer be said to be protective of public health and they should be replaced. A safety buffer or safety factor should also be applied to a new, biologically based ELF limit, and the conventional approach is to add a safety factor lower than the risk level.

While new ELF limits are being developed and implemented, a reasonable approach would be a 1 mG (0.1 μT) planning limit for habitable space adjacent to all new or upgraded power lines and a 2 mG (0.2 μT) limit for all other new construction. It is also recommended that a 1 mG (0.1 μT) limit be established for existing habitable space for children and/or women who are pregnant (because of the possible link between childhood leukemia and *in utero* exposure to ELF). This recommendation is based on the assumption that a higher burden of protection is required for children who cannot protect themselves, and who are at risk for childhood leukemia at rates that are traditionally high enough to trigger regulatory action. This situation in particular warrants extending the 1 mG (0.1 μT) limit to existing occupied space. “Establish” in this case probably means formal public advisories from relevant health agencies. While it is not realistic to reconstruct all existing electrical distribution systems, in the short-term; steps to reduce exposure from these existing systems need to be initiated, especially in places where children spend time, and should be encouraged. These limits should reflect the exposures that are commonly associated with increased risk of childhood leukemia (in the 2–5 mG (0.2–0.5 μT) range for all children, and over 1.4 mG (0.14 μT) for children age 6 and younger). Nearly all of the occupational studies for adult cancers and neurological diseases report their highest exposure category is 4 mG (0.4 μT) and above, so that new ELF limits should target the exposure ranges of interest, and not necessarily higher ranges.

Avoiding chronic ELF exposure in schools, homes and the workplace above levels associated with increased risk of disease will also avoid most of the possible bioactive parameters of ELF discussed in the relevant literature.

It is not prudent public health policy to wait any longer to adopt new public safety limits for ELF. These limits should reflect the exposures that are commonly associated with increased risk of childhood leukemia (in the 2–5 mG (0.2–0.5 μT) range for all children, and over 1.4 mG (0.14 μT) for children age 6 and younger). Avoiding chronic ELF exposure in schools, homes and the workplace above levels associated with increased risk of disease will also avoid most of the possible bioactive parameters of ELF discussed in the relevant literature.

The rapid deployment of new wireless technologies that chronically expose people to pulsed RF at levels reported to cause bioeffects, which in turn, could reasonably be presumed to lead to serious health impacts, is a public health concern. There is suggestive to strongly suggestive evidence that RF exposures may cause changes in cell membrane function, cell communication, metabolism, activation of proto-oncogenes and can trigger the production of stress proteins at exposure levels below current regulatory limits. Resulting effects can include DNA breaks and chromosome aberrations, cell death including death of brain neurons, increased free-radical production, activation of the endogenous opioid system, cell stress and premature aging, changes in brain function including memory loss, retarded learning, performance impairment in children, headaches and fatigue, sleep disorders, neurodegenerative conditions, reduction in melatonin secretion and cancers (BioInitiative Report Chapters 5–10, 12) [1].

This information now argues for thresholds or guidelines that are substantially below current FCC and ICNIPR standards for whole-body exposure. Uncertainty about how low such standards might have to go to be prudent from a public health standpoint should not prevent reasonable efforts to respond to the information at hand. No lower limit for bioeffects and adverse health effects from RF has been established, so the possible health risks of wireless WLAN and WI-FI systems, for example, will require further research and no assertion of safety at any level of wireless exposure (chronic exposure) can be made at this time. The lower limit for reported human health effects has dropped 100-fold below the safety standard (for mobile phones and PDAs); 1000–10,000-fold for other wireless (cell towers at distance; WI-FI and WLAN devices). The entire basis for safety standards is called into question, and it is not unreasonable to question the safety of RF at any level.

A cautionary target level for pulsed RF exposures for ambient wireless that could be applied to RF sources from cell tower antennas, WI-FI, WI-MAX and other similar sources

is proposed. The recommended cautionary target level is 0.1 microwatts per centimeter squared ($\mu W/cm^2$) (or 0.614 V per meter or V/m) for pulsed RF where these exposures affect the general public; this advisory is proportionate to the evidence and in accord with prudent public health policy. A precautionary limit of 0.1 $\mu W/cm^2$ should be adopted for outdoor, cumulative RF exposure. This reflects the current RF science and prudent public health response that would reasonably be set for pulsed RF (ambient) exposures where people live, work and go to school. This level of RF is experienced as whole-body exposure, and can be a chronic exposure where there is wireless coverage present for voice and data transmission for cell phones, pagers and PDAs and other sources of radiofrequency radiation. An outdoor precautionary limit of 0.1 $\mu W/cm^2$ would mean an even lower exposure level inside buildings, perhaps as low as 0.01 $\mu W/cm^2$. Some studies and many anecdotal reports on ill health have been reported at lower levels than this; however, for the present time, it could prevent some of the most disproportionate burdens placed on the public nearest to such installations. Although this RF target level does not preclude further rollout of WI-FI technologies, we also recommend that wired alternatives to WI-FI be implemented, particularly in schools and libraries so that children are not subjected to elevated RF levels until more is understood about possible health impacts. This recommendation should be seen as an interim precautionary limit that is intended to guide preventative actions; and more conservative limits may be needed in the future.

Broadcast facilities that chronically expose nearby residents to elevated RF levels from AM, FM and television antenna transmission are also of public health concern given the potential for very high RF exposures near these facilities (antenna farms). RF levels can be in the 10 s to several 100 s of $\mu W/cm^2$ in residential areas within half a mile of some broadcast sites (for example, Lookout Mountain, Colorado and Awbrey Butte, Bend, Oregon). Like wireless communication facilities, RF emissions from broadcast facilities that are located in, or expose residential populations and schools to elevated levels of RF will very likely need to be re-evaluated for safety.

For emissions from wireless devices (cell phones, personal digital assistant or PDA devices, etc.) there is enough evidence for increased risk of brain tumors and acoustic neuromas now to warrant intervention with respect to their use. Redesign of cell phones and PDAs could prevent direct head and eye exposure, for example, by designing new units so that they work only with a wired headset or on speakerphone mode.

These effects can reasonably be presumed to result in adverse health effects and disease with chronic and uncontrolled exposures, and children may be particularly vulnerable. The young are also largely unable to remove themselves from such environments. Second-hand radiation, like second-hand smoke is an issue of public health concern based on the evidence at hand.

In summary, the following recommendations are made:

- ELF limits should be set below those exposure levels that have been linked in childhood leukemia studies to increased risk of disease, plus an additional safety factor. It is no longer acceptable to build new power lines and electrical facilities that place people in ELF environments that have been determined to be risky (at levels generally at 2 mG (0.2 μT) and above).
- While new ELF limits are being developed and implemented, a reasonable approach would be a 1 mG (0.1 μT) planning limit for habitable space adjacent to all new or upgraded power lines and a 2 mG (0.2 μT) limit for all other new construction, It is also recommended for that a 1 mG (0.1 μT) limit be established for existing habitable space for children and/or women who are pregnant. This recommendation is based on the assumption that a higher burden of protection is required for children who cannot protect themselves, and who are at risk for childhood leukemia at rates that are traditionally high enough to trigger regulatory action. This situation in particular warrants extending the 1 mG (0.1 μT) limit to existing occupied space. “Establish” in this case probably means formal public advisories from relevant health agencies.
- While it is not realistic to reconstruct all existing electrical distributions systems, in the short-term; steps to reduce exposure from these existing systems need to be initiated and should be encouraged, especially in places where children spend time.
- A precautionary limit of 0.1 $\mu W/cm^2$ (which is also 0.614 V per meter) should be adopted for outdoor, cumulative RF exposure. This reflects the current RF science and prudent public health response that would reasonably be set for pulsed RF (ambient) exposures where people live, work and go to school. This level of RF is experienced as whole-body exposure, and can be a chronic exposure where there is wireless coverage present for voice and data transmission for cell phones, pagers and PDAs and other sources of radiofrequency radiation. Some studies and many anecdotal reports on ill health have been reported at lower levels than this; however, for the present time, it could prevent some of the most disproportionate burdens placed on the public nearest to such installations. Although this RF target level does not preclude further rollout of WI-FI technologies, we also recommend that wired alternatives to WI-FI be implemented, particularly in schools and libraries so that children are not subjected to elevated RF levels until more is understood about possible health impacts. This recommendation should be seen as an interim precautionary limit that is intended to guide preventative actions; and more conservative limits may be needed in the future.

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Attachment 2

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Review

Mast cell activation and autism☆

Theoharis C. Theoharides [a,b,c,d,e,*], Asimenia Angelidou [a,e], Konstantinos-Dionysios Alysandratos [a,e], Bodi Zhang [a,b], Shahrzad Asadi [a], Konstantinos Francis [f], Elena Toniato [g], Dimitrios Kalogeromitros [e]

[a] Molecular Immunopharmacology and Drug Discovery Laboratory, Department of Pharmacology and Experimental Therapeutics, Tufts University School of Medicine, Boston, MA 02111, USA
[b] Department of Biochemistry, Tufts University School of Medicine, Boston, MA 02111, USA
[c] Department of Internal Medicine, Tufts University School of Medicine and Tufts Medical Center, Boston, MA 02111, USA
[d] Department of Psychiatry, Tufts University School of Medicine and Tufts Medical Center, Boston, MA 02111, USA
[e] Allergy Clinical Research Center, Allergy Section, Attikon General Hospital, University of Athens Medical School, Athens 12462, Greece
[f] Child Psychiatry Section, Second Department of Psychiatry, Attikon General Hospital, University of Athens Medical School, Athens 12462, Greece
[g] Department of Experimental Medicine and Oncology, Chieti Medical Center, Chieti 67100, Italy

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ABSTRACT

Autism spectrum disorders (ASD) are neurodevelopmental disorders characterized by varying degrees of dysfunctional communication and social interactions, repetitive and stereotypic behaviors, as well as learning and sensory deficits. Despite the impressive rise in the prevalence of autism during the last two decades, there are few if any clues for its pathogenesis, early detection or treatment. Increasing evidence indicates high brain expression of pro-inflammatory cytokines and the presence of circulating antibodies against brain proteins. A number of papers, mostly based on parental reporting on their children's health problems, suggest that ASD children may present with "allergic-like" problems in the absence of elevated serum IgE and chronic urticaria. These findings suggest non-allergic mast cell activation, probably in response to environmental and stress triggers that could contribute to inflammation. *In utero* inflammation can lead to preterm labor and has itself been strongly associated with adverse neurodevelopmental outcomes. Premature babies have about four times higher risk of developing ASD and are also more vulnerable to infections, while delayed development of their gut–blood–brain barriers makes exposure to potential neurotoxins likely. Perinatal mast cell activation by infectious, stress-related, environmental or allergic triggers can lead to release of pro-inflammatory and neurotoxic molecules, thus contributing to brain inflammation and ASD pathogenesis, at least in a subgroup of ASD patients. This article is part of a Special Issue entitled: Mast cells in inflammation.

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1. Prevalence and characteristics of autism spectrum disorders

Autism spectrum disorders (ASD) are pervasive developmental disorders that include autistic disorder, Asperger's disorder and pervasive developmental disorder-not otherwise specified (PDD-NOS) [1]. They are characterized by stereotypic behaviors, variable deficits in language and social skills and a wide range of other behavioral problems. ASD manifest during childhood and at least 30% present with sudden clinical regression of development around 3 years of age [2,3]. Over the last 20 years, there has been an impressive rise in ASD with current prevalence estimates being about 1/100 children [4,5].

In the majority of cases, the cause of ASD is unknown [6], although some possible autism susceptibility genes have been identified [7] and

Abbreviations: ASD, autism spectrum disorders; BDNF, brain-derived neurotrophic factor; BBB, blood–brain barrier; CGRP, calcitonin-gene related peptide; CRH, corticotropin-releasing hormone; CSF, cerebrospinal fluid; FcεRI, high affinity IgE receptor; GI, gastrointestinal; IFN, interferon; LPS, lipopolysaccharide; M-CHAT, Modified Checklist for Autism in Toddlers; MCP-1, chemoattractant protein-1; MIF, macrophage inhibitory factor; NGF, nerve growth factor; NK cells, natural killer cells; NT, neurotensin; PCB, polychlorinated biphenyl; PDD-NOS, pervasive developmental disorder-not otherwise specified; SP, substance P; TGF-β1, transforming growth factor-beta1; TLR, toll-like receptor; TNF, tumor necrosis factor; UP, urticaria pigmentosa; VEGF, vascular endothelial growth factor; VIP, vasoactive intestinal peptide

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* Corresponding author. Molecular Immunopharmacology and Drug Discovery Laboratory, Department of Pharmacology and Experimental Therapeutics, Tufts University School of Medicine, 136 Harrison Avenue, Boston, MA 02111, USA. Tel.: +1 617 636 6866; fax: +1 617 636 2456.
E-mail addresses: theoharis.theoharides@tufts.edu (T.C. Theoharides), Asimenia.Angelidou@tufts.edu (A. Angelidou), Konstantinos-Dionysios.Alysandratos@tufts.edu (K.-D. Alysandratos), Bodizhang@post.harvard.edu (B. Zhang), Shahrzad.Asadi@tufts.edu (S. Asadi), cfrancis@otenet.gr (K. Francis), e.toniato@unich.it (E. Toniato), dicrikal@yahoo.gr (D. Kalogeromitros).

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gene interactions with environmental factors have been suspected [8]. Recent reviews have focused mostly on genomic screens that suggest there are multiple gene interactions in autism; however, no gene abnormality alone can explain the apparent increase in ASD prevalence. Increasing evidence suggests that there are different ASD endophenotypes, even within the ASD spectrum [9].

2. Immune dysregulation

The concept of some immune abnormality in ASD has been debated since the 1990s, when a study reported reduced numbers of CD4+ CD45RA+ lymphocytes (subpopulation responsible for induction of suppressor T cells or regulatory T cells) in autistic subjects ($n=36$) compared to healthy age-matched controls ($n=35$), indicating a functional deficit in the innate immune response [10]. Measurement of natural killer (NK) cell activity in blood samples of autistic children ($n=1027$) revealed that 45% of the subjects exhibited low NK cell activity compared to the controls ($n=113$). The correlation of this finding with low intracellular glutathione, IL-2 and IL-15 levels may indicate the underlying cause for NK cell dysfunction in a subset of autistic children [11]. Gene expression of perforin, granzyme B and interferon-γ (IFNγ) in peripheral blood NK cells of ASD patients ($n=52$) was decreased compared to the control group ($n=27$) under similar stimulation conditions, indicating depressed cytotoxicity [12].

In contrast to possibly depressed cell-mediated immunity, the role of pro-inflammatory molecules appears to be increased in autism. Peripheral blood mononuclear cells from ASD patients ($n=71$) secreted more tumor necrosis factor (TNF) in response to lipopolysaccharide (LPS) as compared to controls ($n=40$) [13]. Plasma levels of IL-12 and IFNγ were increased in autistic individuals [14] and IFNγ plasma levels were later found to be positively correlated with the generation of nitric oxide in autism [15]. IL-6 expression was elevated in the brains of deceased ASD patients [16]; it was detected at low levels in the cerebrospinal fluid (CSF) in subjects with autism ($n=35$) as compared to control subjects with other neurologic disorders, but only TNF receptor II was significantly elevated in the serum [17]. TNF levels were elevated in CSF of patients with ASD but were not elevated in the serum [18]. Elsewhere, there was significant increase in the serum concentration of IFNγ, and a trend towards increased production of IL-6 and TNF in *whole blood* of autistic children [19]. Macrophage inhibitory factor (MIF), a molecule shown to enhance immunity through different mechanisms, was higher in the plasma of probands with ASD than their unaffected siblings and correlated with severity of ASD symptoms [20].

We recently showed that levels of the peptide neurotensin (NT), which is present in both the brain and gut, were elevated in the serum of young autistic patients [21]. NT can stimulate lymphocyte proliferation [22], activate T cells [23], enhance IL-1 production from macrophages [24], and trigger mast cell activation [25]. Unlike NT, substance P (SP) was not elevated as also previously reported [26,27]; β-endorphin was also not elevated, even though it had been reported to be increased in the CSF of a small group of children ($n=9$) with infantile autism [28]. We also recently showed that NT can stimulate mast cells to release mitochondrial DNA extracellularly and that such DNA was significantly elevated in the serum of autistic children [29].

With respect to other neuropeptides, archived neonatal blood was analyzed with immunoaffinity chromatography, and serum levels of vasoactive intestinal peptide (VIP) and calcitonin-gene related peptide (CGRP) were reported to be higher in children with ASD ($n=69$) and those with mental retardation without ASD ($n=60$); in contrast, levels of substance P (SP) and nerve growth factor (NGF) were similar to those of controls [26]. Nevertheless, the same authors using Luminex immunoaffinity arrays later showed no difference in any of these peptides between autistic subjects and controls [27].

There may be a persistently inappropriate immune response of autistic subjects to antigenic stimuli, also observed in their unaffected siblings, suggesting a particular genetic background influenced by environmental triggers [30]. A number of papers have reviewed family or personal history of immune disorders in many children with ASD [31,32], prompting the suggestion that ASD may have a "neuroimmune" component [31–33].

3. "Allergic symptoms" in children with ASD

Many ASD children suffer from "allergic-like" symptoms [34], although their exact prevalence remains unknown compared to the general population. Many of the "allergic-like" symptoms reported by ASD children could be consistent with chronic idiopathic or chronic autoimmune urticaria [35]. A case-control study, nested within a cohort of infants born in California between 1995 and 1999, examined the association of "immune-related conditions" with ASD using health records and reported that prevalence of maternal psoriasis, asthma, hay fever and atopic dermatitis during the second trimester of pregnancy correlated with >2-fold elevated risk of ASD in their children [36]. Increased allergic problems (i.e., atopic dermatitis, asthma and rhinitis, as well as high serum IgE, number of eosinophils and positive skin tests) were present in 70% of Asperger patients ($n=15$) compared to 7% of age-matched healthy controls ($n=15$) [37]. In a National Survey of Children's Health, parents of autistic children ($n=483$) reported more symptoms of allergies (also anxiety/depression), with food allergies being the most prevalent complaint, than those of healthy control children ($n=84{,}789$) [38]. Nevertheless, there are limitations relevant to the subjective nature of parents' perception about allergies, since these were not confirmed by a clinician. A link between allergies and autism is also suggested by a recent preliminary study of children with ASD ($n=245$), which indicated that the strongest association of autism was with a history of allergies [39].

There is also evidence of non-IgE-mediated "allergic symptoms." In a hospital-based case-control study, based on questionnaires completed by the parents and scored blindly by an allergist, 30% of autistic children ($n=30$) had a family history of allergic features compared to 2.5% of age-matched "neurologic controls" ($n=39$) ($p<0.005$); however, there was no difference in serum IgE or skin prick tests to 12 common antigens between autistic subjects and controls [40], suggesting non-allergic mast cell activation. There was also no difference in IgG, IgA or IgM levels [40]. One study reported elevated IgG4 levels in children with autistic disorder ($n=114$) compared to normally developing children ($n=96$) [41]. However, the significance of this finding is not apparent because high levels of IgG4 antibodies to foods during infancy are associated with tolerance later in life [42], while many ASD children are in fact intolerant to foods. Moreover, testing for IgG4 against foods is not recommended for diagnosis of food hypersensitivity. Another study investigated the prevalence of atopic and non-IgE-mediated disorders in ASD children (a) with frequent infections and behavioral problems ($n=26$) and (b) without frequent infections ($n=107$), compared to non-ASD controls ($n=43$). Even though the prevalence of atopic disorders in ASD subjects was similar to that of the controls, non-IgE-mediated food allergy was observed at a significantly higher rate in both ASD subgroups compared to controls [43].

One representative case is that of a 12-year-old Caucasian male with a history of gastrointestinal (GI) complaints, diarrhea and frequent rashes at various parts of the body since birth (Fig. 1), often precipitated by certain foods. Exhaustive clinical testing including immune function, autoimmune indices, serum IgE, tryptase, number of eosinophils, tissue transglutaminase and gliadin antibodies, viral antibody titers were negative. This child was developing normally until 2.5 years of age, at which point he exhibited developmental delay and was diagnosed with regressive autism. At about 8 years old,

he developed hives after eating steak and was suspected of being sensitive to meat carbohydrate components (see Addendum In Press).

A preliminary report indicated that the prevalence of ASD is 10-fold higher (1/10 children) in mastocytosis patients than in the general population (1/100 children) [44]. Mastocytosis is a spectrum of disorders with a prevalence of about 1/4,000 children, which involves proliferation and activation of mast cells in the skin (urticaria pigmentosa, UP) and other organs [45], leading to skin reactions, food allergies often in the absence of positive skin testing, and food intolerance, but also behavioral problems [46,47]. One possible case is that of a 4-year-old Caucasian male who was diagnosed with UP at the age of 1 year. The pediatrician at that time suggested that the skin spots would go away with time; however, they increased after routine vaccination at age 3 years old (Fig. 2A). Soon thereafter, the child regressed and was diagnosed with PDD-NOS. The child also often experienced skin rashes (Fig. 2B), associated with worsening of his behavioral status, even though he tested negative to various antigens on skin prick and RAST tests. It should be noted, however, that this is an atypical case given that the diagnosis was made after 3 years of age, which does not comply with DSM-IV criteria. In addition, one would need to be sure that other potential contributing metabolic disorders, including a mitochondrial disorder, had been ruled out.

4. Non-immune mast cell triggers

Mast cells are critical for allergic reactions [48] but are also important in both innate and acquired immunity [49], as well as in inflammation [50]. Functional mast cell-neuron interactions occur in the GI tract [51] and the brain [52]. Mast cells are involved in GI pathology, inflammation and increased intestinal permeability [53], which may also explain frequent GI-related symptoms in ASD patients [54], especially abnormal intestinal permeability [55].

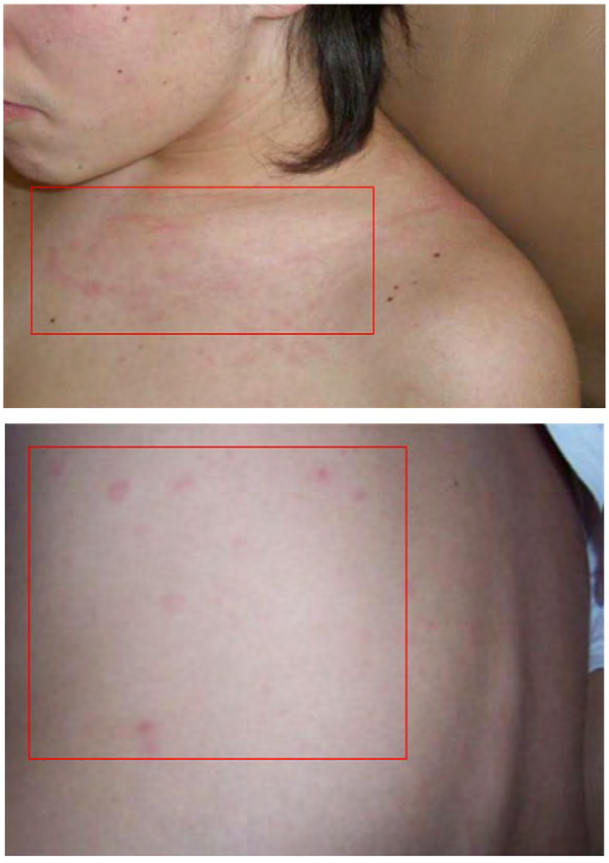

Fig. 1. Photographs of skin areas from a non-atopic, Caucasian male with ASD showing non-specific rashes (boxes) associated with eating steak.

Many substances originating in the environment, the intestine or the brain can trigger mast cell activation [48] (Fig. 3), leading to release of numerous bioactive mediators. These include histamine, prostaglandins, proteases, and vascular endothelial growth factor (VEGF), as well as cytokines, such as IL-6, IL-8, IL-9, IL-13, and TNF. Bacterial LPS activates toll-like receptor-4 (TLR-4) on mast cells and induces selective release of TNF [56]. High levels of TNF were reported in the CSF [18], and high IL-6 gene expression was noted in the brain [16] of autistic patients. CSF and microglia of ASD patients had high levels of macrophage chemoattractant protein-1 (MCP-1) [57], which is also a potent chemoattractant for mast cells [58]. In contrast, ASD plasma levels of transforming growth factor-beta1 (TGF-β1) were low [59], which is important in view of the fact that TGF-β1 inhibits mast cell function and high affinity IgE receptor (FcεRI) expression [60]. TGF-β is also an important mediator released by regulatory T cells [59] and the low plasma TGF-β levels in autistic patients indicate reduced regulatory T cell function in autism.

Mast cells also express viral TLR-3, activation of which by viral double-stranded RNA induces release of IL-6 and TNF without degranulation [61]. The ability of viruses to trigger mast cell activation is especially relevant, since a number of rotaviruses have been isolated from 75% of asymptomatic neonates [62] and could activate mast cells. Environmental toxins linked to developmental neurotoxicity [63], such as polychlorinated biphenyl (PCB) and mercury, have been associated with ASD [64,65], but they also activate mast cells [66,67]. Mast cells can be stimulated by non-allergic triggers to release some mediators selectively, without degranulation [68]. For instance, the peptide corticotropin-releasing hormone (CRH) stimulates selective release of VEGF [69]. CRH is typically secreted from the hypothalamus, but it can also be secreted from nerve endings outside the brain, where it exerts pro-inflammatory effects [70–72]. In fact, CRH acts synergistically with NT to increase vascular permeability [73]. It was recently reported that NT levels are increased in the serum of young children with autistic disorder as compared to normal, age-matched controls [21]. Most recently, we reported that NT induces extracellular release of mitochondrial DNA, which is a potent immunogen and was detected in the serum of young autistic patients [29].

5. The effect of perinatal stress

The effect of CRH may be relevant to ASD, because ASD patients have been reported to have high anxiety levels and cannot handle stress appropriately [74]. Prenatal or perinatal stress may also contribute to the development of ASD through excessive release of CRH. Specifically, CRH is increased in the serum of mothers who delivered preterm babies and correlates with their level of anxiety near the end of gestation [75]. Maternal serum CRH can cross the placenta, and high amounts of CRH could be produced by the placenta itself [76] in response to external or intrauterine stress. Recent reports suggest a potential association between preterm children and autism. In particular, one retrospective study investigated rates of autism in preterm children born in Atlanta, GA (1981–93), who survived to 3 years of age, through the Metropolitan Atlanta Developmental Disabilities Surveillance Program, and showed that preterm birth at <33 weeks gestation was associated with a two-fold higher risk of autism in all infants [77]. Another prospective follow-up assessment on 91 ex-preterm infants (<1500 g at birth) at a mean age of 22 months found 26% of these children to have a positive Modified Checklist for Autism in the Toddlers (M-CHAT) test [78]. A more recent study found that 21% of infants (212/988) born before 28 weeks of gestation screened positive using M-CHAT as compared to 5.7% of healthy children 16–30 months old [79]. Maternal separation stress and CRH are associated with a dysfunctional mucosal barrier in rodents [80]. A short period of restraint [81] or

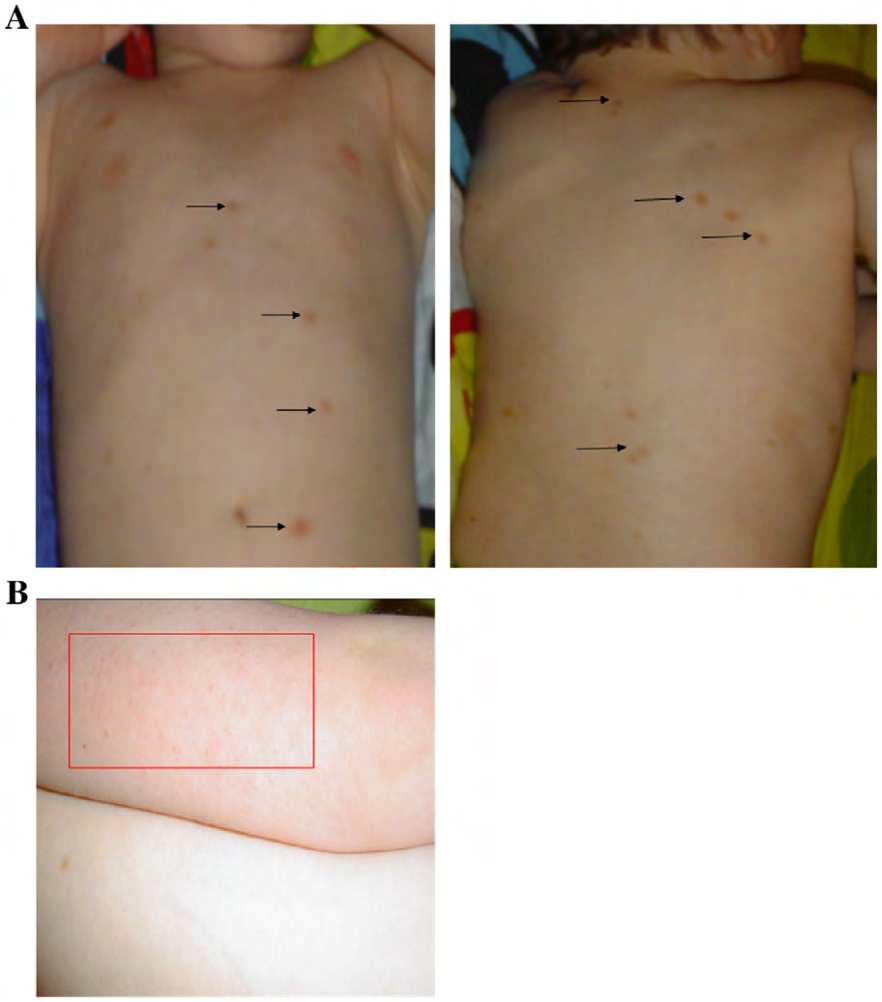

Fig. 2. (A) Photographs of skin areas from a non-atopic, Caucasian male with ASD and UP lesions (arrows), and (B) non-specific rash (box), associated with ASD symptoms.

maternal deprivation stress [82] also increased the severity of experimental autoimmune encephalomyelitis.

The blood–brain barrier (BBB) appears to be compromised in ASD patients as indicated by the presence of serum auto-antibodies against brain proteins (neuron-specific antigens, especially from the cerebellum, cross-reacting with encephalitogenic proteins from milk, Chlamydia pneumoniae and Streptococcus group A) in mothers and children with autism [32,83–86]. In fact, CRH can disrupt the BBB through mast cell activation [87] and also increases intestinal permeability of human colonic biopsies [88].

It is intriguing that mast cell-derived IL-9 induces intestinal permeability and predisposes to oral antigen hypersensitivity in children [89], while it also exacerbates newborn brain toxic lesions [90]. Perinatal mast cell activation, in response to allergic or non-immune triggers, could disrupt the gut–blood–brain barriers [70] through cytokines [87,91] and permit neurotoxic molecules to enter the brain and result in brain inflammation, thus contributing to ASD pathogenesis (Fig. 4). BBB disruption has also been documented in the brain of patients with other inflammatory diseases, such as multiple sclerosis, where it *precedes* any pathological or clinical symptoms [92–94]. This process may worsen by vulnerability due to genetic, metabolic, allergic, autoimmune, environmental and/or other factors.

6. Conclusion

The evidence discussed above does not imply a cause and effect relationship. The issue of "allergies" in ASD still remains poorly defined and controversial. The study designs used to elucidate evidence about atopic and "allergic-like" symptoms in patients with ASD are mostly case-control studies, inherently subject to possible reporting bias of parents. Subjects with ASD susceptibility genes and hypersensitive mast cells may represent a unique subgroup of patients who are more likely to respond to environmental and stress triggers, leading to worsening ASD. It is important to investigate mast cell-associated triggers and mediators in patients with ASD, especially at the time the diagnosis is made. Such efforts could help unveil novel aspects of the pathogenesis of ASD, identify potential biomarkers, as well as lead to new therapeutic approaches. Reduction of stress during gestation and infancy, as well as drugs that could inhibit mast cell activation and prevent BBB disruption or block brain inflammation, may prove useful in at least a subgroup of autistic children.

We have shown that the naturally occurring flavonoids quercetin and luteolin, which are safe [95], can inhibit human mast cell release of inflammatory molecules. Quercetin can reverse acute stress-induced autistic-like behavior and reduces brain glutathione levels in mice [96]. Quercetin also can protect against rat swimming-stress-induced increase in serum lipid hydroperoxide levels [97]. Luteolin inhibits maternal IL-6-induced autism-like behavioral deficits in social interaction in mice [98]. Luteolin also inhibits microglia production of IL-6 [99], can induce anti-inflammatory changes in glial cells [100] and can inhibit cytokine release from peripheral blood monocytes from multiple sclerosis patients [101]. Finally, luteolin (5, 7, 3', 4'-tetrahydroxyflavone) is closely related to 7, 8-dixydroxyflavone, recently shown to mimic brain-derived

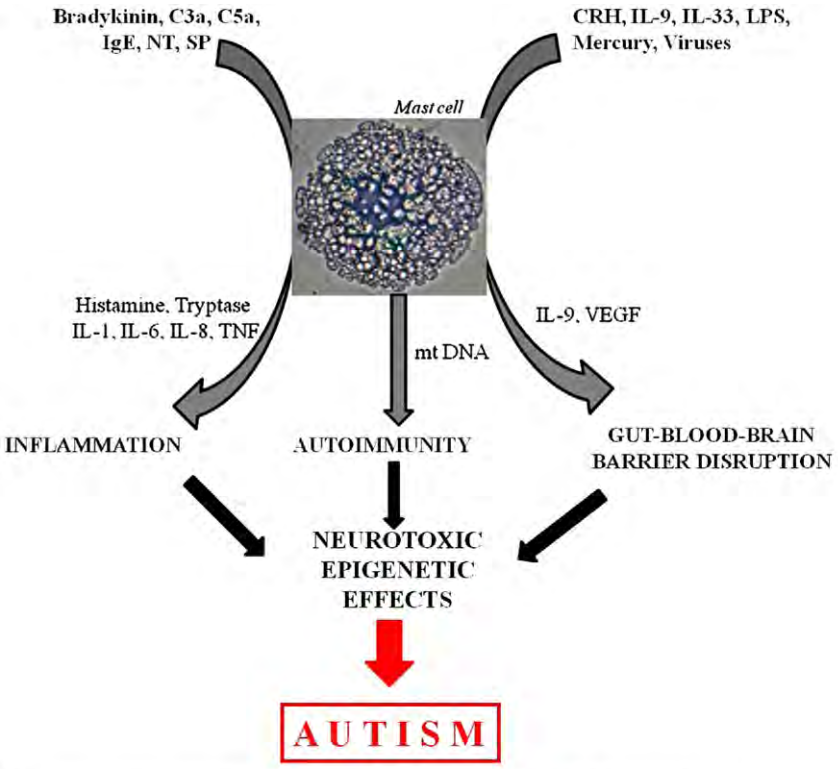

Fig. 3. Schematic representation of mast cell activation by allergic and non-immune triggers, and its possible involvement in the pathogenesis of autism.

neurotrophic factor (BDNF), which is neuroprotective [102]. Luteolin could, therefore, be useful in treating neuroinflammatory diseases, either alone or as an adjuvant to other therapeutic approaches [103]. Unfortunately, flavonoids, especially luteolin are lipophilic and poorly absorbed after oral administration, with significant liver metabolism [104,105]. The unique flavonoid-containing dietary supplement NeuroProtek has been formulated to increase oral bioavailability and holds promise for reducing gut–blood–brain barrier disruption and brain inflammation.

7. Disclosures

The authors declare that they have no competing interests. TCT is the inventor of patent application US 12/534,571 covering the diagnosis and treatment of ASD.

8. Addendum in Press

Additional papers reported increased prevalence of ASD especially after 1986 [106,107]. A recent paper reported increased plasma levels in children with ASD of the chemokines RANTES, MCP-1 and eotaxin [108], all of which are potent chemoattractants for mast cells [109,110,111]. It was also just reported that delayed angioedema and urticaria could develop after eating beef, lamb or pork due to IgE antibodies specific for the meat carbohydarate epitope galactose-a-1,3-galactose [112]. Finally, diagnostic criteria were just proposed for a new entity, "Mast Cell Activation Syndrome" [113], which could explain the findings in many ASD patients who "present with signs and symptoms involving the dermis, gastrointestinal track, and cardiovascular system frequently accompanied by neurologic complaints [113].

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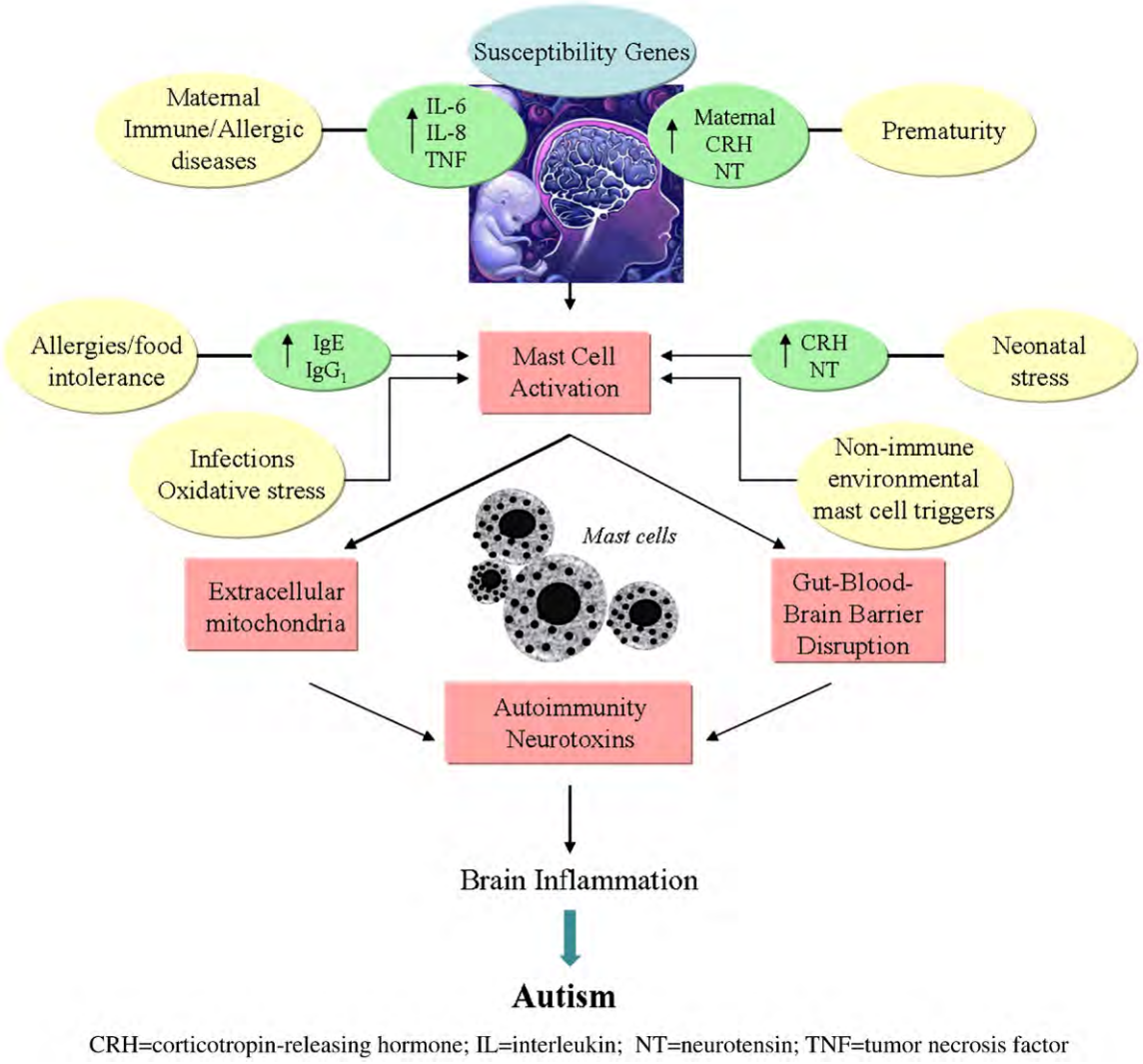

Fig. 4. Schematic representation of different processes involved in perinatal mast cell activation by allergic and non-immune triggers, leading to disruption of the blood–brain barrier, autoimmunity and inflammation that may contribute to the pathogenesis of autism.

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Attachment 3

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Cutaneous mast cells are altered in normal healthy volunteers sitting in front of ordinary TVs/PCs – results from open-field provocation experiments

Olle Johansson, Shabnam Gangi, Yong Liang, Ken Yoshimura, Chen Jing and Peng-Yue Liu

The Experimental Dermatology Unit, Department of Neuroscience, Karolinska Institute, Stockholm, Sweden

Background: Considerable controversy has surrounded the question of possible biological responses to electromagnetic fields (EMFs) generated from visual display terminals (VDTs), such as personal computers (PCs) and ordinary television sets (TVs). The cellular and molecular mechanisms for such potential harmful health hazards have not yet been understood, although clues from the literature include mast cells and histamine. The aim of this study was therefore to investigate possible biological mast cell responses to TV/PC screens.
Methods: Using the indirect immunofluorescence technique, we studied the presence of histamine-containing mast cells in the dermis of healthy volunteers. Cutaneous biopsies taken before and after exposure to ordinary TV/PC screens for 2 or 4 h were investigated in 13 healthy subjects.
Results: Our present *in vivo* study indicates that normal cutaneous mast cells could be altered by exposure from ordinary TV/PC screens. To our great surprise, we found the number of mast cells in the papillary and reticular dermis to increase, to varying degrees, in 5 out the 13 subjects after such an exposure. A migration of mast cells towards the uppermost dermis appeared as the most important event. Thus, the normally upper "empty zone" of the dermis disappeared, and instead, a higher density of mast cells were found in this zone. These cells also seemed to have a tendency to increase in number towards the epidermal-dermal junctional zone and some of them lost their granular content and the cytoplasm shrunk (=degranulation). These findings could only be seen in the exposed skin. Two of the 13 cases instead showed a decrease in mast cell number, but the shift in mast cells towards the upper dermis was still visible. Twenty-four h after the provocation, the cellular number and location were normalized in all subjects.
Conclusions: By definition, normal healthy volunteers are assumed not to react to a TV/PC screen provocation. To our great surprise, this proved not to be true. The present results might lay a foundation to understand the underlying cause of so-called "screen dermatitis" with special reference to mast cells. However, blind or double-blind experiments using patients ought to be further investigated in order to find out the exact cause for the observed changes. Such causes include the effects of surrounding airborne chemicals, stress factors, etc.

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Olle Johansson, Ph.D., Associate Professor, The Experimental Dermatology Unit, Department of Neuroscience, Karolinska Institute, 171 77 Stockholm, Sweden
Tel: +46 8 7287096/+46 8 7287040
Fax: +46 8 303904

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It has previously been assumed that three visual display terminal (VDT)-dependent factors potentially might affect human health: radiation, ergonomics, and stress factors.[1]

The X-ray radiation emitted from the VDT screen was, however, found to be far below accepted risk levels, even under "worst case" conditions, and has not been considered to be associated with any adverse health effects.[1] Considerable controversy has surrounded the question of possible biological responses to low- or high-frequency electromagnetic fields (EMFs). The mechanisms of any harmful health events, such as in so-called "screen dermatitis", have not been understood. Psychological stress factors are believed to be involved in the development of so-called "screen-induced symptoms".[2] Animal experiments have shown disturbances in endogenous opioid systems, with secondary effects on cholinergic systems, after exposure to low-frequency EMFs.[3] Pulsed EMFs imposed on developing chick embryos resulted in an increase in the frequency of abnormal developments.[4] Mammalian cell lines have been shown to respond to extremely low-frequency EMFs with an increase in the activity of a proliferation-regulating enzyme,[5] and stimulation of the calcium influx;[6] furthermore, EMFs could promote peripheral nerve regeneration both *in vivo* and *in vitro*.[7–12] All obtained data thus indicates that EMFs can directly affect biological systems through different, but yet, unclear pathways, including both physiological and biochemical ones.

Most recently, some interesting studies have been published, e.g. Henshaw et al.[13] revealed an enhanced deposition of radon (^{222}Rn) daughter nuclei (^{214}Po; ^{218}Po) in the vicinity of everyday sources of power-frequency EMFs in normal domestic room air. Since countries such as Sweden and the United Kingdom are very rich in ground-based radon as well as radon exposure from building materials, one may ask whether devices like VDTs are true α-emitters?

In this context one has to also mention the findings of Lai & Singh,[14] who investigated the effects of acute (2-h) exposure to pulsed (2 μs pulse width, 500 pulses/s) or continuous-wave 2,450 MHz radiofrequency electromagnetic radiation on DNA strand breaks in the brain cells of rats. An increase in both types of such DNA strand breaks was observed after exposure to either the pulsed or continuous-wave radiation. Again, it is natural to ask what happens with humans using high-frequency devices such as TVs/PCs, mobile telephones, light tubes, etc.?

It has been known for several years that mast cells can be involved in many physiological and pathological reactions in inflammation, allergy, urticaria, psoriasis, itch sensations and pain.[15–17] Cutaneous mast cells are involved in both type I and IV hypersensitivity reactions. In another case-control study (Johansson & Liu, unpublished data), the results in human skin clearly showed that cutaneous mast cells differed both in quantity, quality and distribution pattern in so-called "screen dermatitis" patients as compared to normal healthy volunteers.

Using the same approach in this study, we employed conventional PCs and household TVs as provocation tools to conduct an *in vivo* study on human normal healthy volunteers. The aim of this study was to investigate possible biological mast cell responses to TV/PC screens. By definition, normal healthy volunteers are assumed not to react to such a provocation. To our great surprise, this proved not to be true.

Material and methods

Subjects

Thirteen healthy volunteers (7 male and 6 female, aged 19–34 years old, average 25.7) were selected as the subjects in the study, which was approved by the Committee of Ethics at the Karolinska Hospital. All subjects had no history of dermatoses, allergic diseases or other somatic diseases, and they were all non-smokers.

Test parameters

An ordinary laboratory room without windows was used for the provocation experiment. The room was equipped with 5 conventional PCs (attached to their monitors), 2 ordinary household TVs and 1 portable TV. The temperature in the room was 23–24°C, and the electric and magnetic fields had a strength of 85 V/m, 35 nA and 310 μT/s in the TVs/PCs "OFF"-position, and a strength of 250–500 V/m, 100 nA and >10,000 μT/s in the TVs/PCs "ON"-position,

as measured at the biopsy spot with a Friman Instrument MF-4 (size of measuring plate: 21.5 mm×65.5 mm; 1 m^2 coil (MF-3) and an RC nT-converting filter (no. 169; Friman Datakonsult AB, Stockholm, Sweden). The subjects were seated at a distance of 40 cm from the TVs/PCs with their backs facing the front of the TVs/PCs. The whole provocation lasted 2 or 4 h. Biopsies from the challenged area were taken in pairs at anatomically symmetric sites right before, immediately after the provocation and at 2, 4 or 24 h after the provocation.

Preparation of tissues

Routine punch (Kai Industries, Japan) biopsies (3 or 4 mm in diameter), including epidermis and dermis, were taken under local anaesthesia (Xylocaine, Astra, Sweden). The biopsies were fixed in 4% carbodiimide (1-ethyl-3–3-dimethylaminopropyl-carbodiimide (Sigma, St. Louis, MO, USA) diluted in phosphate buffer (pH 7.4)) for 2 h at 4 °C.[18] The tissue was then rinsed for at least 24 h in 0.1 M Sörensen's buffer containing 10% sucrose, 0.01% NaN_3 and 0.02% Bacitracin and then sectioned on a Microm cryostat to yield 14-µm-thick sections, thawed onto gelatine-coated slides and processed for indirect immunohistochemistry.

Immunohistochemistry

The indirect immunofluorescence technique was employed.[18,19] The sections were kept in an humid atmosphere, incubated with the primary rabbit histamine antiserum (1:2,000; Milab AB, Malmö, Sweden) overnight at 4 °C, rinsed in phosphate-buffered saline (PBS), incubated for 30 min at 37 °C in rhodamine (TRITC)-conjugated goat anti-rabbit IgG (1:80; Boehringer-Mannheim, Mannheim, Germany), rinsed and mounted. All antisera were diluted in PBS containing 0.3% Triton X-100.

The control of the antiserum specificity was performed by pre-absorption with histamine dihydrochloride (0.1 mM; Sigma). In addition, it has been verified by the manufacturer that the antiserum does not cross-react with norepinephrine, serotonin, vasoactive intestinal polypeptide, glucagon or histidine. To test for any possible non-specific binding of the primary antiserum to Fc receptors in the tissue, a normal rabbit serum (1:100) was used instead of the primary antibody. To control for any possible non-specific reactions of the secondary antiserum, PBS was used on certain sections instead of the primary antibody. Other sections were directly only incubated with the secondary antibody. For observation and photography a Nikon Microphot-FXA fluorescence microscope was used. The results were collected from two independent observers.

Results

First, it should be noted that no cutaneous and/or somatic objective or subjective symptoms at all were induced in or reported by the tested volunteers during and after the provocation. This is fully in accordance with the assumption that normal healthy volunteers should not react to the TV/PC provocation situation at all.

The histamine immunoreactivity was only found in cells of the dermis. The immunoreactive cells observed in this study are considered to be mast cells based on their localization, number and cellular morphology, e.g., size and arrangement of granules according to our previous investigations.[18,19] They were seen around dermal appendages such as hair follicles, sebaceous glands, sweat glands and blood vessels, and they all had a similar morphology. Although mast cells were frequently found close to small capillaries and large blood vessels, they were never actually pres-

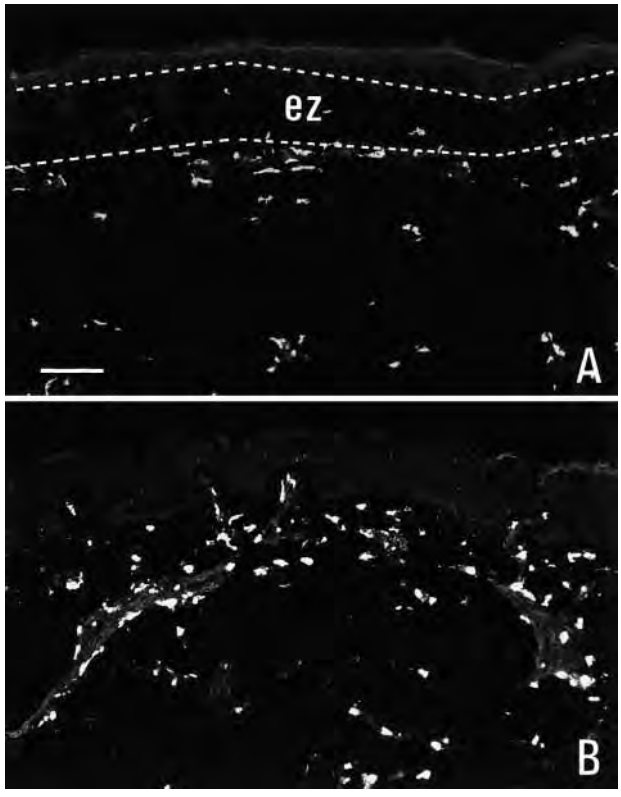

Fig. 1. The distributional alteration of cutaneous mast cells due to the provocation. A) An unexposed biopsy illustrates the relatively empty zone (ez) below the epidermal-dermal junction, where only single cells are seen. Further below this empty zone, mast cells are found in their highest density and gradually decrease towards the deeper dermis. B) In an exposed skin, a cellular migration upwards to the uppermost dermis was induced after exposure, i.e. the normally empty zone disappeared, and, instead, a high density of mast cells is observed in this zone. Bar in A=50 µm. The magnification in A=B.

ent in the vascular wall. The immunolabelling was found exclusively in the cytoplasm of the cells, leaving the ovoid single spherical or oblate spherical nucleus unlabelled, generally displaced to one side of the cells and along the long axis of cell (Fig. 1A). All the positive cells appeared prominently granular and most of them were elongated with cytoplasmic extensions while others were flat or dendritic when examined under high magnification.

In all unexposed biopsies, a relatively empty zone could be seen, which was approximately 100–200 μm wide below the epidermal-dermal junctional zone running parallel to that border (Fig. 1). One could hardly see any mast cells in that zone. This is consistent with our previous results in normal facial skin (Johansson & Liu, unpublished data). Further below the empty zone, mast cells were found in their highest density and gradually decreased towards the deeper dermis.

The main experimental results registered after exposure to the TVs/PCs are shown in Table 1. To our great surprise, the numerical density of mast cells increased in 5 out of the 13 subjects in the papillary dermis and reticular dermis as a response to the provocation; however, the cellular volume seemingly remained unchanged in most cases. Two out of 13 also increased the fluorescence intensity of their histamine granules. Above all, a cellular migration appeared as the most important event in the provocation experiment, i.e. the normally empty zone disappeared, and instead a high density of mast cells was observed in this zone. These cells also seemed to have a tendency to move towards the epidermis and some of them lost their granular content and their cytoplasm shrinked (pointing to a possible degranulation) (Fig. 2).

In contrast to the results mentioned above, two cases showed a numerical mast cell decrease, but the upward movement was still visible. In addition, in one case, the processes of mast cells were found to be

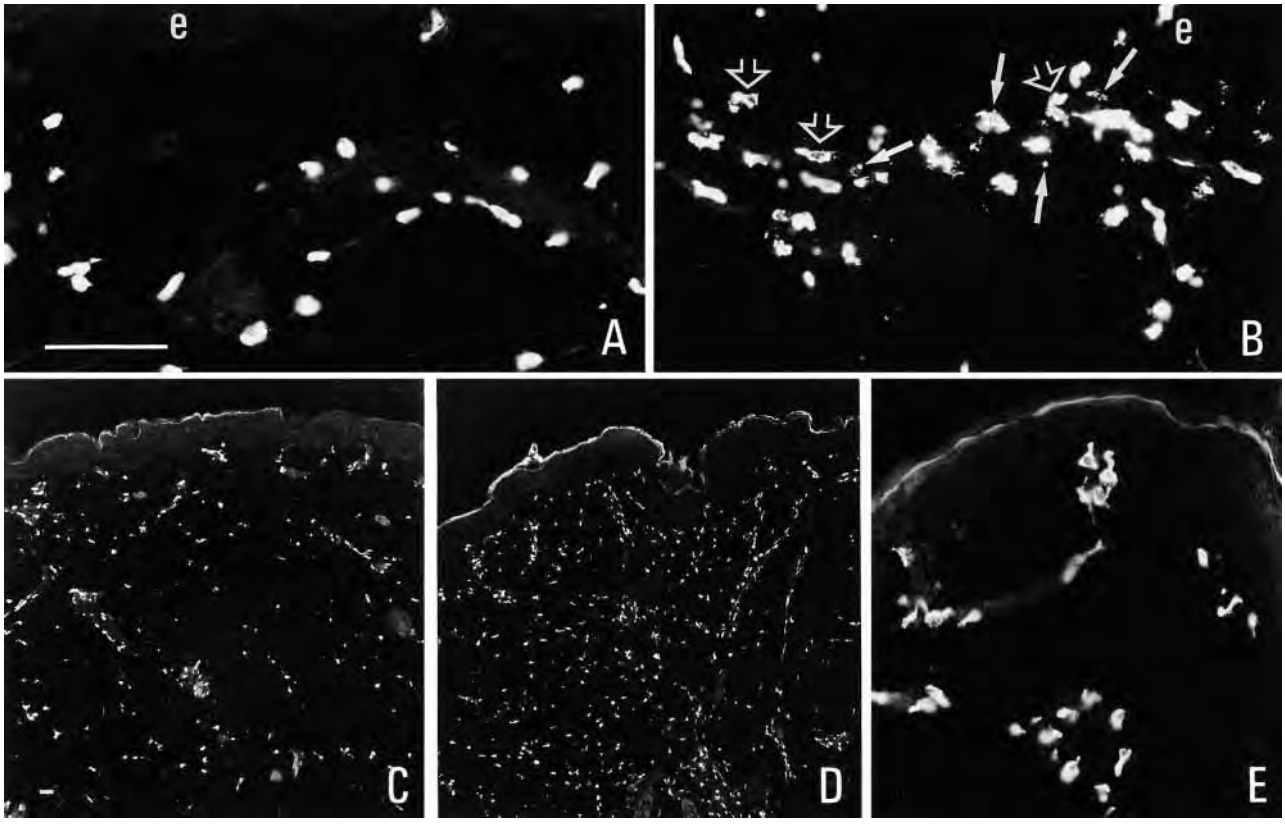

Fig. 2. A typical cutaneous response to TV/PC provocation. Note that biopsies A & C and B & D, respectively, came separately from the same subject. In A, the unexposed biopsy shows the morphology of normal status mast cells. In B, a biopsy from after 4 h of exposure, it is shown that these cells close to the epidermis have degranulated and that their cytoplasm has shrinked. Open arrows point to shrinked mast cells; solid arrows point to discharged histamine granules from mast cells. e=epidermis. C and D are low-power micrographs clearly demonstrating that compared to before (C), after 4-h exposure (D) the number of mast cells has increased and an upward movement of these mast cells has appeared. In E, one case showed an odd morphology of mast cells as a response to the provocation; the processes became more numerous as well as larger, especially in cells that were situated closer to the epidermis. Bars in A and C=50 μm. The magnification in A=B=E, and in C=D.

Table 1. The protocols of cutaneous mast cell variation after 2 or 4 h screen provocation compared with unexposed biopsies

Subject	2 or 4 hours after provocation				After a 24-h delay			
	No.	CV	GI	McM	No.	CV	GI	McM
1.	↑↑	–	-	3+	–	–	–	0
2.	↑↑	↑	–	1+	–	–	–	0
3.	↑↑	–	–	1+	–	–	–	0
4.	↓	–	–	1+	–	–	–	0
5.	–	–	–	1+	–	–	–	0
6.	–	–	↑	0	–	–	–	0
7.	–	↑	–	0	–	–	–	0
8.	–	–	–	0	–	–	–	0
9.	–	↑	↑	2+	–	–	–	1+
10.	↓	–	–	1+	–	–	–	0
11.	↑↑	–	–	1+	–	–	–	1+
12.	–	–	–	0	–	–	–	0
13.	↑↑↑	–	–	2+	↑	–	–	0

–=no change; ↑=increase; ↓=decrease; 0=not present; +=was induced to a mild (1+), medium (2+) or strong (3+) degree; CV=cellular volume; GI=granular intensity; McM=mast cell migration into the uppermost dermis

more numerous as well as larger, especially in cells that were situated closer to the epidermis, as a response to the provocation (Fig. 2E). The morphology of the cutaneous capillaries, sebaceous glands as well as hair follicles always remained unchanged. Finally, it should be noted that 24 h later, all of the subjects with changes reported above revealed a normal pattern.

There was no significant difference between biopsies that were taken instantly after exposure or after 2 or 4 h post-provocation delay. Furthermore, no differences could be seen if the provocation time was 2 or 4 h.

Discussion

Mast cells are effectors of IgE-mediated immune reactions because they have high-affinity receptors for the Fc-portion of IgE.[20] Their ability of rapid response to allergens or poison stimuli may be considered as a first line of defence in protecting the skin from being infiltrated by micro-organisms and other potentially harmful agents. They contain and release, after activation, a wide array of pro-inflammatory mediators affecting structures and cells, and conversely their differentiation and function are affected by their environment.

Histamine is synthesized in mast cells from histidine and is stored within the mast cell secretory granules by forming a complex with the glycosaminoglycan side chains of heparin. In mammalian connective tissue, including the skin, mast cells have generally been regarded as the major source of histamine.[21] It has been found that there were statistically significant correlations between the mast cell number and histamine content.[19,21–24] Therefore, most of the histamine immunoreactivity cells are equal to classical mast cells.

In the present study, we found a special distribution in the papillary dermis, i.e. an empty zone could easily be identified, like in the face (Johansson & Liu, unpublished data), which has not been reported in the back before. This zone may be considered as a 'buffer' zone for immune reactions; once an antigenic substance or stimulus intrude into this area, immunocytes (as well as mast cells) migrate into this zone to participate in the immunological response.

Apart from the fact that mast cells could be provoked to increase their infiltration in the dermis, another prominent finding in this investigation is, of course, the upward migration. It could be induced in most cases (9 out of 13; see Table 1), although two cases showed a decrease of mast cell number. In this context, it may be noted that Donnellan et al.[25] have shown clear-cut effects on a mast cell analogue, RBL-2H3, of EMFs at 835 MHz. The rate of DNA synthesis and cell replication increased, the actin distribution and cell morphology became altered, and the amount of β-hexosaminidase released in response to a calcium ionophore was significantly enhanced, in comparison to unexposed cultures. There were no effects seen on the levels of cytoskeletal protein synthesis or β-actin mRNA. However, the amount of Ras in the membrane fraction of exposed cells increased. The morphological changes persisted following subculture for at least 7 days in the absence of further exposure.[25] This work has now also been extended to yet another mast cell line, namely the HMC-1, and at 864.3 MHz.[26] In their study, the authors reported effects on the localization of the protein kinase C, and expression of 3/588 genes screened. The affected genes included the proto-oncogene c-kit, the transcription factor nucleoside diphosphate kinase B and the apoptosis-associated gene DAD-1. In addition, stress response genes were variably upregulated. No significant effect on cellular morphology or on F-actin distribution was detected. The conclusion of the publication[26] was that the low-power microwave exposure used may act on the HMC-1 cells by altering gene expression via a mechanism involving activation of protein kinase C, and at temperatures (=26.5 °C) well below those known to induce heat-shock responses.

The recent finding that small magnetic particles of magnetite (Fe_3O_4) are present in various biological tissues opens the arena for new speculations on interaction mechanisms.[27] A cluster of cells, denoted magnetocytes or Jurkat cells (a human leukemic T-cell line)[27] and bacteria *Magnetospirillum magnetotacticum* have been reported to contain magnetite, and low-frequency magnetic fields have been shown to increase inositol 1,4,5-triphosphate levels in the Jurkat cell line.[28] Earlier reports from the same group have shown that when a weak 50-Hz magnetic field was applied, the Jurkat cells responded with intracellular

calcium oscillations.[29] The results suggested that the magnetic fields interfered with the signal transduction, although neither target molecules nor molecular mechanisms are at present known. Perhaps some cutaneous mast cells also may contain magnetite and such cells may be involved in possible interactions of environmental EMFs and skin. When there exists an EMF, they might migrate toward the magnetic source, i.e., towards the epidermis, to degranulate their histamine content. Since this effect only happens at the cellular level, it may not be strong enough to immediately cause cutaneous objective/subjective symptoms of long-lasting nature. Support for this idea may be given by the observation that, even if a subject had cellular changes directly after the provocation, 24 hours later they had become normal again (cf. Table 1). But, repeated chronic exposure to magnetic fields may be able to cause symptoms such as itch, smarting, redness, papules, etc.[30] This is correspondent to the fact that mostly when the so-called "screen dermatitis" patients leave their VDT work, the symptoms are relieved or, more or less, disappear.

In conclusion, although the subjects in the present investigation did not report any cutaneous and/or somatic objective or subjective symptoms during as well as after the provocation, 7 out of 13 still had profound cellular changes in their dermal mast cell population. This would mean that normal human skin very well could biologically react to external EMFs generated from TVs/PCs. What this would imply for the whole human being could only, at this stage, be guessed at. Since the subjects did not verbally complain it is not likely that psychological and/or psychosomatic events have taken place, rather the cellular changes point to a UV, microwave, or other high-frequency radiation emitted by the TVs/PCs. The assumption that normal healthy volunteers should not react at all to the TV/PC provocation situation thus proved to be wrong. And, maybe the observed cellular changes are actually just plain radiation damages?

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Attachment 4

Contributions of the environment and environmentally vulnerable physiology to autism spectrum disorders

Martha R. Herbert

TRANSCEND Research Program, Pediatric Neurology, Massachusetts General Hospital, Charlestown, Massachusetts, USA

Correspondence to Martha R. Herbert, TRANSCEND Research Program, Pediatric Neurology, Massachusetts General Hospital, 149 13th Street, Room 10.018, Charlestown, MA 02129, USA
Tel: +1 617 724 5920;
e-mail: mherbert1@partners.org

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Purpose of review

This review presents a rationale and evidence for contributions of environmental influences and environmentally vulnerable physiology to autism spectrum disorders (ASDs).

Recent findings

Recent studies suggest a substantial increase in ASD prevalence above earlier Centers for Disease Control figures of one in 150, only partly explicable by data artifacts, underscoring the possibility of environmental contributors to increased prevalence. Some gene variants in ASD confer altered vulnerability to environmental stressors and exposures. De-novo mutations and advanced parental age as a risk factor for ASD also suggest a role for environment. Systemic and central nervous system pathophysiology, including oxidative stress, neuroinflammation, and mitochondrial dysfunction can be consistent with a role for environmental influence (e.g. from air pollution, organophosphates, heavy metals) in ASD, and some of the underlying biochemical disturbances (such as abnormalities in glutathione, a critical antioxidant and detoxifier) can be reversed by targeted nutritional interventions. Dietary factors and food contaminants may contribute risk. Improvement and loss of diagnosis in some with ASD suggest brain circuitry amenable to environmental modulation.

Summary

Prevalence, genetic, exposure, and pathophysiological evidence all suggest a role for environmental factors in the inception and lifelong modulation of ASD. This supports the need for seeking targets for early and ongoing medical prevention and treatment of ASD.

Keywords

autism, dynamic encephalopathy, environment, glutathione, oxidative stress, pathophysiology

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Introduction

The present review will consider recent documentation of increasing prevalence rates in autism spectrum disorders (ASDs) and what may be contributing to these prevalence rates. It will explore different interpretations of the significance of these reports, gene–environment interactions, and vulnerabilities in physiology in ASD that may be targets of various environmental factors and thereby may be contributing to those portions of the increases in reported prevalence that are not due to other factors, such as broadening of diagnostic criteria and greater awareness of ASD.

Overview: environment and anomalies

Once thought to be rare, ASDs are now reportedly on the rise and are the subject of daily media attention. In addition, there is less consensus than in the past about neural systems being the primary loci of dysfunction in ASD and a growing sense of whole body systems involvement in autism wherein the brain may be impacted in parallel with other systems (Fig. 1) [1,2]. There is beginning to emerge a train of inquiry looking more seriously at potential environmental causes of and mechanisms for rising numbers and systemic features in autism [3,4]. Only in the past few years have funding opportunities started to become available to support these types of inquiries, and it is at least in part for this reason that the relatively small share of the literature devoted to these questions includes studies of varying quality and often with small sample sizes.

Even so, a vantage point with some consistent themes and perspectives is beginning to emerge. We are seeing growing attention to indications of environmental contribution beyond early notice of autism incidence in association with in-utero valproic acid [5] or congenital rubella [6]. Now on the radar screen, we find other evidence for a role of environmental factors in the rising

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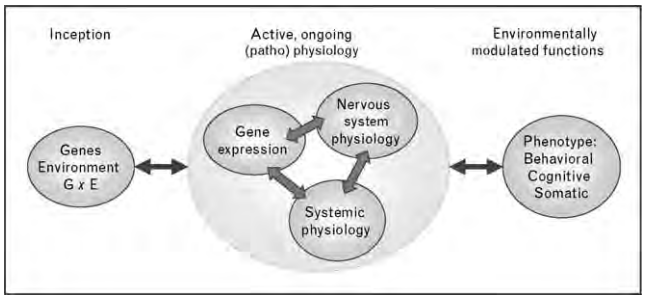
Figure 1 The figure schematizes the inputs from genes, environment, and gene–environment interactions as they impact the organism's gene expression and physiological activities



These activities in turn shape the various levels of phenotype, including behavior, cognitive functioning, and somatic/medical domains.

numbers, such as incomplete monozygotic concordance, differences related to geography, occupation and time of birth; gene–environment interactions; environmental toxins; investigation of genetic and physiological features of the ASD phenotype that may be unusually vulnerable to environmental exposures and stressors; and also unanticipated evidence of plasticity and improvement in response to environmental modulation. Proceedings from a 2007 Institute of Medicine workshop on Autism and the Environment [7•] and a workshop summary [8•] were published. Along with an increased number of articles, there have appeared two books [9••,10••] and one dedicated journal issue [11••,12] focusing centrally on metabolic, immune, and environmental issues in ASD. This review will present a view of this emerging perspective with an emphasis on the linkages being investigated between environment and vulnerable physiology.

Autism prevalence

In the past year, a number of reports suggest that the prevalence of ASD is greater than the one in 150 that was reported by the Centers for Disease Control (CDC) in 2007, using 2000 and 2002 data [13,14]. Baron-Cohen *et al.* [15] generated prevalence estimates in the UK of 94 per 10 000 using the Special Educational Needs register and 99 per 10 000 using a diagnosis survey of children aged 5–9 years in participating schools; when adjusted for a ratio of known:unknown cases of about 3 : 2, their final prevalence estimate was 157 per 10 000, or one in 64. A report from the US Department of Health and Human Services utilizing telephone interview data from the 2007 National Survey of Children's Health found a weighted point prevalence of 110 per 10 000 in the United States, though this study's telephone interview was limited and could have skewed the estimate [16].

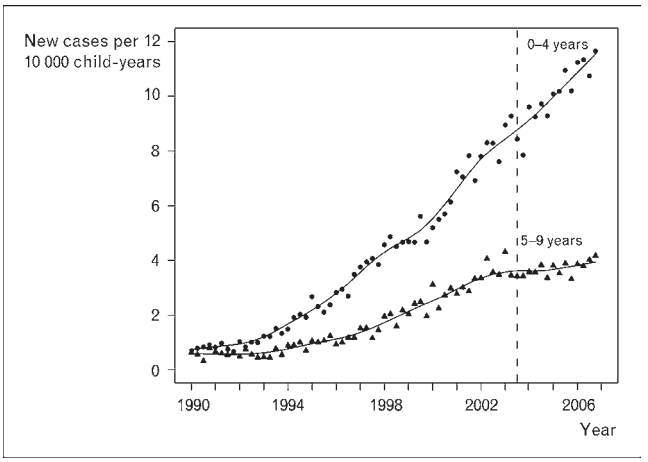
Concerns have been raised about how much of the increases in prevalence represents an actual growth in numbers. Diagnostic substitution – labeling people autistic who previously would have been diagnosed with something else – is one consideration [17–19]. Shortcomings of various data sources, such as administrative data or clinician diagnosis, are also an issue [20–22].

To quantify the extent to which reported increases may be explained by factors other than a true increase in incidence, Hertz-Picciotto and Delwiche [23•] investigated cases in the California Department of Developmental Services databases from 1990 to 2006, during which a 600% increase in incidence rate was observed (Fig. 2). Out of this 600% increase, 24% could be explained by earlier diagnosis, an increase of possibly 56% could have been due to inclusion of milder cases, and based on data from a Finnish study, 120% was considered attributable to changes in the diagnostic criteria; the other 2/3 could not be accounted for by such factors. This analysis minimized the influence of the limitations of administrative data by examining files of individual cases and linking to state birth records, by supplementing with clinical confirmation of diagnoses, and by considering 'age at diagnosis' as a metric that may lag variably behind actual age of onset [24]. The authors acknowledge considerable remaining uncertainty, but nevertheless argue that given the gap between the observed increase and the proportion explained by major factors such as diagnostic substitution, the possibility of a true increase in incidence deserves serious consideration. Two further studies have also shown that diagnostic substitution can only partially account for increased prevalence numbers [25,26].

Genes and environment

Gene–environment interactions could contribute to prevalence increases. In many cases, genes and environment

Figure 2 Annual incidence rates of autism based on the administrative database of the California Department of Developmental Services, 1990–2006



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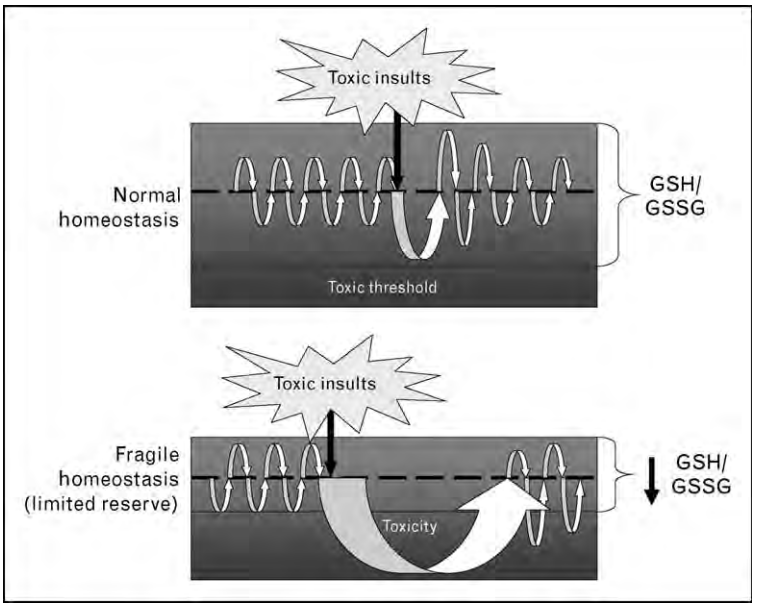
could both be necessary but neither alone sufficient to cause autism. Pessah and Lein [27•] review how low-level chemical exposure can influence some of the same molecular, cellular, and behavioral outcomes that are also influenced by genetics. They focus particularly on environmental agents that interfere with three neurotransmitters and pathways [gamma-aminobutyric acid (GABA), acetylcholine, and calcium signaling pathways and calcium-dependent effectors] already at risk in some individuals with ASD for genetic reasons. The existence of inborn genetic vulnerabilities in such pathways may lower the threshold at which the influence of environmental factors may be felt, leading to an impact of environment that differs across the population based on genetic substrate [28•].

Vulnerabilities are being identified in ASD in a growing number of environmentally responsive or sensitive genes and pathways. The Environmental Genome Project of the National Institute of Environmental Health Sciences (NIEHS) has been investigating such genes and haplotypes [29,30], and informatics resources such as the Comparative Toxicogenomics Database [31] are becoming increasingly detailed and valuable. Aberrant metabolism in environmentally sensitive pathways in individuals with ASD who have no known neurometabolic disease is of growing interest, particularly abnormalities in redox and methylation, given the known impact of toxins on these processes [32•]. Glutathione may be particularly important because, as a critical antioxidant as well as an important endogenous detoxifier, it plays a central role in how the organism handles many types of exposures and stressors (Fig. 3) [33••]. Significant transmission disequilibrium in transmission of three alleles of a human glutathione peroxidase (GPX1) repeat was noted in 103 trios (probands and parents) of autism disorder with undertransmission of ALA6, suggesting a possible protective effect of this allele [34]. An increased frequency of the ALAD2 variant of delta aminolevulinic acid dehydratase in ASD, conferring vulnerability to lead exposure along with a decreased frequency of CPOX (coproporphyrin oxidase) variants associated with vulnerability to lead suggests that ALAD2, particularly in combination with lower glutathione levels, may contribute to lead toxicity as an autism risk factor [35]. Paraoxonase 1 (PON1), which is associated with organophosphate hydrolysis and which has low activity in childhood leading to increased vulnerability in young children [36], was found to be associated with autism in a White-American cohort from the United States where organophosphates have been in more recent use but not in an Italian cohort with less organophosphate exposure [37]. The bioavailability and the catalytic activity of PON1 were significantly impaired in a cohort of 50 children with ASD, despite no association with polymorphisms in the *PON1* gene and a normal distribution of the PON1 phenotype [38], suggesting possible environmental targeting of these functions.

The observation of more frequent de-novo copy number variants in sporadic autism than in cases with affected

Figure 3 Greater cytoxicity from exposures can occur in the setting of impaired glutathione-dependent redox reserves



With a low reserve, there is a fragile homeostasis that shows more vulnerability and lower resilience. Glutathione depletion will increase sensitivity to environmental toxins. With robust glutathione reserves, toxic insults are buffered and may never reach toxic threshold. Depleted glutathione reserve leads to a fragile homeostasis in which a similar toxic insult will lead to toxicity and disorder. Many individuals with autism spectrum disorder (ASD) have reduced glutathione reserve that may render them more sensitive to pro-oxidant environmental exposures. Adapted with permission from [33••].

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first-degree relatives or in controls [39], as well as the presence of hundreds of distinct variants seen only once [40], suggest a potential role for environment. The possibility of de-novo mutations is also raised by the documentation of advanced parental age as a risk factor for autism [41], which could explain some acquired germline mutations. Possible environmental causes were discussed in a literature survey that identified nine pre-conception environmental exposures associated with increased risk for autism and noted that five of these factors (mercury, cadmium, nickel, trichloroethylene, and vinyl chloride) are 'established mutagens' [42]. The impact of such xenobiotics might be amplified by vitamin D deficiency [43] due to the importance of this substance in DNA repair mechanisms [42].

Environment and pathophysiology

Environmental exposures and stressors act through their impact on the organism and they can be studied using markers of exposure but also of susceptibility and effect. Effects may impact brain development, development of other organs and systems, and ongoing physiological processes. A number of clinical and research findings have been encouraging this direction of work. Prominent among these are disturbances in immune function and increases in immune vulnerability that are reviewed elsewhere in this issue. One route of immune disturbance is early life insults from the environment that include xenobiotic-induced developmental immunotoxicity [44,45], as well as prenatal infection [46], prenatal stress [47,48], and immune disruption of the gut–blood–brain barrier [49]. Although we do not have measures at this point that could be considered both sensitive and specific to ASD, it does appear that many of these findings may have clinical significance.

A growing body of literature has documented that oxidative stress, which is well known to be a potential consequence of environmental insult as well as of genetic influences, is increased in ASD [33••,50]. Abnormal sulfur amino acid metabolism consistent with oxidative stress was documented in leukocytes [51]. In one study, abnormalities in these metabolites were identified in children with autistic disorder and pervasive developmental disorder (PDD) but not in Asperger's syndrome [52]. Increased oxidation of cell membrane phosphatidylethanolamine in autism was shown to be mediated by copper and ceruloplasmin, which may thereby be contributory to oxidative stress, reduced phosphatidylethanolamine levels, and abnormal membrane function [53]. A reduction in the ratio of reduced glutathione to oxidized glutathione (GSH/GSSG) indicative of oxidative stress was measured in both cytosol and mitochodria of lymphoblast cell lines from individuals with autism [54]. Parents of children with autism were shown to share similar metabolic deficits in methylation capacity and glutathione-dependent antioxidant and detoxification capacity to those observed in many autistic children [55]. Acetominophen, an over the counter drug in very common pediatric use, especially since the early 1980s when it replaced aspirin in pediatric practice, acutely impairs glutathione metabolism, which suggests it could be a potential risk factor in autism [56]; it was shown (though with disagreement in a letter to the editor) to be associated with autistic disorder in young children with regression in development [57,58]. Treatment in an open-label trial of abnormal glutathione status with methylcobalamine and folinic acid in children with ASD showed efficacy in moving glutathione status less remote from normal, and improvement in Vineland Adaptive Behavior score was used to support a double-blind placebo-controlled trial now underway [54]. However, although these studies suggest metabolic vulnerabilities pertinent to environmental insults, for the most part they have not included direct measures of potential environmental triggers of these pathophysiological alterations; such linkages would likely require larger scale funding and more systematic support of collaboration.

Metabolic alterations consistent with vulnerability to heavy metal and xenobiotic toxicity have also been observed. A reduced zinc–copper ratio was measured in a substantial cohort of children with ASD; such a reduction may indicate inadequate metallothionein function, leading to impaired metal binding and increased vulnerability to metal toxicity [59]. Elevated porphyrins, which have been regarded as markers of xenobiotic and metal (lead, mercury) exposure, have been measured in several cohorts of children with ASD [60,61]. A number of studies have emphasized the lack of connection of vaccines or mercury in vaccines with the increased prevalence of autism [62–67]. One study showed a lack of difference in blood levels of mercury between ASD and control groups, though the authors pointed out that their measure only reflected recent exposures and their findings were not sensitive to more chronic exposures and not pertinent to questions of causation [68]. Others argue that there are legitimate concerns about the impact of even low levels of chronic mercury exposure [69] and that much divisiveness, public suspicion of the health establishment, and possibly harm could have been avoided by an early decision to reduce exposure to thimerosal in vaccinations based on prior removal of this substance from topical medications, as well as development of a more coherent environmentally responsive research agenda for autism [70].

There are also findings possibly consistent with oxidative stress and immune activation in the central nervous system. Pertinent neuropathological findings, although preliminary and in small samples, include elevated

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cerebellar 3-nitrotyrosine [71], reduced neuronal density with increased glial density and lipofuscin in language-related cortex [72], and immunocytochemical detection of three markers of oxidative injury and lipid peroxidation in ASD brain tissue [73]. An earlier landmark paper documenting innate immune activation and abnormal cytokines in post-mortem brain tissue [74] received some support by independent documentation of increased innate and adaptive immune activation in ASD brain tissue [75]; such neuroimmune changes can have a relationship with environmental toxins [76] from exposures such as air pollution [77–79]. The great heterogeneity in ASD may relate at least in part to the many different types of such contributors that could contribute etiologically to autism's defining behavioral characteristics.

Various brain imaging findings can also be interpreted as consistent with central nervous system tissue disturbances such as immune activation or oxidative stress. Although the highly replicated phenomenon of early rapid brain enlargement in a substantial subset of individuals with autism [80,81] has led to the inference that this size increase would be accounted for by a greater number of neurons and myelinated axons in ASD brains, imaging findings are beginning to suggest the opposite. The strong predominance of findings in magnetic resonance spectroscopy is of reduced density of metabolites [82•]. This, along with the reduced fractional anisotropy and increased diffusivity in diffusion imaging of white matter [83,84], suggests a reduction rather than an increase in neuronal and white matter integrity, cell number or density; tissue changes that could lead to such signal could derive from oxidative stress, neuroinflammation, or edema. Such potentially environmentally mediated tissue pathophysiology might also contribute to reduced cerebral perfusion, with recent single photon emission computed tomography (SPECT) studies [85,86] supporting prior documentation of hypoperfusion in a dozen and a half earlier papers.

Dietary factors are also under consideration as environmental contributors to ASD. A several-fold reduction in the proportion of ω-3 fatty acids in lipid intake over the past few generations, and potential exacerbation of the impact of this deficiency by gastrointestinal disturbances in ASD [87], may contribute to abnormal fatty acid profiles in ASD [88] that could affect neuronal processing [89], though rigorous evidence for the efficacy of essential fatty acid supplementation in ASD is still weak [90]. Nutritional insufficiencies that may reduce the availability of substrates for neuronal metabolism and increase vulnerability to oxidative stress [91] may result from self-restriction of intake common in ASD [92], and this may be further complicated by ingestion of toxicants and heavy metals as food contaminants [91]. Earlier work documented abnormal clostridial colonization in regressive ASD as well as transient behavioral improvement with eradication of these organisms [93,94]. Based on this work and concerns more broadly about rises in clostridial infection [95], as well as the impact of gut microbiota on human health and environment on the microbiota [96], rodents were injected with propionate (a major byproduct of clostridia as well as a common food preservative) and manifested autistic-like manifestations at multiple levels, including social isolation, reduced play behavior, oxidative stress, and a brain neuroinflammatory response [97,98]; this model system may be applicable to many other environmental exposures.

The dynamic nature of autism symptoms and their severity, including both regression in early childhood and improvement or even loss of diagnosis, suggest a potential role for environmental factors in modulating the pathophysiology underlying autism well after the period of in-utero and early postnatal development [1]. A surprising report of improvements in core features of ASD in the setting of fever [99] prompted much reflection on underlying neurobiological mechanisms such as cytokine alteration or lipid membrane fluidity changes that might permit such dynamism in what had previously been considered a static encephalopathy [100,101•]. One provocative paper suggested that this phenomenon may derive from developmental dysregulation of the locus ceruleus-noradrenergic system, that is widely distributed and can cause rapid state alterations, and that may be transiently restored to normal regulation during fever [102]. The phenomenon of loss of diagnosis and 'recovery' has also been discussed, not only in widespread anecdotal reports but now also in academic literature, with a review of the implications of 'recovery' [103•] and case series documenting loss of rigorously documented diagnosis [104]. One study reported that recovery occurs in 19% of cases of early autism diagnosis, which may reflect some combination of true improvement, maturation, and overdiagnosis [105].

Conclusion

A line of inquiry is developing aimed at making sense of the increasing prevalence of ASD, the failure to fully account for this increase by factors such as diagnostic substitution, a growing body of evidence demonstrating the role of gene–environment interactions in ASD, environmentally vulnerable physiology in ASD, and a wide range of contributory environmental factors. All of these support the need to increase our attention to environment and vulnerable physiology in ASD. Environmental influences on physiology might begin *in utero* and might thereby contribute to alterations in brain and other body systems development and involve epigenetic changes. But given the documentation of potentially environmentally modulated active pathophysiology later in childhood

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and into adulthood, it is reasonable to devote more attention to environmental influences that could modulate encephalopathy, that is, physiological brain function, in ASD in an ongoing and active fashion [101•,106], starting preclinically in infancy before it is possible to identify clear autism symptoms, and continuing further throughout the lifespan. Through environmental modification, including medical intervention, it may be possible to avoid or limit the triggering or aggravation of vulnerabilities and thereby to reduce both prevalence and suffering in this complex and challenging syndrome.

Acknowledgements

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References and recommended reading

Papers of particular interest, published within the annual period of review, have been highlighted as:

- • of special interest
- •• of outstanding interest

Additional references related to this topic can also be found in the Current World Literature section in this issue (pp. 000–000).

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Attachment 5

What causes autism? Exploring the environmental contribution

Philip J. Landrigan

Department of Community and Preventive Medicine, Department of Pediatrics, Children's Environmental Health Center, Mount Sinai School of Medicine, New York, USA

Correspondence to Philip J. Landrigan, MD, MSc, Mount Sinai School of Medicine, One Gustave L. Levy Place, Box 1057, New York, NY 10029, USA
Tel: +1 212 824 7018; fax: +1 212 996 0407;
e-mail: phil.landrigan@mssm.edu

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Purpose of review

Autism is a biologically based disorder of brain development. Genetic factors – mutations, deletions, and copy number variants – are clearly implicated in causation of autism. However, they account for only a small fraction of cases, and do not easily explain key clinical and epidemiological features. This suggests that early environmental exposures also contribute. This review explores this hypothesis.

Recent findings

Indirect evidence for an environmental contribution to autism comes from studies demonstrating the sensitivity of the developing brain to external exposures such as lead, ethyl alcohol and methyl mercury. But the most powerful proof-of-concept evidence derives from studies specifically linking autism to exposures in early pregnancy – thalidomide, misoprostol, and valproic acid; maternal rubella infection; and the organophosphate insecticide, chlorpyrifos. There is no credible evidence that vaccines cause autism.

Summary

Expanded research is needed into environmental causation of autism. Children today are surrounded by thousands of synthetic chemicals. Two hundred of them are neurotoxic in adult humans, and 1000 more in laboratory models. Yet fewer than 20% of high-volume chemicals have been tested for neurodevelopmental toxicity. I propose a targeted discovery strategy focused on suspect chemicals, which combines expanded toxicological screening, neurobiological research and prospective epidemiological studies.

Keywords

autism, developmental neurotoxicity, national children's study, toxicity testing, vaccines

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Introduction

Autism is a complex, serious, biologically based disorder of brain development first described in 1943 by Kanner [1]. Social deficits, abnormalities in communication, repetitive behaviors, and cognitive inflexibility are the characteristic features [2]. There is no specific biochemical indicator or distinct neuroanatomical abnormality that defines autism, and the diagnosis is based on clinical and behavioral assessment.

Cases of autism vary from mild to profound and in the relative prominence of particular features and comorbidities. Approximately 50% of autistic children have intellectual disability, some have abnormally increased brain size, one-third have had at least two epileptic seizures by late adolescence, and about half have severely impaired speech [3]. Yet some children with autism, notably those with Asperger's syndrome, have highly developed intellectual skills, sometimes in specific areas such as mathematics. Because of this heterogeneity, the term 'autism spectrum disorder' (ASD) has come into use. ASD encompasses autistic disorder (DSM 299.00), Asperger's syndrome (DSM 299.80) and pervasive developmental disorder – not otherwise specified (PDD-NOS) (DSM 299.80).

The causation of autism is the subject of intense inquiry [4–7,8•]. Genetic factors are clearly important. Gene mutations, gene deletions, copy number variants (CNVs) and other genetic anomalies are all persuasively linked to autism [9]. But none accounts for more than a relatively small fraction of cases. The hypothesis therefore arises that early environmental exposures may also contribute to causation, perhaps acting in concert with genetic susceptibilities. It may further be hypothesized that variation in the interplay between different environmental exposures and inherited vulnerabilities may account for the observed heterogeneity in the autism phenotype.

The article explores the possible contribution of early environmental exposures to causation of autism, with particular focus on the possible role of toxic chemicals.

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It proposes a strategy for discovery of currently unrecognized and potentially preventable causes of autism.

Epidemiology of autism

The prevalence of autism currently reported in the US is 6–7 cases per 1000 children [10]. This reported prevalence is substantially higher than that of a decade earlier. Similar increases have been noted in the UK, Europe and Japan [11,12]. The CDC survey that established the current US rate found no significant difference between Caucasian and African–American children. It confirmed previous reports that ASD is 3–5 times more common in boys [13].

The reported increase in prevalence of autism has triggered vigorous debate as to whether the trend reflects a true increase in incidence, or is merely a consequence of expansion in the definition of ASD and greater awareness, improved diagnosis and better reporting [11]. This highly controversial question is not yet settled [14]. A recent critical analysis concludes that increases in recognition, changed diagnostic criteria, and changing public attitudes about autism have played a major role in catalyzing the upward trend in reported prevalence. This analysis observes, however, that the possibility of a true rise in incidence cannot be excluded [12].

Genetic factors in autism

Genetic and familial factors are unquestionably involved in causation of autism [4]. Families with multiple cases have been described. Autism has repeatedly been seen in sibs and twin pairs. Concordance in monozygotic twins is reported to be as high as 70% [15], and, when the broader phenotype of autism is considered, concordance in monozygotic twins approaches 90%. Concordance rates for autism in dizygotic twins appear no higher than among singleton siblings. Families with autistic children may contain members with 'autistic traits' such as social isolation or tendency toward repetitive behavior [13]. Autism occurs in a number of genetic conditions, among them Fragile X syndrome, Down syndrome, Cohen syndrome, Angelman syndrome [16] and Rett syndrome [17].

Ongoing research into the genetics of autism has employed the following three main strategies [18]:

(1) Family-based and case–control evaluations of candidate genes [19,20]. These studies have identified numerous candidate loci, most consistently on chromosomes 7q, 15q and 2q [19,20]. They have also identified specific mutations associated with ASD, notably in *SHANK3*, a gene that encodes a synaptic scaffolding protein; in *NLGN 3/4*, also involved in synapse formation; and in *PTEN* [16].
(2) Cytogenetic studies. Cytogenetic studies have identified abnormalities on chromosome 15q [19].
(3) Genome-wide association screens [9,21]. These studies – the most recent generation of genetic investigations into the causation of autism – have identified large-scale genetic duplications, deletions and CNVs associated with ASD. These include CNVs in *CNTN4*, a gene involved in development of neuronal networks; in *NRXN1*, involved in synaptogenesis [21]; and a recurrent microdeletion on chromosome 16p [22,23•]. Each of these microdeletions accounts for approximately 1% of cases of ASD.

At the present time, genetic factors are thought to account for 7–8% of autism cases, but this fraction will likely increase as genetic research advances and additional genetic causes are discovered.

Despite rapid advances in understanding the genetic contribution to autism, a purely genetic explanation for causation has difficulty in explaining certain clinical and epidemiological aspects of autism, among them the occurrence of sporadic cases, wide heterogeneity in clinical presentation, discordant development in monozygotic twins, and occurrence within families of members with fully developed autism side by side with others who manifest only 'autistic traits' [7,20]. This situation therefore raises the possibility that environmental exposures could also play a role in causation of autism [7,20]. These factors could act in concert with inherited susceptibilities or through inducing epigenetic changes [24•].

Plausibility for an environmental contribution to causation of autism

Support for the possibility that there is an environmental contribution to causation of autism comes from the following two sources:

(1) Current understanding of the exquisite vulnerability of the developing human brain to toxic exposures in the environment [25]; and
(2) Historically important, proof-of-concept studies that specifically link autism to environmental exposures experienced prenatally.

Vulnerability of the developing human brain to toxic exposures

Long and tragic experience that began with studies of lead [26] and methylmercury [27] has documented that toxic chemicals can damage the developing human brain to produce a spectrum of neurodevelopmental disorders ranging from overt toxicity at high levels of exposure down to subclinical dysfunction [28–31].

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The developing human brain is understood today to be exquisitely susceptible to injury caused by toxic chemicals in the environment [32]. This vulnerability is greatest during embryonic and fetal life, and may be especially great in the first trimester of pregnancy [33–35]. There exist windows of susceptibility in early development that have no counterpart in the mature brain [36].

A growing list of chemicals is now implicated in causation of neurodevelopmental disabilities, including:

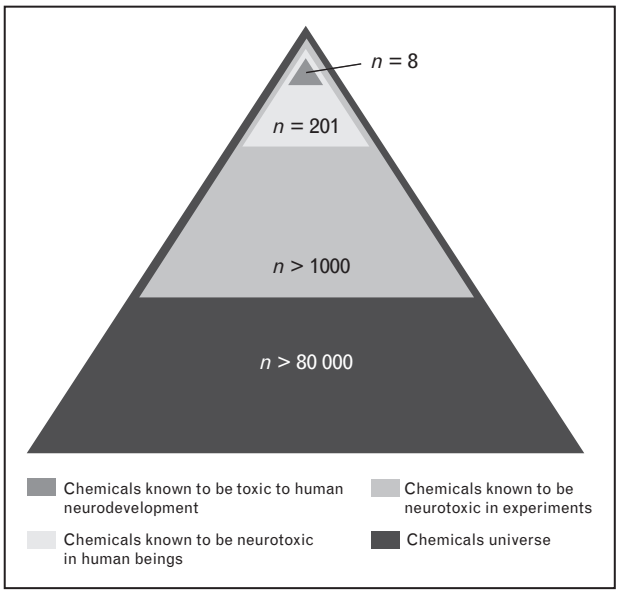
(1) Lead [26,28–30];
(2) Methylmercury [27,31];
(3) Polychlorinated biphenyls (PCBs) [37,38];
(4) Arsenic [39,40];
(5) Manganese [41];
(6) Organophosphate insecticides [42–44,45••,46];
(7) DDT [47];
(8) Ethyl alcohol [48].

Can other chemicals cause developmental neurotoxicity?

This short list of chemicals currently identified as human developmental neurotoxicants may be only the currently visible tip of a potentially much larger problem (Fig. 1).

Children today are at risk of exposure to 3000 synthetic chemicals produced in quantities of more than 1 million pounds per year, termed high-production-volume (HPV) chemicals. HPV chemicals are found in a wide array of consumer goods, cosmetics, medications, motor fuels and building materials. They are common in hazardous waste sites [49]. They are routinely detected in air, food and drinking water. Measurable quantities of several hundred HPV chemicals are found in the blood and urine of nearly all Americans, as well as in human breast milk and the cord blood of newborn infants [50]. Fewer than 20% of HPV chemicals have been tested for potential to cause neurodevelopmental toxicity [51].

Figure 1 The iceberg (triangle) of developmental neurotoxicants



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A recent systematic review of the world's literature undertaken to identify chemicals potentially toxic to the developing human brain produced a list of approximately 200 industrial chemicals documented to be neurotoxic in adult humans [33]. These are primarily industrial chemicals – metals, solvents, and pesticides – and nearly half are HPV materials. This search also produced a second list of approximately 1000 chemicals that have not been examined in humans, but that are neurotoxic in experimental models.

Given current understanding of the great vulnerability of the developing brain to toxic chemicals, likelihood is high that many of the materials identified through this search have potential to cause injury to the developing brain and to produce neurodevelopmental disorders, possibly autism among them.

Direct evidence for environmental causation of autism

The most strongly positive, 'proof-of-concept' evidence to support the hypothesis that environmental factors contribute to causation of autism comes from clinical and epidemiological studies that link autism with specific environmental exposures.

(1) Thalidomide: An increased incidence of autism is reported among children exposed prenatally to thalidomide [52]. In a population of 100 Swedish thalidomide embryopathy cases, at least four met full diagnostic criteria for autism [53]. On the basis of the pattern of concomitant somatic malformations, the time of critical exposure was calculated to be 20–24 days post conception [54].
(2) Misoprostol: Misoprostol is a prostaglandin analogue, licensed in the US for the prevention of gastric ulcers. It is widely used in some countries as an abortifacient. A case series from Brazil describes a group of seven children with ASD, of whom four (57.1%) had prenatal exposure to misoprostol [55]. The relevant exposures were reported to have occurred in the first trimester of pregnancy following unsuccessful abortion attempts; mean exposure was in the sixth week post conception.
(3) Valproic acid: Children exposed prenatally to the anticonvulsant valproic acid exhibit patterns of somatic malformation similar to those of thalidomide

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embryopathy, but of lesser severity. These include neural tube defects, cardiac malformations, craniofacial anomalies and limb defects. They can also develop autism [56]. Autism was reported in 11% of 57 children whose mothers took valproic acid in early pregnancy. An even larger number of these children had some autistic traits. On the basis of the pattern of somatic malformations, the time of critical vulnerability was calculated to be in the first 3–4 weeks post conception [57•]. In-utero exposure of rats to valproic acid has been shown to produce behavioral abnormalities analogous to autism [57•].

(4) Prenatal rubella infection: Clinical and epidemiological studies have linked maternal rubella infection in early pregnancy with autism [58]. In these studies, autism occurred in conjunction with other anomalies typical of the congenital rubella syndrome, including eye defects, deafness, mental retardation and cardiac malformations. Risk for autism appeared greatest when infection occurred in the first 8 weeks post conception.

(5) Chlorpyrifos: Chlorpyrifos is an organophosphate insecticide widely used until a few years ago to control insects in schools and homes in the US and still used extensively in agriculture. Chlorpyrifos was first recognized to be a developmental neurotoxicant in experimental studies, in which perinatal exposure of newborn rodents to low doses of chlorpyrifos was shown to cause reduced numbers of neurons, decreases in intelligence and persistent alterations of behavior [59].

Prospective assessments of infants exposed *in utero* to chlorpyrifos have reported exposure-related decreases in duration of gestation and in body weight at birth, as well as an exposure-related increase in the number of abnormally primitive neonatal reflexes [43,60,61]. Continuing follow-up of these children through 24–36 months demonstrated significant developmental delays [62], cognitive deficits, and increased risk for attention deficit–hyperactivity disorder (ADHD). Most recently these studies have found, on the basis of maternal report, an increased incidence of PDD-NOS [47,62].

In each of these examples the environmental exposures relevant to autism appear to have occurred prenatally, indeed very early in the first trimester of pregnancy [53,63]. These findings have substantial implications for understanding the environmental contribution to causation of autism and for the design of research that seeks to discover these causes [64••].

Vaccines and autism

Childhood immunization is a factor that has received much scrutiny as a potential environmental cause of autism. Claims of a link between vaccines and autism first arose in the late 1990s in the UK, the US and other countries and were triggered by clinical observation of onset of autism in the days immediately following vaccination [65]. In the UK, these claims focused on the measles–mumps–rubella (MMR) vaccine [66]. In the US, they focused on thimerosal, a preservative containing ethyl mercury that was added to multidose vials of many vaccines to prevent microbial contamination.

To address the issue, a series of studies was undertaken in the US, the UK, Europe and Japan. None of these studies have found any credible evidence for a link between vaccines and autism [12]. Key findings are:

(1) In the UK, there was a steady year-to-year increase in the reported number of cases of autism from the 1980s into the late 1990s. There was no evidence of a change in this trend line following introduction of MMR vaccination in 1988. In a British series of 498 cases of autism, there was no difference in age at diagnosis of autism between vaccinated children and children never vaccinated. There was no temporal association between MMR vaccination and onset of autism [67,68].

(2) In California, continuous increase in the rate of diagnosed autism occurred from the 1980s into the 1990s, but did not correlate with immunization patterns. Thus, autism cases increased from 44 per 100 000 live births in 1980 to 208 per 100 000 live births in 1994 (a 373% increase), whereas in the same time period MMR coverage increased from only 72 to 82% [69].

(3) In Yokohama, Japan, the MMR vaccination rate declined significantly between 1988 and 1992, and no MMR vaccine was administered in 1993 or thereafter. Despite declining immunizations, cumulative incidence of ASD increased significantly each year from 1988 through 1996 and rose especially dramatically beginning in 1993. Overall incidence of autism nearly doubled in those years [70].

(4) In Denmark, a comparison of autism rates in 440 655 immunized children versus 96 648 unimmunized children in the years 1991–1998 found no differences in incidence or prevalence between the two groups. There was no association between age at immunization or season at immunization and rate of autism [71].

(5) In Finland, a retrospective study in 535 544 1–7-year-old children vaccinated between November 1982 and June 1986 found no increases in incidence of autism during the 3-month period following immunization and no temporal clustering of autism hospitalizations [72].

(6) In the UK, a prospective population-based cohort study that has followed more than 14 000 children

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from birth found no evidence that early exposure to thimerosal had any deleterious effect on neurologic or psychological outcome [73].

(7) In the US, an analysis of neuropsychological function in 1047 children found no consistent correlation between neuropsychological functioning at age 7–10 years and early exposure to thimerosal-containing vaccines [74].

Taken together, this extensive series of high-quality, peer-reviewed studies has failed to show any association between autism and childhood immunization. Fear of autism does not justify failure to vaccinate children against life-threatening diseases [75].

Need for an autism discovery strategy

Although vaccines and their components are not credible causes of autism, the possibility remains open that there exist unrecognized environmental causes of autism [33]. Most likely these are to be found among the HPV chemicals to which pregnant women and children today are routinely exposed. The rationale for seeking environmental causes of autism is that, once discovered, these causes are potentially preventable [76].

A successful strategy for discovering the environmental causes of autism will need to be highly interdisciplinary. It will need to bring together researchers from outside the traditional autism research community [12]; from a wide array of disciplines including toxicology, epidemiology, developmental psychology, developmental neurobiology, neuropathology, molecular genetics, genomics, proteomics, functional neuroimaging and medical informatics.

Three key components of a proposed autism discovery strategy are:

(1) Toxicological studies: To identify chemicals that are developmental neurotoxicants and therefore have potential to contribute to causation of autism, a highly targeted toxicological search is urgently needed. A logical starting point for this search would be the 1200 chemicals identified as neurotoxic through the literature review described above [33]. Highest priority should be assigned to examining the chemicals on these lists that are already known to be neurotoxic in either humans or animals and that are most widely distributed in children's environments. Chemical classes that fulfil these criteria are organophosphate pesticides, organohalogens, phthalates and phenols, such as Bisphenol A.

New, more rapid screening tools for detection of developmental neurotoxicity need to be applied in this search [77,78•]. Current testing methods are slow and cumbersome and leave too many common chemicals untested. Also, to better detect the potential of chemicals to injure the developing brain, toxicity testing protocols need to expand to include examination of neurobehavioral function [79]. Current test protocols rely mainly on such crude parameters as brain weight and gross morphology [80,81] and are therefore relatively insensitive.

(2) Neurobiological research: To understand the cellular and molecular mechanisms involved in environmental causation of autism, a broad range of neurobiological studies need to be undertaken. These studies will discover how toxic chemicals interact with the developing brain, and identify the ways in which chemicals interact with the genome to produce changes in brain structure and function.

A possible approach to such studies would be to expose animals in early gestation to chemicals known to cause autism, such as thalidomide or valproic acid, and then to study the cascade of genetic, molecular and cellular effects that these exposures produce in offspring. That work has the potential to identify perturbations in signaling pathways that are critical in the genesis of autism. Those pathways could then be queried in relation to other synthetic chemicals in new, high-throughput toxicological testing systems.

(3) Prospective epidemiological studies: Large-scale, prospective epidemiological studies such as the recently launched US National Children's Study are extraordinarily powerful engines for discovery of the environmental causes of autism. The National Children's Study is the largest study of children's health ever undertaken in the US. It will follow 100 000 children, a statistically representative sample of all children born in the United States from conception to age 21 [81,82]. It is the first large-scale prospective study of children's health to specifically measure children's environmental exposures, prenatally as well as after birth, using a combination of maternal and infant biological markers and direct sampling of the ambient environment. It will collect samples for genetic analysis from each mother and child.

The National Children's Study will attempt to link children's prenatal and postnatal environmental exposures with the subsequent appearance of disease and dysfunction. It will examine gene–environment interactions. It is hypothesis-driven and will specifically seek environmental causes of autism and other neurodevelopmental disorders. Findings from the toxicological and neurobiological studies described above could inform and focus the National Children's Study by identifying particular classes of chemicals as targets for investigation. Given the currently reported prevalence of autism in the US, the study can be expected to include nearly 700

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children with autism. It will provide an unparalleled opportunity to examine interactions between genetic and environmental factors in the genesis of autism.

Conclusion

Much attention in recent years has focused on understanding the genetic contribution to causation of autism. This elegant research has identified a series of genetic factors and will likely discover still more. But none of these anomalies accounts to date for more than a small fraction of cases, and there is substantial imbalance between the extensive and highly sophisticated information on the genetics of autism and the scarcity of investigation into potential environmental causes. This situation raises the possibility that unsought environmental exposures contribute to causation of autism.

To discover the undiscovered environmental causes of autism, an interdisciplinary autism discovery strategy is proposed that combines toxicological screening, neurobiological research and prospective epidemiological study. Likelihood is high that this strategy will identify new environmental causes of autism, causes that can in theory be prevented. Potential for breakthrough discovery is high.

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Bioelectromagnetics 13:119–129 (1992)

Bursting Responses of *Lymnea* Neurons to Microwave Radiation

M.A. Bolshakov and S.I. Alekseev

Institute of Biological Physics, USSR Academy of Science, Puschino, Moscow Region, USSR

Microelectrode and voltage-clamp techniques were modified to record spontaneous electrical activity and ionic currents of *Lymnea stagnalis* neurons during exposure to a 900-MHz field in a waveguide-based apparatus. The field was pulse-modulated at repetition rates ranging from 0.5 to 110 pps, or it was applied as a continuous wave (CW). When subjected to pulsed waves (PW), rapid, burst-like changes in the firing rate of neurons occurred at SARs of a few W/kg. If the burst-like irregularity was present in the firing rate under control conditions, irradiation enhanced its probability of occurrence. The effect was dependent on modulation, but not on modulation frequency, and it had a threshold SAR near 0.5 W/kg. CW radiation had no effect on the firing rate pattern at the same SAR. Mediator-induced, current activation of acetyl-choline, dopamine, serotonin, or gamma-aminobutyric-acid receptors of the neuronal soma was not altered during CW or PW exposures and, hence, could not have been responsible for the bursting effect. © 1992 Wiley-Liss, Inc.

Key words: molluscan neurons, firing rate, mediator-receptor interaction

INTRODUCTION

The effect of high-frequency electromagnetic (HFEM) fields on molluscan neurons has been reported in a number of papers [Wachtel et al., 1975; Seaman and Wachtel, 1978; Arber and Lin, 1985a, 1985b; Arber et al., 1985; Bolshakov, 1985; Bolshakov and Alekseev, 1987]. Most studies have revealed a decrease in firing rates at specific absorption rates (SARs) above 7–10 W/kg, but this decrease has not been accompanied by a substantial alteration in the amplitude of action potentials. The response of neurons to pulse-modulated microwaves has been more complex. A dependence on modulation frequency as well as SAR has been found [Bolshakov and Alekseev, 1987]. In particular, modulated, low-intensity HFEM fields were associated with more pronounced changes in the firing rate as compared with a CW field at the same average values of the SAR.

Our paper is concerned with the appearance of bursting in the spontaneous activity of pacemaker neurons in response to PW and CW radiation by microwaves.

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Address reprint requests to M.A. Bolshakov, Institute of Biological Physics, USSR Academy of Science, Puschino, Moscow Region, USSR.

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We also observed evidence of an effect of HFEM fields on various receptor systems of neuronal soma.

MATERIALS AND METHODS

Materials

All solutions were freshly prepared. Acetylcholine (ACh) was obtained from Serva (Germany), dopamine (DA) and serotonin (5-HT) from Sigma (USA), gamma-aminobutyric acid (GABA) from Reanal (Hungary), and pronase from Boehringer (Germany). Other chemicals were of analytical grade and were used without further purification.

Preparation of Nerve Cells

The study of microwave effects on firing rate was conducted on BP-4-identified neurons [Zherelova, 1971] that had been isolated from large parietal ganglia of *L. stagnalis*. These pacemaker neurons have a small number of synaptic inputs that provide steady rates of firing.

After connective tissue was removed, an isolated brain preparation was attached to the wax base of a support chamber with cactus needles. The chamber was filled with 0.5 ml of physiological solution of the following content (in mM): NaCl, 80; KCl, 1.6; $CaCl_2$, 4; $MgCl_2$, 4; and Tris, 2. The pH was 7.5.

In experiments with mediator-induced ionic currents, unidentified neurons (80–100 μm in diameter) from large parietal ganglia were used. The neurons for perfusion were isolated as follows. Nerve ganglia were dissected and soaked in 0.35% pronase solution for 30 min. at room temperature, then transferred to the physiological solution for the next 30 min. A large parietal ganglion was opened. The neuropil with cells attached was freed from its covering sheath. Undamaged neurons were separated from neuropil by a glass pipette and placed in an experimental chamber that contained a physiological solution of the same formulation as mentioned above. A plastic pipette with a hole at its tip served as a suction electrode. It was filled with a solution of KCl (80 mM) and Tris (10 mM) at a pH of 7.3. The pipette hole was 30–40 μm in diameter and offered a DC resistance of 100–120 kohms. The neuron selected was sucked into the pipette. A small area of neuronal membrane embedded in the hole was moved aside to permit internal perfusion.

Microwave Exposure

The chamber with a ganglion or a perfused neuron was inserted through a access port into the center of a waveguide that operated in the TE_{10} mode. A cover for the access port was made of a metallic mesh (1 × 1 mm) that allowed us to maneuver a microelectrode into the chamber and to view the neuronal preparation. The waveguide system with an experimental chamber is shown in Figure 1. The microwave source was a 900-MHz generator. Both CW and PW radiations were used. Modulation frequencies ranged from 0.5 to 100 pps in the firing-rate experiments (the pulse rates were 0.5, 2.5, 6, 16, 40, and 100 pps). In the mediator-induced, ionic-current experiments, a 16-pps modulated field was used. The pulse-period to pulse-duration ratio was 25 in all cases. All experiments and SAR measurements were performed at 18–20°C. To determine SARs, elevations of temperature of the physiological solution at different output powers were measured with a thermistor near the

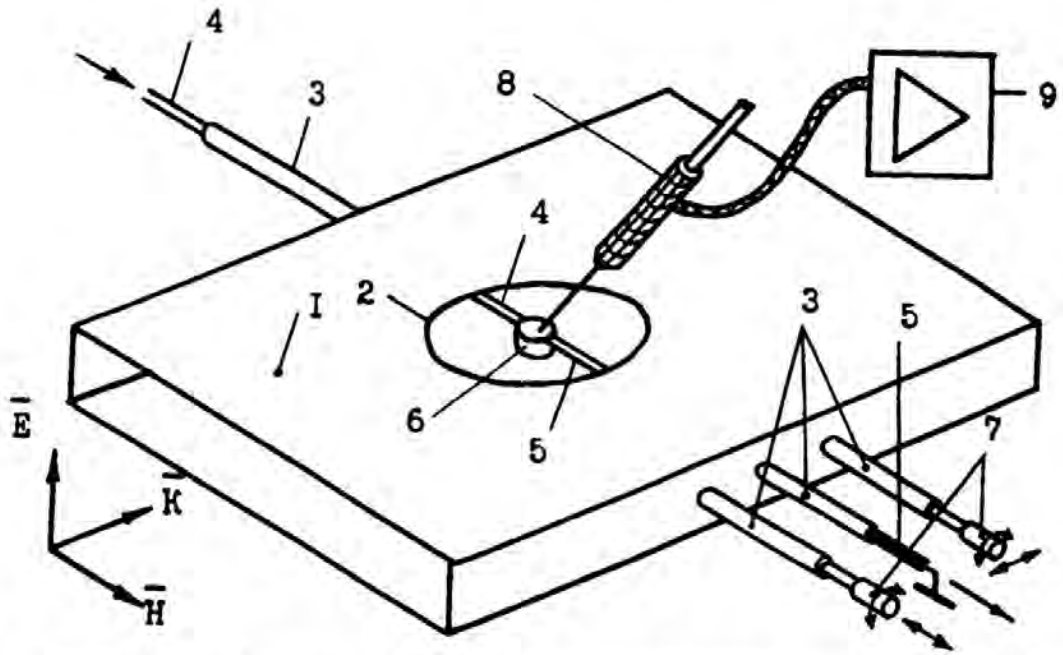

Fig. 1. Waveguide system as modified to expose a neuronal preparation to pulsed or continuous, 900-MHz microwaves while recording its spontaneous electrical activity by a microelectrode. **1:** Waveguide section. **2:** Access port. **3:** Metallic tubes to filter low-frequency currents [Tyazhelov et al., 1977]. **4 and 5:** Inlet and outlet for physiological bathing solution, which enclose the indifferent electrode. **6:** Holding chamber for neuronal preparation. **7:** Devices for orienting the holding chamber in one of two directions, as indicated. **8:** Shielded microelectrode holder. **9:** Microprobe.

cell immediately after irradiation. The SAR in W/kg was calculated from the equation:

$$SAR = 4200 \cdot \Delta T/t$$

where $\Delta T/t$ is the increment of temperature after a 1–3 min. exposure. The output-power vs. SAR plot obtained in the SAR range of 4–20 W/kg was nearly a straight line and could be extrapolated to a zero point. SARs within the range of 2–4 W/kg were determined by direct measurements of temperature during exposure. Lower values of the SAR (with $\Delta Ts < 0.1$ °C) were estimated from the extrapolated output-power vs. SAR plot. The SAR values were varied, as a rule, from 0.5 to 4 W/kg and from 0.5 to 15 W/kg, respectively, for pulse-modulated (average value) and CW fields. The temperature of a preparation was controlled by flow of the physiological solution through the chamber. At a flow rate of 2 ml/min and a microwave power level providing an SAR of 20 W/kg in a resting solution, the temperature did not rise by more than 0.1 °C. A desired elevation of temperature in the chamber was achieved by regulating the flow rate of the physiological solution after heating it 3 °C above the ambient temperature. Temperatures proximal to a nerve-cell preparation were monitored by a thermistor-based thermometer. At a flow rate of 2 ml/min. the initial rate of incrementing temperature was about 0.2 °C/s.

Firing Rate and Current Measurements

A conventional microelectrode technique was used to make intracellular recordings. Glass microelectrodes filled with 0.1-M KCl instead of 2-M KCL had a DC resistance between 30 and 40 megohms. They were inclined 45° with respect to the

E-field vector. As a rule, the tip of a microelectrodes was immersed by 4–5 mm into the physiological solution covering a cell. At a distance of 5 mm from a microelectrode's tip, the width of the glass wall of a microelectrode was less than 20 μm, which provided a low-capacity impedance to microwave-induced currents. Pick-up of microwave-induced currents by the microelectrodes was minimal. Exposure to CW fields at an SAR of 2–4 W/kg did not markedly influence the spontaneous firing activity of neurons, which confirmed out supposition that induced currents were minimal. The microelectrode was connected to a microprobe of a Dagan preamplifier system (Model 8500). The output signal was recorded by a chart recorder and a tape recorder (TEAC, Japan) to permit off-line computer analysis. Artifactual potentials were less than 0.5 mV during exposures that resulted in SARs of 10 W/kg.

Membrane currents were recorded by the Dagan-8500 system via the whole-cell, voltage-clamp technique. The holding potential on the membrane was set between −60 and −70 mV. Any artifactual currents that may have been present during exposures were so low as to be undetectable. The voltage-clamp and perfusion techniques, and the microwave-exposure and data-acquisition systems were identical to those described in detail by Alekseev et al. [1986].

RESULTS

Bursting Response of Neurons to Microwave Exposure

Most BP-4 neurons exhibited a steady rate of spontaneous activity under physiological conditions. However, about 25 percent of the neurons exhibited burst-like irregularities that were interspersed during otherwise uniform periods of firing (Fig. 2). The bursts arose with some periodicity (after ~290-s intervals). As a rule, a burst lasted for 5–10 s during which 10–30 spikes appeared instead of the 2–5 spikes associated with normal activity.

The firing-rate patterns of both types of neurons were not changed noticeably during CW exposures at SARs that ranged from 0.5 to 4.0 W/kg. In contrast, PW exposures within the same range of SARs caused specific changes in the firing rate, as reported by Bolshakov and Alekseev [1987]. In addition, the PW irradiation elicited spike bursting in neurons that otherwise exhibited normal spontaneous activity. Bursts occurred at the very beginning of an exposure but did not reappear during 10-min. exposures. The latency of bursting activity was about 42 s (±22 s). PW radiation also shortened the interburst interval in neurons with irregular spiking activity. To evaluate these changes, the PW field was applied for 4–6 min. after the appearance of two bursts, and then a new inter-burst interval was measured. The results obtained are summarized in Table 1. The bursting activity was essentially independent of modulation frequency in the range tested (0.5–100 pps) and at SARs of 1–4 W/kg. At SARs below 0.5 W/kg, statistical analysis was confounded by the increasing irregularity and variability of the bursting end point. We interpret this instability to indicate that the threshold SAR for observation of the rapid changes of firing rate is near 0.5 W/kg. A CW exposure at an SAR that ranged from 0.5 to 15 W/kg did not induce the bursting effect.

Effects on Mediator-Induced Ionic Currents in the Nerve-Cell Membrane

It is possible that the bursting response of a nerve cell may result from application of an HFEM field on one of the various receptor systems of the cell's mem-

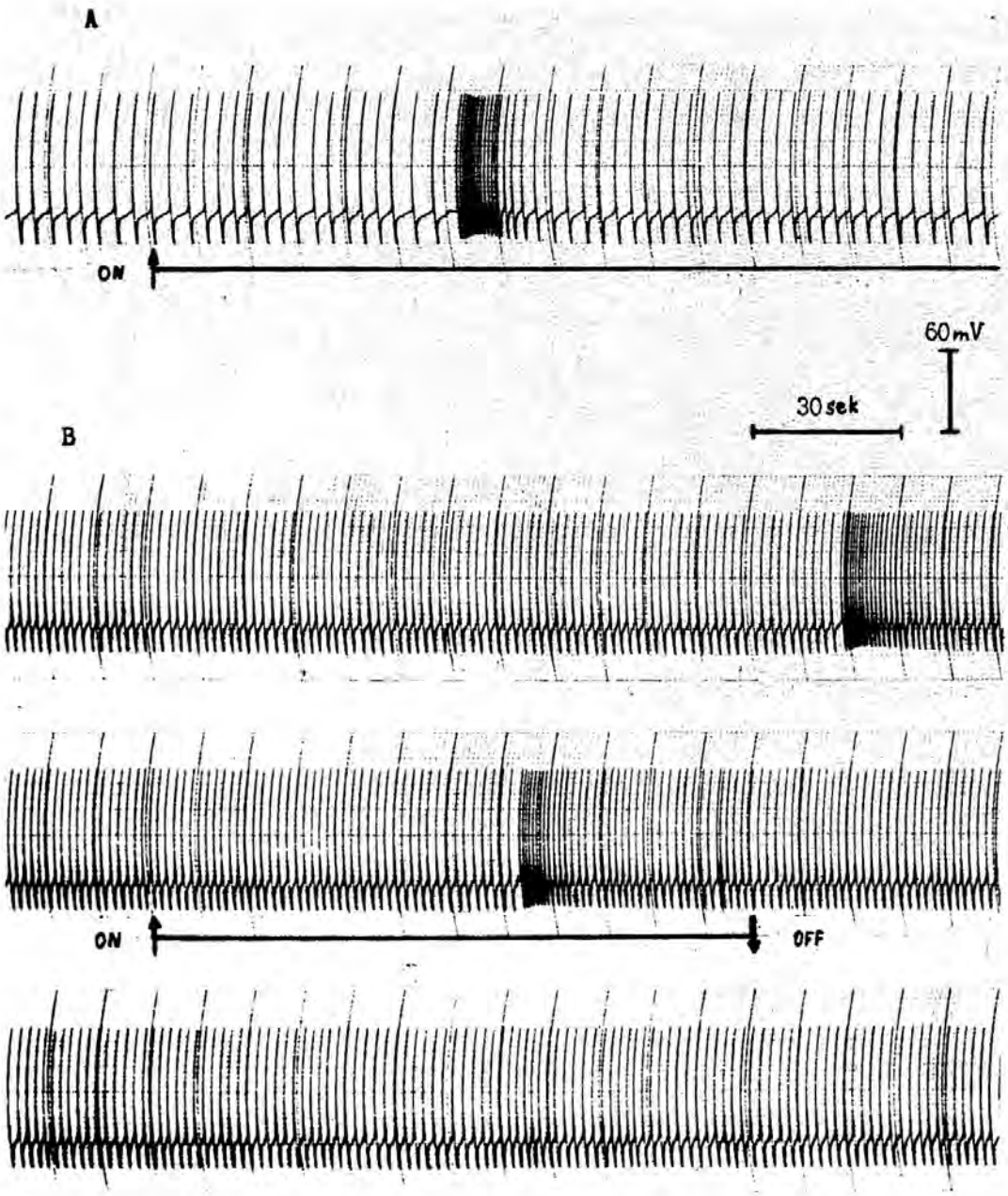

Fig. 2. Bursting responses of neurons to pulsed-wave (PW) irradiation. **A:** A burst of spikes from a neuron that exhibited a uniform firing rate before and briefly after initiation of microwave irradiation at an average SAR of 2 W/kg and a pulse-repetition rate of 16 pps. The latency of onset of bursting after the onset of irradiation was approximately 1.5 min. **B:** The effect of microwave irradiation on a neuron with an irregular rate of firing (2 W/kg and PW at 16 pps).

brane. This possibility was noted in a report by Fray and Wesler [1983]. If so, detectable changes in ionic currents activated by mediators might be found. Accordingly, we studied effects of microwave irradiation on the ionic currents that result from activation of ACh, DA, 5-HT, and GABA receptors.

We found that ionic currents induced by various mediators depended on mediator concentration and cell size. The properties of these currents were similar to those of corresponding currents reported earlier [Akopyan et al., 1980; Andreev et al., 1984; Gershenfeld and Paupardin-Tritsh, 1974; Jarowsky and Carpenter, 1978]. Mediators were applied to the cell by substitution of bathing solutions. To prevent strong desensitization by the induced currents, concentrations of the mediators were chosen

TABLE 1. Latencies in Seconds to Microwave-Induced Appearance of Bursting Activity During Otherwise Uniform, Low Rates of Neuronal Firing*

Time condition	Mean	SE	SAR in W/kg	PRR in pps	N
Latency	55	30	4	0.5	16
Latency	44	26	2	2.5	14
Latency	47	17	2	6.0	42
Latency	42	22	2	16.0	21
IBI (before exposure)	290	70	—	—	50
IBI (during exposure)	140	21	2	16.0	15
Latency	37	20	2	40.0	22
Latency	49	24	2	100.0	17

*Also shown are means of inter-burst intervals (IBI) of neurons that displayed irregular rates of firing. Means and standard errors (±SEs) are shown for pulse repetition rates (PRR) of the 900-MHz microwave irradiation and the number of neuronal preparations studied (N).

to be within 5×10^{-2} to 10^{-3} mM, which induced currents between 10^{-9} and 10^{-8} A. Short applications of mediator were used (5–10 s). Nevertheless, the currents were gradually declining during successive applications of the mediator. In many cases the time dependence in the decline of peak-current was a straight line with a slope that depended on the kind of mediator. Figure 3B (curve 1) demonstrates the change of the DA-induced current. It had a slowly declining slope and was chosen to illustrate this characteristic. Exposure to microwaves was initiated only after determination of the slope, to insure correct evaluation of changes of current.

The effect of PW exposure on DA-induced currents is shown in Figure 3A and B; the exposure had virtually no effect. Neither PW nor CW fields had reliable effects on currents induced by the other mediators. These results are presented in the Table 2. We note that at an SAR of 20 W/kg and with an interrupted flow of bathing solution, increases of current could be observed. These increases correlated well with those produced by the elevations of temperature during exposures (2.0 to 2.1°C, 10-min. durations).

The data demonstrate that low-to-moderate levels of microwave irradiation produced little, if any, effect on receptor-channel conductance or binding of mediator to receptor. This conclusions is almost certainly true for ACh, DA, 5-HT and GABA receptors of molluscan soma.

DISCUSSION

Some molluscan neurons have an endogenous bursting pattern under physiological conditions. The bursting activity results from rhythmical changes of the bursting pacemaker potential (BPP). The mechanisms underlying the endogenous burst activity are still not completely understood. It is known that BPPs in molluscan neurons are characterized by a cyclical variation in sodium (and/or calcium) and potassium conductances [Faber and Klee, 1972; Junge and Stephens, 1973; Gola, 1974; Smith et al., 1975]. There is evidence that BPPs can be modified by application of exogenous calcium [Johnston, 1976] and do not depend on the cyclic activity of an electrogenic pump [Junge and Stephens, 1973]. Several studies have shown that BPPs

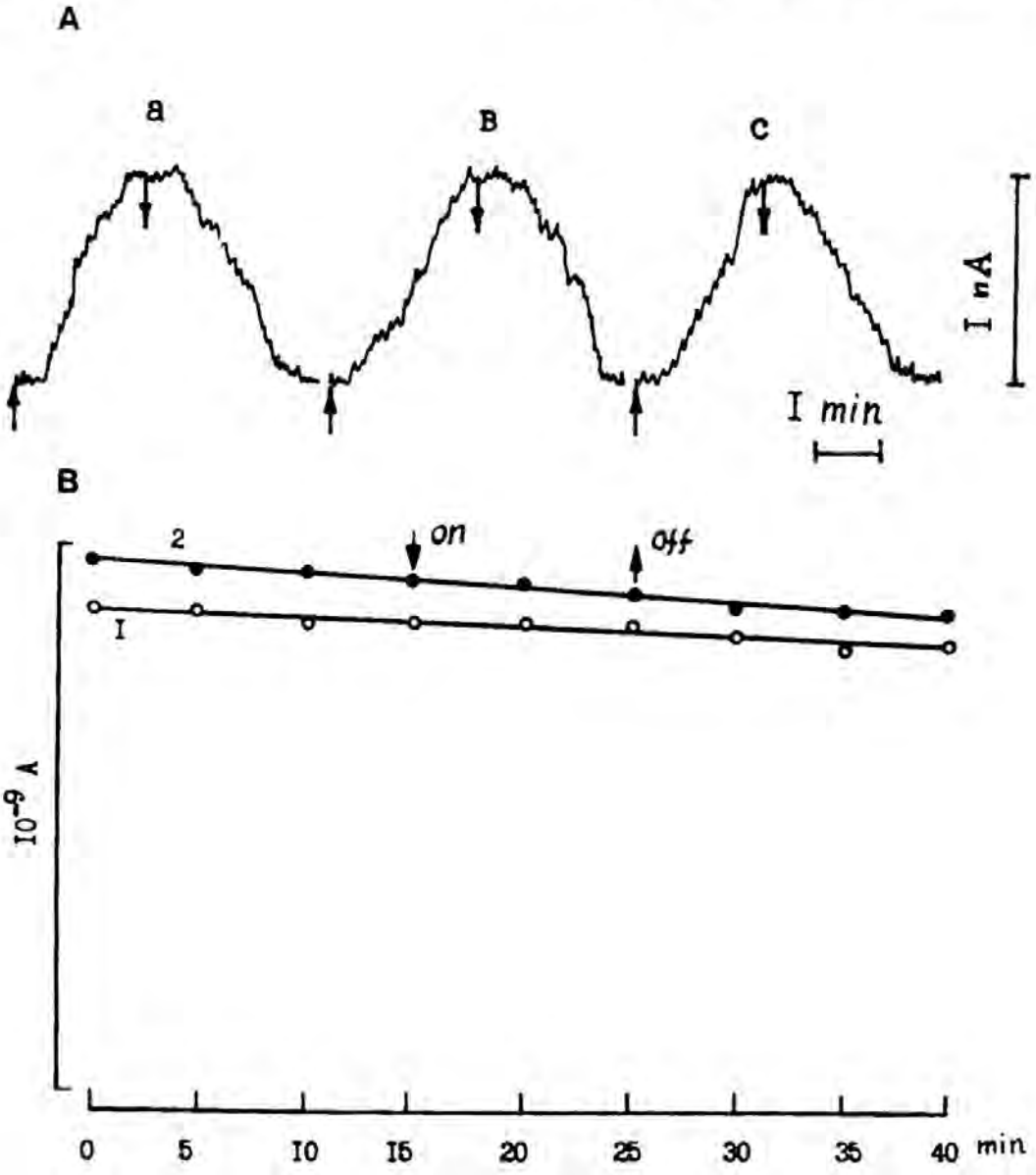

Fig. 3. Dopamine (DA) induced currents in neurons during PW irradiation at an average SAR of 4 W/kg. **A:** Recordings of DA-induced currents before irradiation (a); after a 10-min. exposure (B); and 10-min. after cessation of exposure (c). The arrows indicate the onset and offset of application of the DA at 2×10^{-5} M. **B:** Changes in the peak current induced by application of the DA to a control preparation (I) and to an irradiated preparation (2). The interval between the arrows denotes the period of microwave exposure.

can be modulated by external chemical agents. Certain convulsant agents have induced BPP-like activity in normally silent neurons [Klee et al., 1973].

It is likely that the burst-like responses of the neurons to microwave exposure were initiated by transient depolarization of the cell membrane via modification of ion-transport systems and/or facilitated release from cell membranes of some physiologically active agent(s), including calcium ions. Ion transport through the membrane is usually controlled by various types of ionic channels, electrogenic pumps, and receptors. Therefore, it is important to elucidate both the cellular and physical mechanisms by which microwave irradiation induces the bursting effect.

Seaman and Wachtel [1978] were probably the first to observe bursts of spikes in the regular firing of pacemaker neurons in *Aplysia californica*. They termed this

TABLE 2. Effects of 16-pps-Modulated, 900-MHz Microwaves on Mediator-Induced, Transmembrane Ionic Current*

Mediator	SAR	N	Mean	±SEM
ACh	0.5	6	1.00	0.02
DA	0.5	7	1.03	0.04
ACh	2.0	6	0.98	0.03
DA	2.0	6	1.02	0.04
5-HT	2.0	5	1.00	0.06
GABA	2.0	5	1.00	0.02
ACh	4.0	7	0.99	0.02
DA	4.0	4	1.03	0.04
5-HT	4.0	5	1.01	0.04
GABA	4.0	5	0.99	0.02

*After application of acetylcholine (ACh), dopamine (DA), serotonin (5-HT), or gamma-aminobutyric acid (GABA), neuronal preparations were exposed to pulsed microwaves for 15 min. The mean level of current and its associated standard error of the mean (SEM) are shown as normalized to values of a matched set of controls. The pulsed microwave field had essentially no effect on levels of induced current as SARs increased from 0.5 to 4.0 W/kg.

effect a rapid increase in the firing rate. The change in the firing rate was attained 25 s after the onset of irradiation. Later, Wachtel et al. [1982] demonstrated that the increase in the firing rate can be elicited by a temperature rise of 1 °C within several seconds. They concluded that a cell system sensitive to the rate of temperature change is responsible.

In our *Lymnea* neurons, bursts were not elicited by conventional heating at various rates as high as 0.2 °C/s. On the contrary, conventional heating resulted in a decrease of the firing rate, which depended on the rate of temperature incrementing [Bolshakov and Alekseev, 1986]. This finding is in accord with findings of Carpenter and Alving [1968] and Carpenter [1981], who demonstrated that the decrease of the firing rate is related to thermal activation of the electrogenic pump. Later, this result was confirmed by Bolshakov and Alekseev [1986] for *Lymnea* BP-4 neurons. Firing rates decreased significantly under rapid heating. At a thermal incrementing rate of 0.2 °C/s, the firing ceased completely within 30 s from the onset of heating. All these findings indicate that the mechanisms underlying the bursting response in *Lymnea's* neurons are not related to the integral of ΔT or to activation of the electrogenic pump.

Alekseev et al. [1986, 1987] reported that CW or PW irradiation resulted in an increase of potassium and calcium currents in *Lymnea's* neurons. These effects were a consequence of microwave heating of the external solution. No athermal effects were observed. As a rule, ionic currents had a temperature coefficient (Q_{10}) ranging from 1.5 to 2.5. So it seems at low levels of microwave exposure ($\Delta Ts < 0.1$ °C) that passive ion-transport changes are very small, and that they are not involved in the formation of the bursting effect.

We anticipated the possibility that HFEM fields might affect mediator-receptor interactions in the neuronal soma. There is evidence that some protein subunits of olfactory receptors are shed from membrane to bathing solution after exposure to

microwaves [Philippova et al., 1988]. However, the mediator-induced ionic currents of our neurons were not altered by microwave irradiation. These data are consistent with those obtained by D'Inzeo et al. [1988], who reported that the open time and the conductance of ACh-activated channels are not affected by microwaves. It is known that olfactory receptors function by intracellular mediators rather than by regulated channels, as is the case with ACh, DA, 5-HT, and GABA receptors. The results lead us to conclude that this difference in microwave effects is due to the differences in the two types of receptors.

Another way to evoke bursting is to increase the concentration of mediator (hormone) in the extracellular space within a ganglion. A rapid increase of firing rate was demonstrated by Vulfius and Zeimal [1968] in experiments in which ACh was applied to neurons. On the other hand, Gandhi et al. [1987] found that microwave irradiation increased the concentration of ACh in rat brain. The results of McArthur et al. [1977] also imply the release of transmitter substances during exposure to 960-MHz fields. Taking into account the reports by Liburdy and Penn [1984] and Philippova et al. [1988], one can speculate that there might be microwave-induced shedding of some protein subunits or polypeptides from neuronal membranes. These substances could play a role as physiologically active agents. Although there are no data that confirm the possibility of microwave-induced release of mediators, calcium ions or other substances in *Lymnea's* neurons could greatly influence the pattern of firing.

Given our range of SARs, we found that pulsed waves but not continuous waves can elicit bursting responses of neurons. Within the limits of experimental error the effect did not depend on the modulation frequency, but it was sensitive to the SAR. During shorter inter-pulse intervals of PW irradiation, the peaks of field strength were much greater than those of CW exposures at the same mean values of the SAR. The peaks of field strength may have been the cause of the microwave effect. The bursting effect was not elicited by CW irradiation at any SAR at levels as high as 15 W/kg. Both the peak strength of the pulsed field and the pulsing per se are factors that may determine the physical basis of the bursting effect.

Only a few comments can be directed to the question of mechanisms. It seems that a "windowed" effect similar to that found by Bawin et al. [1975] is unlikely because a pronounced frequency dependence of the bursting response was not observed in our studies. However, a pulse-modulated field can induce mechanical oscillations in the chamber. One hypothesis is that the effect of the pulse-modulated field is due to stimulation of neurons by mechanical oscillation or vibration. Under certain conditions mechanical oscillations induced by pulsed fields are strong enough to produce biological effects [Tigranyan and Shorokhov, 1985]. Further study is needed to identify with a high degree of confidence the mechanisms underlying the bursting responses of the neurons to microwave irradiation.

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Attachment 7

Changes of Clinically Important Neurotransmitters under the Influence of Modulated RF Fields—A Long-term Study under Real-life Conditions

Klaus Buchner and Horst Eger

This follow-up of 60 participants over one and a half years shows a significant effect on the adrenergic system after the installation of a new cell phone base station in the village of Rimbach (Bavaria).
After the activation of the GSM base station, the levels of the stress hormones adrenaline and noradrenaline increased significantly during the first six months; the levels of the precursor dopamine decreased substantially. The initial levels were not restored even after one and a half years. As an indicator of the dysregulated chronic imbalance of the stress system, the phenylethylamine (PEA) levels dropped significantly until the end of the study period.
The effects showed a dose-response relationship and occurred well below current limits for technical RF radiation exposures. Chronic dysregulation of the catecholamine system has great relevance for health and is well known to damage human health in the long run.

Keywords: cell phone base station, long-term study, stress hormones, radiofrequency radiation, GSM transmitter, far-field radiation

---------- Introduction

Despite the distribution of numerous wireless transmitters, especially those of cell phone networks, there are only very few real-life field studies about health effects available. In 2003, the Commission on Radiation Protection was still noticing that there are no reliable data available concerning the public's exposure to UMTS radiation near UMTS base stations (1).

Since the 1960s, occupational studies on workers with continuous microwave radiation exposures (radar, manufacturing, communications) in the Soviet Union have shown that RF radiation exposures below current limits represent a considerable risk potential. A comprehensive overview is given in the review of 878 scientific studies by Prof. Hecht, which he conducted on behalf of the German Federal Institute of Telecommunications (contract no. 4231/630402) (2, 3).
As early as the 1980s, US research projects also demonstrated in long-term studies that rats raised under sterile conditions and exposed to "low-level" RF radiation showed signs of stress by increased incidences of endocrine tumors (4, 5).

Concerned by this "scientific uncertainty" about how radiofrequency "cell tower radiation" affects public health, 60 volunteers from Rimbach village in the Bavarian Forest decided to participate in a long-term, controlled study extending about one and a half years, which was carried out by INUS Medical Center GmbH and Lab4more GmbH in

Original study in German: BUCHNER K, EGER H (2011) Umwelt-Medizin-Gesellschaft 24(1): 44-57.

Zusammenfassung

Veränderung klinisch bedeutsamer Neurotransmitter unter dem Einfluss modulierter hochfrequenter Felder - Eine Langzeiterhebung unter lebensnahen Bedingungen

Die vorliegende Langzeitstudie über einen Zeitraum von eineinhalb Jahren zeigt bei den 60 Teilnehmern eine signifikante Aktivierung des adrenergenen Systems nach Installation einer örtlichen Mobilfunksendeanlage in Rimbach (Bayern).

Die Werte der Stresshormone Adrenalin und Noradrenalin steigen in den ersten sechs Monaten nach dem Einschalten des GSM-Senders signifikant; die Werte der Vorläufersubstanz Dopamin sinken nach Beginn der Bestrahlung erheblich ab. Der Ausgangszustand wird auch nach eineinhalb Jahren nicht wieder hergestellt. Als Hinweis auf die nicht regulierbare chronische Schieflage des Stresshaushalts sinken die Werte des Phenylethylamins (PEA) bis zum Ende des Untersuchungszeitraums signifikant ab. Die Effekte unterliegen einem Dosis-Wirkungs-Zusammenhang und zeigen sich weit unterhalb gültiger Grenzwerte für technische Hochfrequenzbelastung. Chronische Dysregulationen des Katecholaminsystems sind von erheblicher gesundheitlicher Relevanz und führen erfahrungsgemäß langfristig zu Gesundheitsschäden.

Schlüsselwörter: Mobilfunk-Basisstationen, Langzeituntersuchung, Stresshormone, Mobilfunkstrahlung, Fernfeld

in cooperation with Dr. Kellermann from Neuroscience Inc.[1].

Common risk factors such as external toxic agents, parameters of the catecholamine system (6) were determined prior to the activation of the GSM transmitter and followed up in three additional tests for a period of more than 18 months. The informed consent of all participants included the condition that the data were to be published anonymously.

---------- Materials and Methods

Study Setting and Selection of Study Subjects

In spring 2004, a combined GSMD1 and GSMD2 cell transmitter (900 MHz band) was installed on Buchberg mountain in D-93485 Rimbach (Lower Bavaria) with two sets of antenna groups each. The installation height of the antennas for both systems is 7.9 m; the horizontal safety distance along the main beam direction is 6.3 or 4.3 m, respectively. At the same tower, there is also a directional antenna at 7.2 m (7).

1) INUS Medical Center, Dr. Adam-Voll Str. 1, 93437 Furth im Wald, Tel.: 09973/500 5412, www.inus.de; Lab4more GmbH, Prof. Dr. W. Bieger, Paul-Heyse-Straße 6, 80336 München, Tel.: 089/54321 730, info@lab4more.de; NeuroScience Inc., Dr. Kellermann, 373 280th Street - Osceola, WI 54020 - USA, Tel.: +1/715/294-2144, www.neuroscienceinc.com.

Shortly after it had become known that the wireless transmitters were to be installed, all inhabitants of Rimbach had been asked to participate in a mass screening. The municipality has approximately 2,000 inhabitants. In 60 volunteers (27 male, 33 female) aged between 2 and 68, the levels of adrenaline, noradrenaline, dopamine, and PEA (phenylethylamine)—which cannot be consciously regulated—were determined in their urine at the end of January/beginning of February 2004 (shortly before the activation of the antennas and the RF emissions beginning) as well as in July 2004, in January 2005, and in July 2005.

Most of these study participants signed up immediately after an informational gathering in late January 2004, at which the course of action by the cell phone service providers was criticized. Others signed up following a call for participation in the local paper. Since Rimbach is a small municipality, mouth-to-mouth propaganda also played a role. Participation was made attractive to the volunteers because a lab test that usually would be very expensive was offered for a small fee. Since the study required to show the status of the biological parameters over a given time period, only those study subjects participating in all four tests are included.

The data presented below come primarily from volunteers who have a certain interest in the life of their community and their health. Other persons joined the stress hormone investigation because of the recommendation of, or request by, their fellow citizens. This does not meet the requirements for a random sample. The result of this study, however, is hardly affected because Rimbach is a very small municipality. Therefore, the social contacts that lead to participation are very important. Most probably they do not affect the blood parameters. Furthermore, numerous large families participated as a whole whereby the health status of the individual family members did not play any role. For this reason, but especially because of the population structure, the study includes many children but only a few adolescents and young adults: there are hardly any opportunities for occupational training in Rimbach. In contrast, the municipality is attractive to young families with many children.

Sample Collection

The second morning urine was collected at INUS Medical Center on Mondays between 9:00 and 11:00 a.m. We made sure that each participant's appointment was always scheduled for the same time and that the time of breakfast or the state of fasting was the same for each participant at all tests. On the same day, the samples were sent by express to *Labor Dr. Bieger* in Munich where they were processed. In addition, samples were also sent to a laboratory in Seattle for control analyses (8-11).

Medical History

Medical doctors of the INUS Medical Center took a thorough medical history of each participant. At the initial test, the following data were also gathered: exact address, average time spent at home, indoor toxins, stress due to heavy-traffic roads, and the number of amalgam fillings. The latter number also included fillings that had already been removed. A nine-year-old child was noted to be electro-

sensitive to the effects of household wiring and connected appliances. All other study participants declared themselves to be not electrosensitive.

When taking their medical history, participants were also questioned about subjective symptoms and chronic diseases at the start of the study and during its course; if overweight, this was also noted. In this study, overweight in adults is defined as a weight greater than the "body height in cm minus 100 plus 5 kg tolerance."

Consistency checks for the parameter "overweight," however, indicate that—especially with regard to children—different criteria have been applied during the taking of the medical history. These data, therefore, can only serve as a reference point. They are listed here anyhow since they can provide suggestions for further studies.

All atopic disorders such as:

1. Hay fever, neurodermatitis, allergies, asthma, eczema are referred to as "chronic disorders;" as well as
2. All chronic inflammations such as interleukin- or COX-2-mediated problems;
3. All autoimmune diseases such as rheumatism, multiple sclerosis (MS);
4. All chronic metabolic disorders such as diabetes, liver diseases, intestinal diseases, kidney diseases.

Out of the 16 chronically affected participants 12 had allergies.

It was also asked whether there were DECT, Wi-Fi, or Bluetooth devices in the house or apartment during the study period from late January 2004 until July 2005. Also included were those devices present only for part of the study period, but not those turned off at night.

Exposure Level Measurements

For the most part, Rimbach municipality is located at one side of a narrow V-shaped valley. The cell phone base station is situated almost right across from the village center on the other side. RF radiation levels were measured at the outside of the residences of all study participants, wherever possible with direct line of sight of the transmitter. Because the municipality is located on a slope, great differences were noted inside homes—depending on whether or not a line of sight to the transmitter existed. In three cases, it was possible to measure the exposure levels at the head end of the bed. In these cases, the peak value of the power density was lower by a factor of 3.5 to 14 compared to measurements in front of the house with direct line of sight to the transmitter. The exact location of DECT, Wi-Fi, and Bluetooth base stations (if present) as well as possible occupational exposures, etc. were not determined by most participants.

At first, the measurements were taken with a broadband RF meter HF38B of Gigahertz Solutions, for which the manufacturer guarantees an error margin of max. ±6 dB (+ 7 decimal places; but this error can be mostly eliminated by selecting the appropriate measurement range). However, an inspection revealed that the error margin was less than ±3 dB. In addition, the broadband RF meter

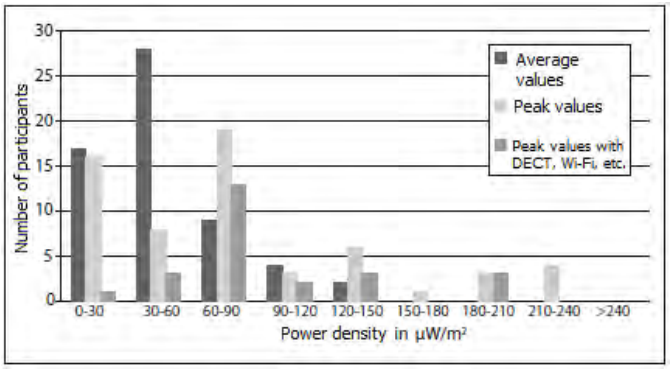

Fig. 1: Classification of participants based on average or peak value of the GSM power density level

HF59B (±3 dB, ±5 decimal places) was used at several points. With this RF meter, relevant frequencies can be analyzed with variable filters, the ELF modulation frequencies via fast Fourier analysis.

By using broadband RF meters, the testing effort and expense are reduced compared to spectrum analyzers. Thus, it was possible to take measurements at a greater number of points, and as a result, it was easier to determine the maxima and minima of the power density levels. Furthermore, the accuracy of high-quality broadband RF meters is similar to that of spectrum analyzers.

In this study, only cell phone signals are considered: not DECT, Wi-Fi, or Bluetooth devices inside homes or emissions from broadcast or TV stations at *Hohenbogen*, a mountain above Rimbach. For the most part, the emissions from the latter transmitters remained stable during the study period, whereas the focus of this study is on changes in exposure levels. For almost all sample measurements, the portion of the exposure due to the transmitter at *Hohenbogen* was at maximum 35 µW/m^2 (peak value). It was higher in the residences of only two study participants: 270 µW/m^2 (average) or 320 µW/m^2 (peak), respectively. At these residences, the GSM exposure was approximately 10 µW/m^2.

For the assessment, the peak values of the signals are used because, in the case of GSM radiation, they are less dependent on the usage level than average values. The peak value of the power density for all study participants from Rimbach was on average 76.9 µW/m^2 (Tab. 1).

In Figure 1 the exposure of the participants is given as power density levels in increments of 30 µW/m^2.

Classification of Participant Group and Exposure Levels

Sixty persons participated in the study; their age distribution is shown in Figure 2 according to year groups. In order to capture the effect of the cell phone base station, other environmental factors must be excluded as much as possible. It is vitally important to ensure that no major differences between high-exposure and low-exposure persons influenced the results.

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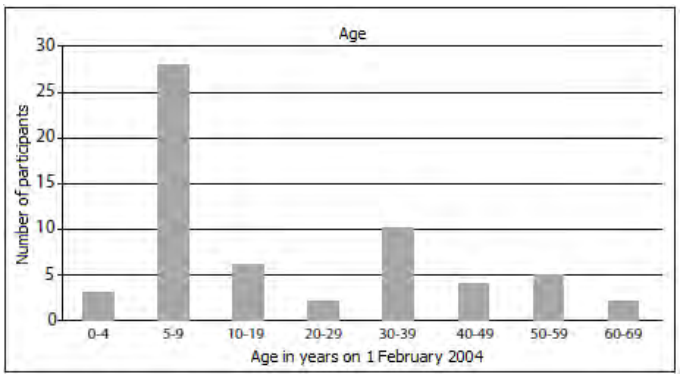

Fig. 2: Age distribution of study participants on 1 February 2004

	All	<=60 µW/m²	60-100 µW/m²	>100 µW/m²
Participants	60	24	20	16
Power density, avg (µW/m²)	76.9	21.7	68.1	170.7
Healthy adults	20	9	5	6
Sick adults	9	6	2	1
Healthy children	24	9	7	8
Sick children	7	0	6	1
Overweight	14	7	3	4
Amalgam number	12	5	3	4
Evaluation of amalgam/person	120	76.4	32.7	240
Street	8	0	8	0
Indoor toxins	17	7	6	4
DECT, Wi-Fi, Bluetooth	25	4	14	7

Tab. 1: Data on the 60 study participants who are classified into exposure groups 0 - 60 µW/m², 60 - 100 W/m², and above 100 µW/m², based on relevant peak values of GSM exposure in front of their residence.

Additional information:

Power density, avg (µW/m²) means: average peak value of GSM exposure level in the relevant category;
Healthy adults: adults without chronic diseases. Participants who were born after 1 February 1994 are referred to as children, all others as adults;
Sick adults: adults with chronic diseases;
Healthy children: children without chronic diseases;
Sick children: children with chronic diseases;
Overweight: see text;
Amalgam number: number of participants who had at least one amalgam filling (which may have been removed prior to the study period);
Evaluation of amalgam/person: For each tooth with an amalgam filling of a participant, the size of the filling (values from 1 to 3) is multiplied with the number of years this filling has been placed prior to the date of the initial test of this study (rounded up to the nearest whole number). The value in the table is the sum of these numbers for all amalgam fillings of a person in the respective category divided by the number of participants with amalgam fillings (= "amalgam number");
Street: number of participants who live at a busy street;
Indoor toxins: number of participants who have had contact with toxins, varnishes, preservatives, etc. at home or at work;
DECT, Wi-Fi: number of persons who had DECT, Wi-Fi, Bluetooth or the like at home at the end of January 2004 or later.

As shown in Table 1, the group with exposure levels greater than 100 µW/m² included fewer chronically ill persons and fewer residences at heavy-traffic roads, but considerably higher amalgam exposures by dental fillings compared to the average of the participants. These differences, however, cannot explain the observed development of the blood parameters as will be shown further below. It should also be noted that the number of children in the group of <= 60 µW/m² is considerably lower than in the other two groups.

Statistics

Because of the large individual differences in blood values, their asymmetrical distribution, and because of the many "outliers," the assessment presented here focuses on the following problem: "Did the level of a given substance predominantly increase (or decrease, respectively) in the test subjects?" For this problem, the so-called signed-rank paired Wilcoxon test (12) is applied. How to determine the confidence intervals of medians is described in an easy-to-understand form in (13).

Due to the rather large differences in individual values, we refrained from carrying out additional statistical analyses, especially those with parametric methods.

---------- Results

1 Clinical Findings

Adrenaline, noradrenaline, and dopamine as well as phenylethylamine (PEA) levels were determined at the time when the medical history was taken at INUS Medical Center. Out of the 60 participants, eleven had sleep problems until the end of 2004. During the study period (until July 2005), eight additional cases with these problems were reported. At the end of January 2004, only two participants complained about headaches; eight additional cases were reported thereafter. For allergies, there were eleven cases in the beginning and 16 later; for dizziness five and eight; and for concentration problems ten and fourteen. Due to the limited number of participants, no meaningful statements can be made about changes during the study period regarding the conditions tinnitus, depression, high blood pressure, autoimmune diseases, rheumatism, hyperkinetic syndrome, attention deficit hyperactivity disorder (ADHD), tachycardia, and malignant tumors. (Tab. 2)

Symptoms	Before activation of transmitter	After activation of transmitter
Sleep problems	11	19
Headache	2	10
Allergy	11	16
Dizziness	5	8
Concentration problems	10	14

Tab. 2: Clinical symptoms before and after activation of transmitter

2 Adrenaline

The adrenaline level trends are shown in Figure 3. After the activation of the transmitter from January until July 2004, a clear increase is followed by a decrease. In participants in the exposure category above 100 µW/m^2, the decrease is delayed.

Since the distribution of the adrenaline levels is very asymmetrical as shown in Figure 4, the median values are better suited for evaluation than the average values. However, there is no significant difference between the trend of the median and the trend of the average values (Tab. 3). But it stands out that, in the lowest exposure group with a power density below 60 µW/m^2, median values do not decrease between July 2004 and January 2005.

The statement "The adrenaline values of study subjects increased after the activation of the transmitter, i.e. between January and July 2004" is statistically confirmed ($p<0.002$), as well as the statement "The adrenaline level of the study participants decreased from July 2004 to July 2005" ($p<0.005$). In the lowest exposure group, the increase is the smallest. Until the end of the study period, these values do not drop.

A certain dose-response relationship can be observed for the increase in adrenaline levels from January 2004 until July 2004. The increase in medians was 2.3 µg/g creatinine for all subjects. At an RF radiation level up to 60 µW/m^2, creatinine was 1.0 µg/g, and by contrast, for power density levels between 60-100 µW/m^2 it was 2.6 µg/g.

For subjects in the exposure group above 100 µW/m^2, creatinine levels were found to be 2.7 µg/g, i.e. this value did not increase. We refrain from any additional statistical analysis because, as shown further below, the increase in adrenaline levels was mainly observed in children and chronically ill participants whose numbers were not sufficient to be broken down into further subgroups.

		January 2004	July 2004	January 2005	July 2005
All	Average	8.56	10.79	8.84	9.14
	Median	7.44	9.75	8.40	7.45
	CI	5.9 - 8.4	6.6 - 11.7	6.1 - 10.0	6.5 - 9.6
0-60 µW/m^2	Average	8.9	10.3	7.7	9.0
	Median	6.4	7.4	7.8	7.4
	CI	3.8 - 10.3	4.6 - 13.2	3.4 - 9.4	5.5 - 11.1
60-100 µW/m^2	Average	7.9	10.4	8.4	9.0
	Median	7.4	10.2	8.1	7.2
	CI	5.3 - 10.0	6.6 - 12.8	5.0 - 11.2	6.4 - 9.7
>100 µW/m^2	Average	8.9	12.0	11.1	9.6
	Median	8.2	10.9	10.6	8.6
	CI	5.3 - 10.9	5.7 - 19.6	5.8 - 15.2	4.9 - 13.4

Tab. 3: Results for adrenaline levels in µg/g creatinine
CI = 95% confidence interval of median

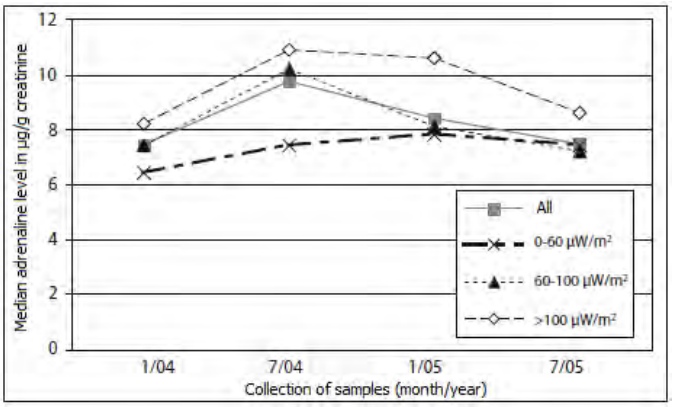

Fig. 3: Median adrenaline levels for all participating citizens of Rimbach whose cell phone base station exposure was above 100 µW/m^2, between 60 and 100 µW/m^2, or up to 60 µW/m^2. The power density levels refer to peak values of the GSM radiation exposure in front of a given residence.

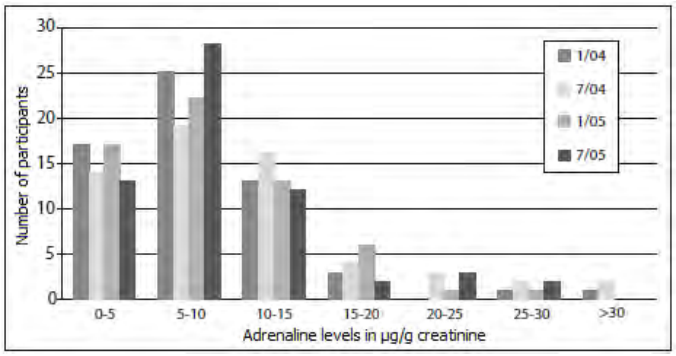

Fig. 4: Distribution of adrenaline levels in µg/g creatinine

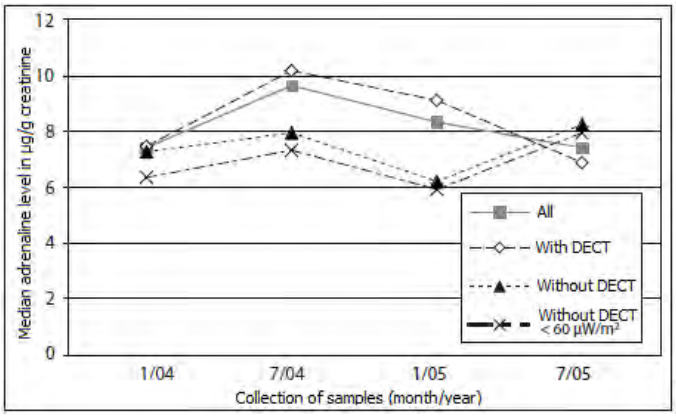

Fig. 5: Median adrenaline levels for all participating citizens of Rimbach who have a DECT phone, Wi-Fi, Bluetooth, or similar device, for those who do not have such wireless devices, and for the lowest exposure group without indoor wireless transmitters and with a GSM power density level up to 60 µW/m^2.

The impact of indoor wireless devices such as DECT, Wi-Fi, and Bluetooth (the latter are not specifically mentioned in the graphs) are shown in Fig. 5. Within the first year after the activation of the GSM transmitter, i.e. until and including January 2005, the group with indoor wireless devices shows the strongest responses.

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It is possible that in the less exposed subjects seasonal fluctuations or other factors such as "overshooting" of the values could have played a role.
It should be noted here that both the average as well as the median adrenaline values increased after the activation of the transmitter and decreased again after one year. This, however, only applies to exposure levels >60 µW/m^2. Chronically ill subjects and children showed especially strong responses; except for some "outliers," no effect was observed in healthy adults.

The adrenaline level of overweight subjects and those with an amalgam burden hardly changed during the study period (Fig. 6). In contrast, chronically ill subjects showed especially strong responses above average. In fact, the increase in the median values between January and July 2004 for all study subjects was predominantly caused by children and chronically ill subjects; adults without any chronic disease show a flat curve. During this period, an increased adrenaline level between 5 and 10.3 was measured in three healthy adults. Because of these "outliers," the average values for healthy adults clearly increased in contrast to the median values.

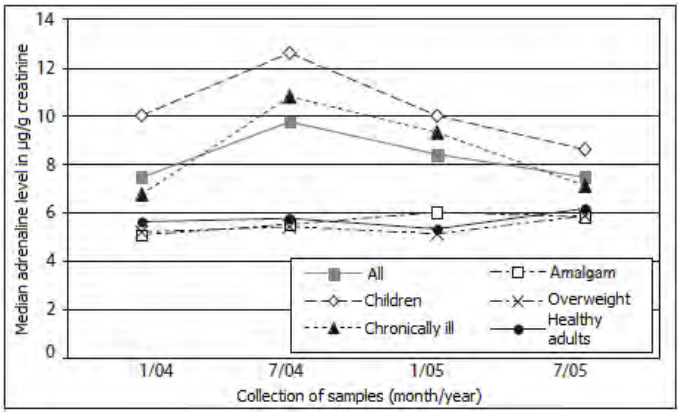

Fig. 6: Median adrenaline levels for participating children, for chronically ill subjects, for those with amalgam burden, and overweight subjects in Rimbach in comparison to the median levels of all study subjects and adults without chronic disease

The lower sensitivity of subjects with an amalgam burden can be explained by the fact that the effect occurs more often in children and that children according to our definition are younger than 10 years. They have hardly any fillings with amalgam.

3 Noradrenaline

The results for noradrenaline are similar to those for adrenaline (Tab. 4, Fig. 7). The statement that individual noradrenaline levels from January to July 2004 increased is statistically well supported with $p<0.001$. The fact that the levels dropped between July 2004 and July 2005 is also well supported with $p<0.0005$. Like in the case of adrenaline, the period under investigation is July 2004 to July 2005 to take the delayed decrease in the high exposure group into consideration. According to Table 4, the median of all noradrenaline levels increased from January to July 2004 for 11.2 µg/g creatinine; for exposures up to 60 µW/m^2, there were 2.2 µg/g creatinine, at 60-100 µW/m^2 12.4 µg/g creatinine, and above 100 µW/m^2 12.3 µg/g creatinine. As in the case of adrenaline, the increase for the last two groups is almost the same. Again, it is not possible to statistically verify a dose-response relationship. In Figure 7, a dose-response relationship

		January 2004	July 2004	January 2005	July 2005
All	Average	55.8	64.9	57.7	55.7
	Median	49.8	61.0	52.2	53.5
	CI	44.3-59.1	53.3-72.2	45.0-60.3	41.9 -60.5
0-60	Average	54.7	59.3	56.5	53.5
µW/m^2	Median	45.2	47.4	48.7	48.1
	CI	35.1-67.8	36.3-75.6	40.1-60.0	36.3-65.6
60-100	Average	51.4	63.6	49.1	55.9
µW/m^2	Median	47.5	59.9	45.8	54.8
	CI	38.0-59.1	53.1-74.8	40.5-58.4	34.9-66.5
>100	Average	62.9	74.9	70.1	58.8
µW/m^2	Median	58.8	71.1	71.6	56.3
	CI	49.9-87.3	54.9-91.6	48.7-89.1	36.9-81.6

Tab. 4: Results for the noradrenaline levels in µg/g creatinine
CI = 95% confidence interval of the median

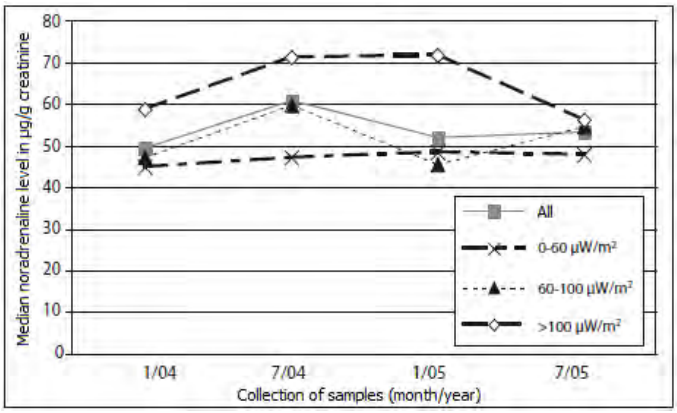

Fig. 7: Median noradrenaline levels in all participating citizens of Rimbach as a function of GSM power density levels (peak values)

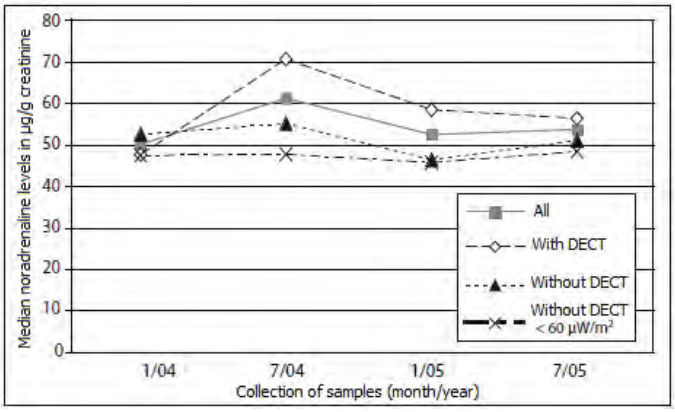

Fig. 8: Median noradrenaline values for subjects who had a DECT phone or other wireless devices at home, for those without indoor wireless devices, as well as for subjects without indoor wireless devices and with a GSM radiation exposure up to 60 µW/m^2 (peak value measured in front of residence)

is seen, whereby the dot-dashed line serves as reference for persons with very low exposures. It stands out that the "recovery period," i.e. the decrease in values in 2005, drags on for longer in subjects in the exposure group with GSM radiation levels above 100 µW/m^2. This also corresponds with the behavior of the adrenaline levels.

In comparison with adrenaline, noradrenaline plays a somewhat greater role in residences where wireless devices existed before the beginning of this study (Fig. 8).

The trend in Figure 9 shows that children and chronically ill subjects in contrast to overweight subjects express strong responses to cell tower radiation. The ratios, however, are not as clearly visible as with adrenaline. Especially in overweight subjects, they indicate a slow response to GSM radiation.

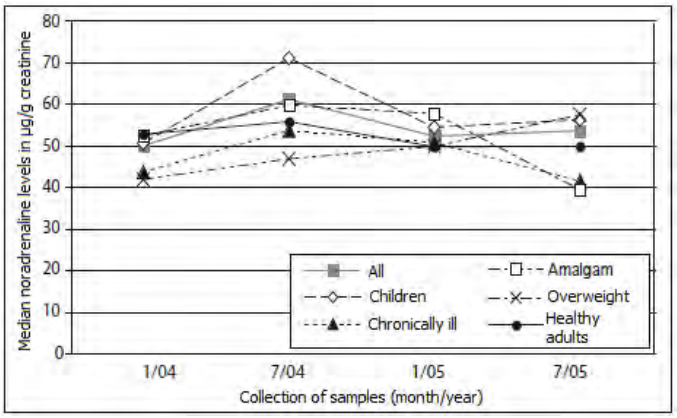

Fig. 9: Median noradrenaline levels of children, chronically ill subjects, those with amalgam burden and overweight subjects in Rimbach in comparison to the median values of all study subjects and healthy adults

Noradrenaline and adrenaline, however, responded very similarly.

4 Dopamine

For dopamine, inverse effects to those for adrenaline and noradrenaline were observed. The median dopamine levels decreased from 199 to 115 µg/g creatinine between January and July 2004 (Tab. 5). The fact that the dopamine levels of the study subjects decreased during this period is highly significant ($p<0.0002$). Thereafter, the median increased again: In January 2005, it was at 131 µg/g creatinine, in July of this year 156. This increase is also significant (for increase between July 2004 and July 2005 $p<0.05$).

This, too, is a dose-response relationship: from January to July 2004, the median for all subjects decreased for 84 µg/g creatinine, in the exposure group up to 60 µW/m^2 for 81, in the exposure group above 100 µW/m^2 even 153 µg/g (see Tab. 5 and Fig. 10). This dose-response relationship is statistically significant based on the signed-rank Wilcoxon test (12) with $p<0.025$. The following statement applies: "The decrease in dopamine levels for exposure levels up to 100 µW/m^2 is smaller than at exposure levels above 125 µW/m^2."

In subsequent laboratory tests, the dopamine levels do not return to the same level as in January 2004. From Figure 11, it is obvious that the correlation with prior exposures to indoor wireless devices is small.

		January 2004	July 2004	January 2005	July 2005
All	Average	233	158	138	164
	Median	199	115	131	156
	CI	168-273	86-160	111-153	145-175
0-60 µW/m^2	Average	217	183	130	148
	Median	189	108	116	147
	CI	142-273	80-254	90-157	129-167
60-100 µW/m^2	Average	242	161	140	178
	Median	223	150	131	175
	CI	137-335	94-168	93-164	126-207
>100 µW/m^2	Average	244	115	147	170
	Median	244	91	151	156
	CI	139-316	48-202	117-169	138-209

Tab. 5: Results for dopamine levels in µg/g creatinine
CI = 95% confidence interval of median

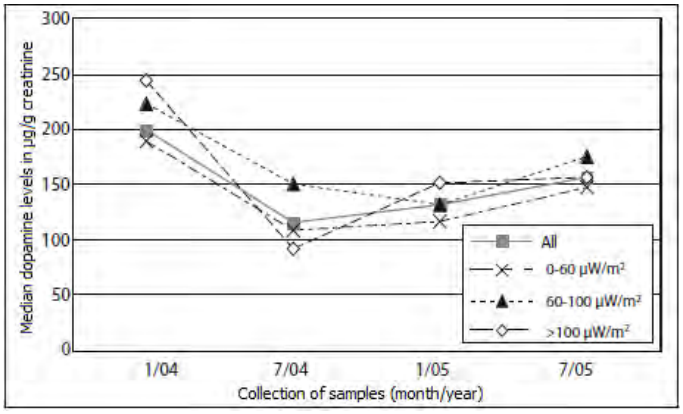

Fig. 10: Median dopamine levels for different GSM power density levels

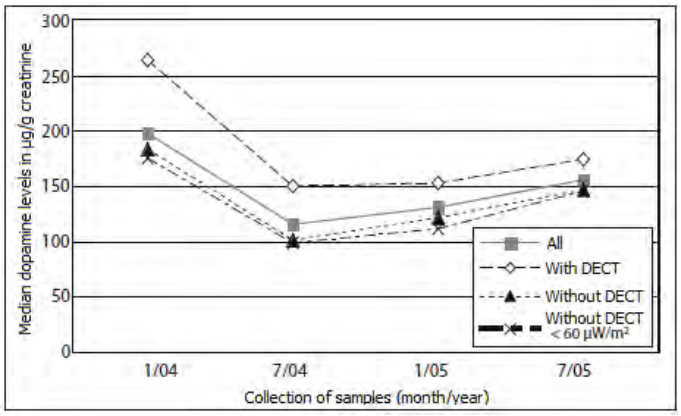

Fig. 11: Median dopamine levels for all participating citizens of Rimbach, for those with and without DECT phone, Wi-Fi, or Bluetooth, and for those without indoor wireless devices who had a GSM exposure level below 60 µW/m^2 (peak value).

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It is to be emphasized that the lowest exposure group without such indoor wireless devices and with a GSM power density level < 60 µW/m^2 responds almost as strongly as all other study subjects. This is consistent with the data in Figure 10: the data suggest that the effect of the radiation on the dopamine levels can already be observed at very low power density levels; however, it still can increase at levels above 100 µW/m^2.

Figure 12 shows that the radiation effect is somewhat more pronounced in children compared to the average, i.e. the gradient of the curves between the first two data points is somewhat greater. However, the difference is far too small to be statistically significant.

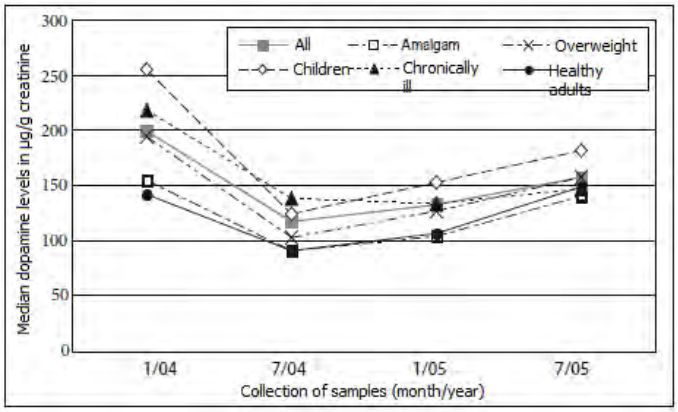

Fig. 12: Median dopamine levels of children, the chronically ill, with amalgam burden, overweight subjects, and healthy adults in Rimbach

In summary, dopamine levels decreased after the activation of the GSM transmitter and were not restored to the initial level over the following one and a half years. A significant dose-response relationship is observed. In children, the decrease is somewhat more pronounced than in adults.

5 Phenylethylamine (PEA)

Phenylethylamine (PEA) levels respond more slowly to the radiation compared to the substances investigated so far (Tab. 6, Fig. 13). Only in the exposure group above 100 µW/m^2 GSM radiation do the PEA levels decrease within the first six months. Thereafter, hardly any differences can be discerned between PEA values of the various power density levels investigated here.

The decrease of PEA levels between July 2004 and July 2005 is highly significant ($p<0.0001$)

Similar to adrenaline and noradrenaline, a previous exposure to indoor wireless devices intensifies the effect of the GSM radiation (see Fig. 14). The subjects of the low-exposure groups without indoor wireless devices do respond in a time-delayed fashion, but after six months they respond just as clearly as the subjects of the highest exposure group. In this regard, the PEA levels behave like those of dopamine in contrast to adrenaline and noradrenaline, which only respond to stronger fields.

		January 2004	July 2004	January 2005	July 2005
All	Average	725	701	525	381
	Median	638	671	432	305
	CI	535 -749	569 - 745	348 - 603	244 - 349
0-60 µW/m^2	Average	655	678	523	329
	Median	604	653	484	243
	CI	477 - 835	445 - 835	279 - 675	184 - 380
60-100 µW/m^2	Average	714	699	535	451
	Median	641	678	426	330
	CI	492 - 746	569 - 790	310 - 804	293 - 438
>100 µW/m^2	Average	843	739	514	371
	Median	780	671	413	305
	CI	451 - 1144	334 - 822	338 - 748	157 - 513

Tab. 6: Results for phenylethylamine (PEA) levels in ng/g creatinine
CI = 95% confidence interval of median

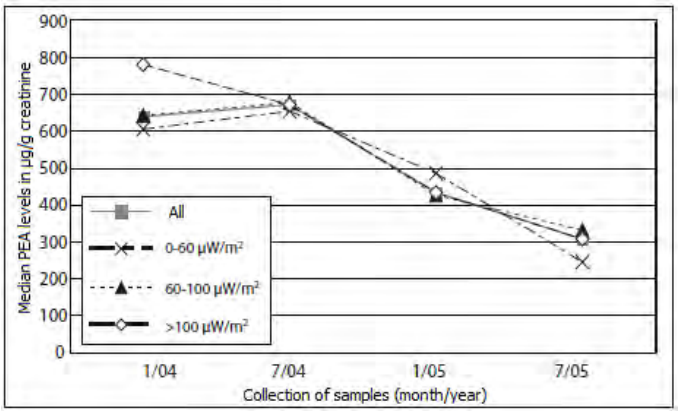

Fig. 13: Median phenylethylamine (PEA) levels for various GSM power density levels

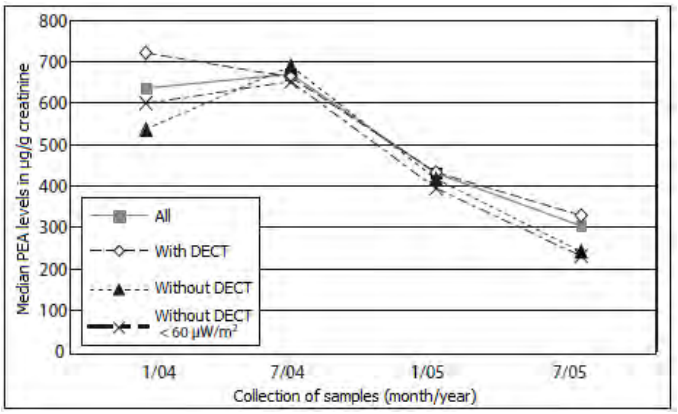

Fig. 14: Median phenylethylamine (PEA) concentrations in µg/g creatinine of subjects with and without indoor wireless devices at home and subjects without indoor wireless devices with a GSM power density level below 60 µW/m^2

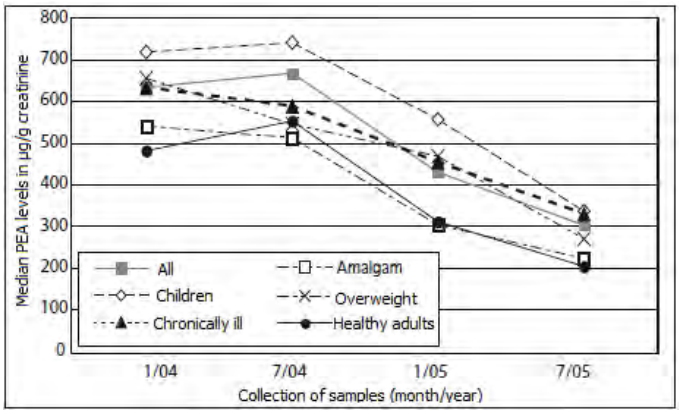

Fig. 15: Median phenylethylamine (PEA) concentrations in µg/g creatinine of children, the chronically ill, with amalgam burden, and overweight subjects, as well as health adults in Rimbach

In children, the effect of GSM radiation on their PEA levels is no greater than in the average of the study subjects; healthy adults also do not respond substantially differently. In contrast to the other substances looked at so far, the group of overweight subjects does respond particularly rapidly to PEA.

---------- Summary of Results

Adrenaline and noradrenaline levels increase during the first six months after the GSM transmitter had been activated; thereafter, they decrease again. After an exposure period of one and a half years, the initial levels are almost restored. Only at power density levels above 100 µW/m^2 is this decrease delayed for several months. In contrast, dopamine levels decrease substantially after the exposure begins. Even after one and a half years, the initial levels are not restored. Six months after the activation of the transmitter, PEA levels decrease continuously over the entire exposure period. Only in the exposure group above 100 µW/m^2 is this effect observed immediately. All findings were observed well below current exposure limits (14).

Wireless devices used at home such as DECT, Wi-Fi, and Bluetooth amplify the effect of the GSM radiation. In the case of adrenaline and noradrenaline, almost exclusively children and chronically ill subjects (here mostly subjects with allergies) are affected. However, the response of chronically ill subjects to dopamine and the response of children to PEA are very similar to those found in the average of the study subjects. Except for PEA, overweight subjects show only very weak responses to GSM radiation.

---------- Discussion

Catecholamine System and Phenylethylamine (PEA)

The survival of mammals depends on their ability to respond to external sources of stress. An established, well-researched axis of the human stress system represents the catecholamine system (6, 15, 16). It can be activated by psychic or physical stressors. Impulses mediated by nerves are responsible for an induction of the catecholamine biosynthesis at the level of tyrosine hydroxylase as well as dopamine beta-hydroxylase, whereby the effect is based on an induction of both enzymes. Many biochemical regulatory mechanisms tightly control catecholamine synthesis (8, 15, 17). Chronic dysregulation always leads to health problems in the long run. The development of high blood pressure under continuous stress serves as a clinical example; so-called "beta blockers" directly block the action of adrenaline and noradrenaline on the target receptors, and it is impossible to imagine medication-based therapy without them (15).

PEA can be synthesized from the essential amino acid phenylalanine either via tyrosine, dopamine, noradrenaline, and adrenaline or via a direct biochemical path (15) (Fig. 16). The sympathetic-mimetic effect of PEA was first described by Barger in 1910 (18).

PEA is also synthesized from phenylalanine and is considered a superordinate neuromodulator for the regulation of catecholamine synthesis (19-22).

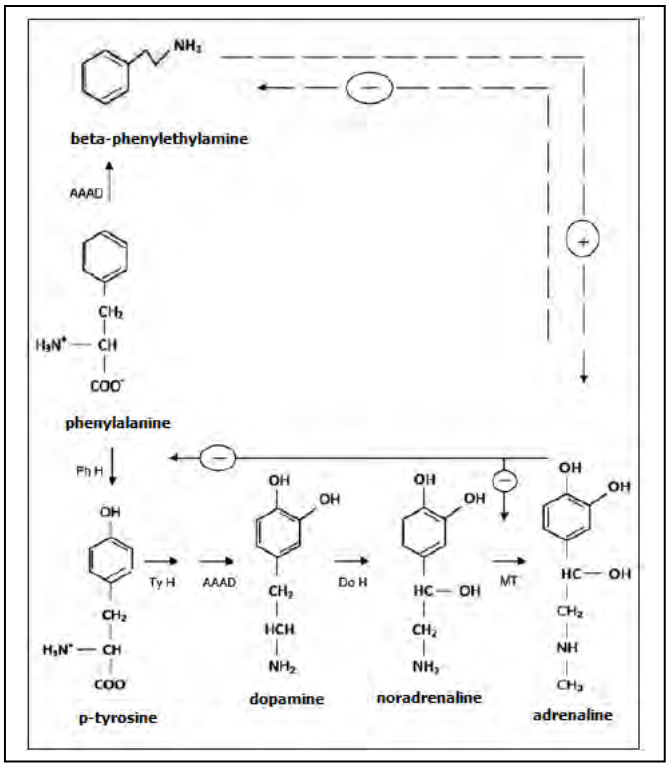

Fig. 16: Chemical structure of derivatives of the essential amino acid phenylalanine and the simplified synthesis pathways of catecholamines or phenylethylamine, respectively, simplified according to Löffler (15).

Abbreviations
AAAD: aromatic l-amino acid decarboxylase,
DoH: dopamine beta-hydroxylase,
PhH: phenylalanine hydroxylase,
MT: n-methyltransferase,
TyH: tyrosine hydroxylase
—(—)— ---- known feedback loop, - - (---) - - postulated feedback loop

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In 1976, Zeller described the physiological relationships (23) and points out that PEA is released by the brain via electrical stimulation (24).

The effect mechanism of PEA in the catecholamine system is the center of current pharmaceutical research efforts. In molecular biological terms, intracellular TAAR (trace amine-associated receptor) G-protein-coupled receptors that mediate modulatory effects of PEA are verified (20).

For high nanomolar to low micromolar PEA concentrations, in vivo studies have shown amphetamine-like effects. During an increase of PEA, an increased amount of noradrenaline and dopamine is also released and the reuptake of these substances is impaired (25, 26).
According to Burchett, the following effects of PEA amplifying the catecholamine effect are assumed to be known: Direct agonist action via increased release of transmitters, reuptake inhibition, and stimulation of transmitter synthesis as well as inhibition of monoamine oxidase (MAO) (19). PEA's high lipophilia—a prerequisite for the permeability of membrane barriers such as the blood-brain barrier—is of note here; PEA levels in the brain, serum, and urine correlate quite well (10, 21, 25, 27).

The clinical relevance of changed PEA levels is well documented for mental illnesses. Endogenous depression is associated with lowered PEA levels, whereby the transition from depression to maniac episodes is accompanied by an increase in PEA levels (28-32).

The therapeutic increase in the PEA level has a positive impact on the course of the disease. Phenylalanine improves the effectiveness of antidepressants; PEA by itself is a good antidepressant—effective in 60% of the cases of depression.

In persons with ADD/ADHD (attention deficit hyperactivity disorder), PEA levels are substantially lower; the ADHD treatment with methylphenidate (Ritalin®) normalizes PEA excretion in the urine of responders (33, 34).

Contributing Factors

Laboratory tests of catecholamine have been established for years. Increased values are found in disorders such as pheochromocytoma, neuroblastoma, and arterial hypertension, whereby it is impossible for a subject to consciously regulate these values. Especially urine tests offer a sufficient level of sensitivity and specificity because urine contains 100 to 1000 times higher levels than blood plasma. The intraindividual variation coefficient ranges from 7% to 12% from one day to another; stored under appropriate conditions, the stability of the samples can be guaranteed without problems (8).

In Rimbach, urine samples were always collected at the same time of the day so that a circadian dependence could be ruled out. Other contributing factors such as increased physical activity as well as large meals were also ruled out by collecting the urine in the morning. Seasonal factors of the samples collected twice in winter and summer should have been reflected as undulating levels in the testing results. Only in the adrenaline levels of the lower exposure groups (Fig. 5) can such a corresponding correlation be found. All other data did not indicate any seasonal influences.

In the study presented here, the selection of the participating citizens of Rimbach was not based on random assignment, but on self-selection. We can assume that the subjects, especially the adults, had informed themselves about the issue of cell tower radiation. However, because it is impossible to consciously regulate these levels, this self-selection should not make any difference in this study.
Especially in children below age ten, it is not thought possible to maintain a chronic state of anxiety for one and a half years due to an abstract term such as cell tower radiation.

This study limits itself to the following type of questions: "Did the level of a given substance predominantly increase or decrease during the study period?" Independent of each model, this question can be clearly answered with the Wilcoxon test and the indication of the confidence interval. The corresponding results are statistically very well supported. Any statements beyond this—e.g. the dependence of levels on certain parameters—cannot be made because with 60 study subjects the number of cases is too small to establish the same type of statistical significance.

The great advantage of the "Rimbach data" is that prior to January 2004 the exposure levels were very low because there was no cell phone tower and because only a few citizens had installed DECT, Wi-Fi and similar devices. In addition, due to the testing equipment with a measurement accuracy of less than ± 3 dB combined with repeated control measurements, the classification of the exposure groups can be considered to be verified.

For the stress hormones adrenaline and noradrenaline, the increase occurred only after the installation and activation of the transmitter, and thereafter, levels continued to decrease but did not fully normalize.
For dopamine, significant differences in the dose-response relationship according to exposure group could be shown after the activation of the new cell tower antenna. Also, the consistently decreasing levels of the hypothetically superordinate regulatory PEA do not support the hypothesis that the stress factor for the observed changes in the adrenergic system would exclusively be found in the realm of psychological factors.

Mode of Action of Microwave Radiation

There is a wide range of evidence to interpret the newly emerging microwave exposures as an invisible stressor.

Microwaves are absorbed by living tissue. The frequencies used for cell phone technologies have a half-life penetration depth of several centimeters, whereby cell membranes constitute no obstacle (35).

Microwaves cause enzymes to malfunction directly by, for example, monomerization (36). Thus, it is conceivable that enzymes of the catecholamine system could be affected directly.

Intracellular processes are changed, and cellular mitosis is disturbed by forces acting on the cellular spindle apparatus (37, 38). The human body is required to provide a higher level of repair services that is comparable to a chronic state of stress. A decrease in adenosine triphosphate (ATP) due to microwave exposure could be demonstrated by Sanders in intracerebral tissue already in 1980 (39).

Within current exposure limits, Friedman could show the stress caused by microwaves in the cell membranes of a cell model (40). The oxygen radicals formed by NADH have an activating effect on subsequent intracellular cascades that amplify the membrane effect by a factor of 10^7, which in turn substantially change intracellular processes (17). Even reproductive impairments due to microwaves are mediated by the formation of free radicals (41).

In industry, more and more microwave devices are being used for chemical peptoid syntheses, which allow for a 100 times faster and more precise production even without any measurable heating (42). The toxic effects of free radicals formed by microwaves are used in such technical applications as water purification (43).

In several studies, the chronic symptoms of residents near cell tower antennas were described (44-48). Interestingly, the expansion of wireless networks corresponds with the increase in prescription expenses for methylphenidate, a drug whose chemical structure is related to PEA and which is indicated in cases of attention deficit disorder (ADD) (49).

Long-term studies over five years suggested an increased cancer incidence due to microwave exposure (50, 51). Since the catecholamine system is directly linked with the nervous system within the psychoneuroimmunological framework beside its organ-specific effects, the observed increase in cancer incidence can now also be understood from a pathophysiological perspective (6, 15, 52, 53).

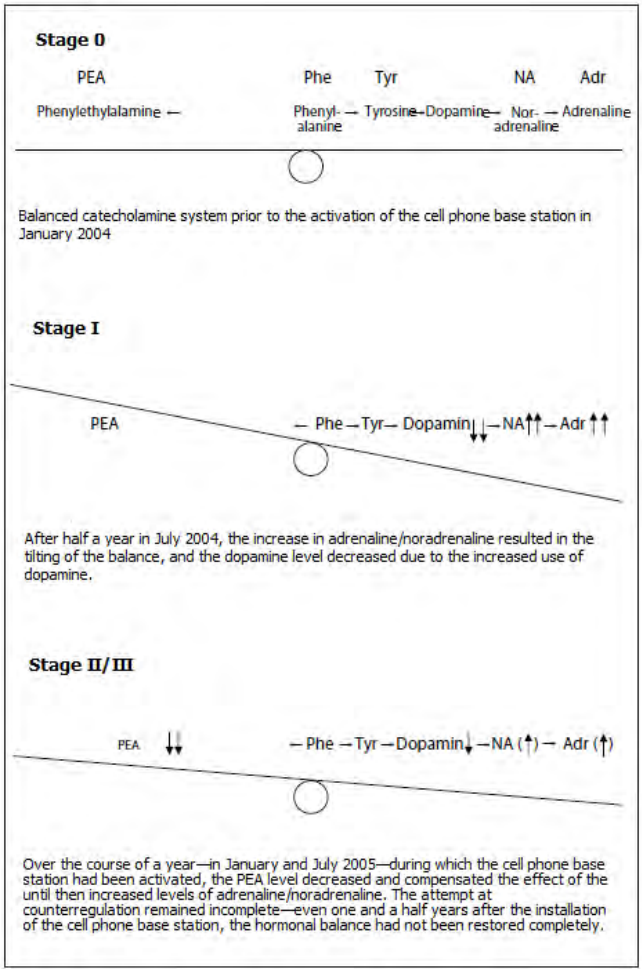

Fig. 17: Stage-like course of the stress response in Rimbach

Hypothesis of the Course of the Stress Response in Rimbach

Significant research on the stress-response axis was carried out in the 1950s. Selye established the nowadays generally accepted theory of the general adaptation syndrome of the human body to a stressor (16). He distinguished between three stages in the stress response, which can be found again in the description of the microwave syndrome according to Hecht (2, 3). Thus, after the stages of alarm and resistance, the last stage of exhaustion sets in (Fig. 17). The parameters investigated in the Rimbach study follow this pattern.

STAGE I—Activation Stage

The results of the long-term study presented here show an immediate activation of the adrenergic system. After the activation of the cell phone base station under investigation, the parameters adrenaline and noradrenaline increase significantly within a period of one and a half years. Because of the increased production of the final hormones noradrenaline/adrenaline, the use of dopamine increases, and as a result, the dopamine level decreases. The decrease in the dopamine level is the more pronounced, the higher the GSM radiation exposure level is at the residence of the individual participants.

STAGE II—Adaptation Stage

After this sympathicotonic activation stage, the body tries to compensate the increase in adrenaline and noradrenaline. In order to inhibit the overshooting catecholamine production and to ensure a stable regulation, the phenylethylamine level (PEA level) decreases. Here the decrease in PEA starts in the highest exposure group first.

STAGE III—Premorbid Stage

According to our hypothesis, the effects of adrenaline and noradrenaline are inhibited by feedback mechanisms at the expense of a chronically, over six continuous months, lowered PEA level. However, the attempt at counterregulation remains incomplete—even one and a half years after the installation of the cell phone base station; the hormonal balance had not been restored completely. The PEA level remains at a low level, which is to be interpreted as evidence for the beginning of exhaustion.

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---------- Conclusion

Thus, the following hypothesis is proposed: Although participants maintained their usual lifestyle, they developed chronic stress with a primary increase in adrenaline/noradrenaline and a subsequent decrease in dopamine in response to the microwave exposure from the newly installed cell phone base station. During the stage of counterregulation, the "trace amine" PEA decreases and remains decreased.
This is of considerable clinical relevance because psychiatric symptoms also exhibit altered PEA levels. In Rimbach, the increase in sleep problems, cephalgia, vertigo, concentration problems, and allergies could be clinically documented after the cell phone base station had been activated. The newly developed symptoms can be explained clinically with the help of disturbances in the humoral stress axis (53).

After having exhausted the biological feedback mechanisms, major health problems are to be expected. The possible long-term consequences of remaining caught in the exhaustion stage have already been described by Hecht and Selye (3, 16).

Thus, the significant results presented here not only provide clear evidence for health-relevant effects in the study subjects of Rimbach after a new GSM base station had been installed there, but they also offer the opportunity to carry out a causal analysis. This has already been successfully done in the "shut-down study" of Schwarzenburg, Switzerland (54). In Rimbach, the documented levels should return to normal once the relevant base station is shut down.

Epidemiological Evidence

There is current epidemiological evidence for the considerable clinical relevance of the dysfunction of the humoral stress axis with its endpoints of PEA decrease and adrenaline increase, as documented by us.

1. Decreased PEA levels can be found in a large portion of ADD/ADHD patients. As therapy methylphenidate is used, a substance that is structurally related to PEA. Between 1990 and 2004, the boom time of cell phones, prescription costs for this medication had increased by a factor of 86 (49, 55).

2. As part of the German Mobile Telecommunication Research Programme, approximately 3000 children and adolescents were studied in Bavaria for their individual cell phone radiation exposure levels in relation to health problems. Among the various data sets, the data set regarding behavioral problems showed a significant increased risk for both adolescents (OR: 3.7, 95%-CI: 1.6-8.4) and also children (OR: 2.9, 95%-CI: 1.4-5.9) in the highest exposure group (56). For the first time, the "Rimbach Study" provides a model of explanation in biochemical terms.

3. Pheochromocytomata are adrenaline- and noradrenaline-secreting tumors of the adrenal gland (57). This type of tumor due to microwave exposure has already been demonstrated in animal experiments in 1985 (5). The increase of this disease in the US population is highly significant. Concurrent with the increase in local microwave exposures due to an increased number of base stations and use of wireless communication technologies, the number of cases have increased from 1,927 to 3,344 between 1997 and 2006 (58, 59).

It is a physician's responsibility—not bound by directives—to work toward the preservation of the natural basis of life regarding human health (60). Now it is the duty of the responsible agencies (public health department, Bavarian State Ministry of the Environment and Public Health as well as other federal ministries) to investigate the current situation.

Note

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Contact:

Professor Dr. rer. nat. Dr. habil. Klaus Buchner
Straßbergerstraße 16
80809 München

Dr. med. Horst Eger (Korrespondenz)
Ärztlicher Qualitätszirkel „Elektromagnetische Felder in der Medizin - Diagnostik, Therapie, Umwelt'; (Code-Nr. 65143 KV Bayern)
Marktplatz 16
95119 Naila
E-mail: horst.eger@arcormail.de

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Attachment 8

SECTION 10

Effects of Electromagnetic Fields From Wireless Communication upon the Blood-Brain Barrier

Leif G. Salford, MD, PhD
Senior Professor Department of Neurosurgery
Lund University

Henrietta Nittby, MD, PhD
Department of Neurosurgery
Lund University Hospital

Bertil R. R. Persson PhD, MD h.c.
Prof. em. Department of Medical Radiation Physics
Lund University

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I. INTRODUCTION

The Blood-Brain Barrier

Some organs of crucial importance for the function of our bodies are protected from exposure to potentially harmful compounds in the blood. Thus the brain, the eyes (which are protrusions of the brain), the testes and the follicles of the ovaries have special barriers between the capillaries and the tissue. In the normal brain, the passage of compounds over this barrier, the Blood-Brain Barrier (BBB), is highly restricted.

The BBB is a hydrophobic barrier formed by the vascular endothelial cells of the capillaries in the brain with tight junctions between them leaving no openings between the vessel lumen and the surrounding brain. The existence of the mammalian BBB was discovered in the late 19th century by the German bacteriologist Paul Ehrlich and his student, Edwin Goldman. Paul Ehrlich found, that when he injected dyes into the systemic blood circulation, the brain tissue did not take up any of the stain. A barrier surrounding the brain tissue at the site of the brain micro vessels seemed to be a logic explanation to these findings.

There is scientific evidence that the BBB exists not only in vertebrates, but also in insects (1), crustaceans and cephalopod molluscs (such as the cuttlefish) (2) and in elasmobranchs (cartilaginous fishes such as sharks) (3) and helices (landsnails) (4), maintaining ionic integrity of the neuronal bathing fluid.

The BBB seems to be present very early in the foetal development. Also, at an early stage, there seems to be a cerebrospinal fluid barrier, which excludes cerebrospinal fluid (CST) protein from the brain extracellular space (5).

BBB Anatomy and Physiology

The tight junctions of the BBB are composed of tight junction proteins (occludin, claudin and zonula occludens, where the zonula occludens is the intracellular peripheral membrane protein that anchors claudin and occludin to the actin cytoskeleton (6). An important part is

the binding of claudin proteins on opposing membranes, where claudin-5 in particular is crucial in the BBB (7). Astrocytes are surrounding the outer surface of the endothelial cells with protrusions, called end feet, and are implicated in the maintenance, functional regulation and repair of the BBB. The astrocytes form a connection between the endothelium and the neurons and constitute a second barrier to hydrophilic molecules (see Figure 1).

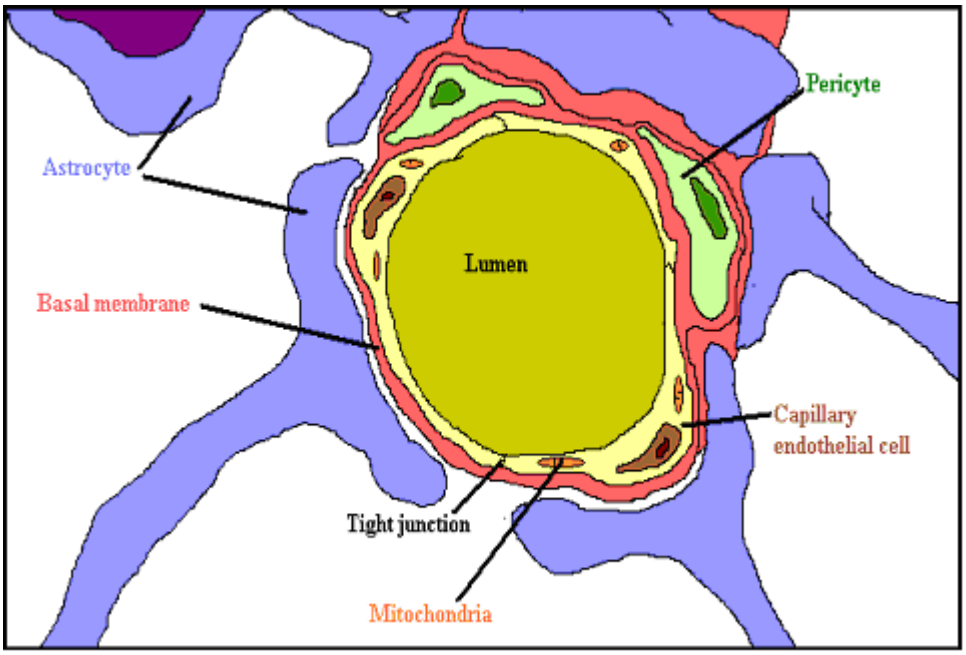

Fig. 1. The mammalian BBB

Other periendothelial accessory structures of the BBB include pericytes and a bilayer basal membrane which surrounds the endothelial cells and pericytes. The basement membrane (basal lamina) supports the ablumenal surface of the endothelium and may act as a barrier to passage of macromolecules. The pericytes are a type of macrophages, expressing macrophage markers with capacity for phagocytosis but also for antigen presentation. In fact, the pericytes, which cover about 25% o the capillary surface (8), seem to be in a position to significantly contribute to central nervous system (CNS) immune mechanisms (9). The pericytes also have other functional roles: with their capability for contractility they seem to serve as a smooth muscle equivalent, and through regulation of endothelial cells they maintain the stability of blood vessels (9). Additionally, the pericytes seem to be highly involved in many diseases, both infectious and autoimmune, and also in other diseases such as Alzheimer's by production

of amyloid. Also, by regulating their vascular permeability, the pericytes are supposed to play an important role in inflammatory diseases (9).

Physiologically, the microvasculature of the central nervous system (CNS) differs from that of peripheral organs. It is characterized not only by its tight junctions, which seal cell-to-cell contacts between adjacent endothelial cells, but also by the low number of pinocytotic vesicles for nutrient transport through the endothelial cytoplasm and its lack of fenestrations, and the five-fold higher number of mitochondria in BBB endothelial cells compared to muscular endothelia in rat (10). All this speaks in favour of an energy-dependent transcapillary transport. These above-described membrane properties of the BBB control the bidirectional exchange of molecules between the general circulation and the central nervous system. By at least four mechanisms, the endothelial cells directly control the flux of solutes into the brain parenchyma. Firstly, the tight junctions and low number of pinocytotic vesicles guarantee that proteins cannot pass freely into the brain parenchyma.
Secondly, solutes which are not highly lipid soluble, or which do not bind to selective transporters with high affinity, are excluded from free exchange. By means of this lipid solubility, carbon dioxide and oxygen, among many others, are able to enter the brain interstitial fluid passively, whereas the passage of, for example sugars and many amino acids, depends on other, active mechanisms. Thirdly, the BBB has a capacity to metabolize certain solutes, such as drugs and nutrients (11). Fourthly, active transporters maintain the levels of certain solutes at specific values within the brain interstitial fluid, made possible by active transport against the concentration gradients. These enzyme systems are differently distributed between the luminal and the ablumenal membranes of the endothelial cells, thus gaining the BBB polarity properties. For example, Na^+-K^+-ATPase is located on the antilumenal membrane (12).

It has been proposed that the active transport across the brain capillaries might be the most important mechanism for the regulation of the internal milieu within the brain parenchyma. Also, it has been proposed that this mechanism, requiring energy to function properly, might be the one most sensitive to disease and that interference with this active transport could play an important part in the neurological dysfunction seen in many metabolic disorders (12).

It is important to have information on possible differences between homo and other mammals. The mammalian brain at large seems to have a uniform anatomy of its BBB constituents

preserved through the evolution, and very little information about differences between mammalian species has been available. However, recently very interesting observations have been published. Humans have evolved protoplasmic astrocytes that are both larger (27-fold greater volume) and far more elaborate than their rodent counterparts. These astrocytes reside near blood vessels, and their processes contribute to the BBB (13). When the end feet of human and rodent protoplasmic astrocytes are compared, it is shown that nearly all astrocytes in both species contact the vasculature, but in the human brain, the end feet completely encompass the vessels while the rodent astrocytes form rosettes of end feet around the vasculature. The number of mithochondria is however equally abundant in human and rodent end feet (14).

Comparisons between mammalian species concerning enzymatic functions in the BBB are few in number. Similarities are described: mouse *vs* human (15) and rat *vs* human (16), while differences are demonstrated between rodent and dog BBB leading to the conclusion that the canine BBB may be preferable to that of the rat as a model for studies of glucose transport relevant to human brain (17).

In summary, the BBB serves as a regulatory system that stabilizes and optimizes the fluid environment of the brain's intracellular compartment (18-20). The intact BBB protects the brain from damage, whereas the dysfunctioning BBB allows influx of normally excluded hydrophilic molecules into the brain tissue. This might lead to cerebral oedema, increased intracranial pressure, and in the worst case, irreversible brain damage.

II. DISRUPTION OF THE BLOOD-BRAIN BARRIER

The normal selective permeability of the BBB can be altered in several pathological conditions such as epileptic seizures (21) or extreme hypertension (22)and also transient openings of the BBB might lead to permanent tissue damage (22). Considering the ensuing leakage of substances from the blood circulation into the brain tissue, harmful substances might disrupt the cellular balance in the brain tissue and in the worst case, even carcinogenic substances might pass into the brain tissue. It has also been shown that an increased permeability of the BBB is seen in cases of oxidative stress (23), where BBB dysfunction and

neurodegeneration were shown to be mediated through an excitotoxicity mechanism by the serine protease tissue plasminogen activator, with NO and $ONOO^-$ as downstream mediators (23).

Opening of the BBB thus can have detrimental effects and since it has been shown for a few decades that EMFs have the potency to increase the permeability of this barrier, a major debate is going on in society with increasing intensity. In the following, we try to clarify the actual status of the available evidence in the field.

Early Studies

In early studies on the effects of low-intensity EMFs on the BBB, various compounds were injected intravenously, followed by EMF exposure and comparisons of the penetration into the brain tissue between sham and exposed animals.

Frey et al. (25) found increases in the BBB permeability of rats to fluorescein after 30 min of exposure to both pulsed and continuous waves (CWs) at 1.2GHz with average power densities of $0.2mW/cm^2$. Similar observations were made in a study with 180 animals by Oscar and Hawkins (26). Exposure of anaesthetized rats for 20 min to 1.3GHz of pulsed EMFs with average power densities of $0.3mW/cm^2$ resulted in leakage of 14C-mannitol, dextran, and inulin into the cerebellar brain tissue, as well as inulin and dextran leakage from capillaries into hypothalamic and medullar tissue. Also, BBB permeability to mannitol was investigated in un-anaesthetised rats, which were exposed to pulsed radiation or sham exposed for 20 min. The animals were sacrificed at different time intervals after the exposure. BBB permeability was seen in the groups sacrificed 8 min and 4 h after exposure, but to a much lesser extent in those sacrificed after 8 h. Finally, the permeation of mannitol through the BBB was found to be a very definite function of exposure parameters such as power density, pulse width, and the number of pulses per second. However, in later studies, Oscar et al. (27) emphasised that changes of BBB permeability after microwave exposure partly could be explained by an increase of local cerebral blood flow. In accordance with this, they concluded that their initial findings (26) might be of less magnitude than originally thought (Table 1).

Effects of Radiofrequency/Microwave Radiation upon the BBB – A summary of Previous Studies

Table 1. BBB permeability after EMF exposure. (From Nittby et al. (24))

Reference	EMF Frequency (MHz)	Modulation, pulses per second (pps)	Duration of exposure	SAR (W/kg)	Effect on BBB permeability?	Total number of animals included in the study	Tracer or studied effect	Remark
Findings by the Lund Group								
Salford et al. 1994	915	CW and pulse-modulated with repetition rates of 8, 16, 50 and 200 /s	2 hours	0.016-5 W/kg	Yes	246 Fischer 344 rats	Albumin extravasation	
Persson et al. 1997	915	217, 50 Hz and CW	2-960 min	0.0004-0.95 W/kg	Yes	1002 Fischer	Albumin extravasation	

				average whole-body		344 rats		
Salford et al. 2003	915	GSM	2 hours	0.002-0.2 W/kg	Yes		Albumin extravasation and dark neurons	Effect was seen 50 days after the exposure
Eberhardt et al. 2008	915	GSM	2 hours	0.0002-0.2 W/kg	Yes	96 Fischer 344 rats	Albumin extravasation and dark neurons	Albumin extravasation 14 days after exposure, dark neurons 28 days after exposure
Mobile phone exposure								
Fritze et al. 1997	900	GSM	4 hours	0.3 to 7.5 W/kg	Yes		Albumin	Albumin extravasation only reported for SAR-values of 7.5 W/kg
Töre et al. 2001	900	GSM	2 hours	0.12; 0.5 and 2.0 W/kg	Yes	70 Sprague-Dawley	Albumin leakage, seen with fluorescein-labelled proteins	Albumin extravasation at SAR-values

								of 0.5 and 2.0 W/kg
Neubauer et al. 1990	2450	100 pps	30-120 min	Average 2 W/kg	Yes		Rhodamine-ferritin complex	No leakage at 1 W/kg at short-term exposure of 15 min
Tsurita et al. 2000	1439	TDMA	1 hour daily, for 2 or 4 weeks	Average whole-body 0.25 W/kg; peak in the brain of 2 W/kg	No	36 Sprague-Dawley rats	Evans blue, albumin	
Kuribayashi et al. 2005	1439	TDMA, 50 pps	90 min daily, for 1 to 2 weeks	Average brain power densities of 2 or 6 W/kg; average whole-body 0.29 or 0.87 W/kg	No	40 Fischer 344 rats	Three BBB-related genes; FICT-dextran and albumin extravasation	

Finnie et al. 2001	898.4	GSM	1 hour	Whole-body of 4 W/kg	No	60 mice	Albumin extravasation	
Finnie et al. 2002	900	GSM	1 hour daily, 5 days a week for 104 weeks	Average whole-body 0.25; 1.0; 2.0 and 4.0 W/kg	No	207 mice	Albumin extravasation	
Franke et al. 2005b	1800	GSM	1 to 5 days	Average 0.3 W/kg	No		Sucrose permeation	In vitro model of BBB
Schirmacher et al. 2000	1800	GSM	4 days	Average 0.3 W/kg	No		Sucrose permeation	In vitro model of BBB
Franke et al. 2005a	1966	UMTS	1 to 3 days	Average 1.8 W/kg	No		Sucrose and albumin permeation	In vitro model of BBB
Cosquer et al. 2005	2450	500 pps	45 min	Average whole-body 2 W/kg	No	Rats	Scopolamine methylbromide extravasation	Indirect investigation of BBB opening by performance in radial arm maze

RF exposure of other kinds								
Frey et al. 1975	1200	1000 pps and CW	30 min	0.2 mW/cm^2	Yes	Rats	Fluorescein	
Oscar and Hawksins 1977	1300	50-1000 pps	20 min	0.3 mW/cm^2	Yes	180 Wistar rats	Leakage of mannitol, dextran and inulin	
Preston et al. 1979	2450	CW	30 min	0.1 – 30 mW/ cm^2	No	Rats	Mannitol	
Merritt et al. 1978	1200 and 1300	1000 pps and CW	30 min	2-75 mW/ cm^2 and 0.1-50 mW/cm^2	No	Sprague Dawley rats	Fluorescein, mannitol, serotonin	Tried to replicate findings by Frey et al. (1975) and Oscar and Hawkins (1977)
Ward et al. 1982	2450	CW	30 min	10-30 mW/ cm^2	No	Rats	Sucrose and inulin	
Ward and Ali 1985	1700	CW and 1000 pps	30 min	0.1 W/kg	No	Rats	Sucrose and inulin	
Albert and	2450	CW	2 hours	2.5 W/kg	Yes	80 Chinese	Horseradish peroxidase	Reversible

Kerns 1981						hamsters		process with no HRP permeation after 1-2 recovery
Gruenau et al 1982	2800	CW and 500 pps	30 min	1-40 mW/cm^2	No	31 rats	Sucrose	
Lin and Lin 1980	2450	500	20 min	0.04-80 W/kg	No	Wistar rats	Evans blue and sodium fluorescein	
Lin and Lin 1982	2450	25-500	5-20 min	0.04-240 W/kg	No	51 Wistar rats	Evans blue	BBB permeability only at SAR of 240 W/kg, which is a thermal effect
Goldman et al. 1984	2450	500		240 W/kg	No		Rubidium-86	Hyperthermia induced BBB permeability
Williams et al. 1984a	2450	CW	30-180 min	4-13 W/kg	No	32 Fischer 344 rats	Fluorescein	BBB permeability only at

								hyperthermic levels > 41°C
Williams et al. 1984b	2450	CW	30-180 min	4-13 W/kg	No	20 Fischer 344 rats	HRP	
Williams et al. 1984c	2450	CW	30-90 min	13 W/kg	No	24 Fischer 344 rats	Sucrose	
Williams et al. 1984d	2450	CW	30-180 min	4-13 W/kg	No	66 Fischer 344 rats	Fluorescein, HRP, sucrose	BBB permeability only at brain temperatures > 40°C
Quock et al. 1986	2450	CW	10 min	24 W/kg		Mice	Domperidone	BBB permeability due to temperature increase
Quock et al. 1987	2450	CW	10 min	24 W/kg		Mice	Domperidone	BBB permeability due to temperature increase

Moriyama et al. 1991	2450	CW			21 Sprague Dawley rats	HRP	BBB permeability due to temperature increase
Nakagawa et al. 1994	2450	CW			Japanese monkeys		BBB permeability due to temperature increase

MRI exposure			**Magnetic field**				
Shivers et al. 1987		23 min	0.15 T static magnetic field	Yes		HRP	Standard MRI procedure
Preston et al. 1989		23 min	4.7 T static magnetic field	No	Rats	Sucrose	Standard MRI procedure
Prato et al.	65	23 min x 2	0.15 T static	Yes	43	Diethylenetriaminepentaacetic	Standard MRI

1990			magnetic field		Sprague Dawley rats	acid (DTPA)	procedure
Prato et al. 1994		23 min x 2	1.5 T static magnetic field	Yes	50 rats		Standard MRI procedure
Garber et al. 1989			0.3-0.5 T static magnetic field	Yes	Rats	Mannitol	Standard MRI procedure
Adzamli et al. 1989				No			Standard MRI procedure
ELF exposure							
Öztas et al. 2004	50	8 hours daily for 21 days	0.005T	Yes	34 Wistar rats	Evans-blue	BBB disruption in diabetic rats, but not in normoglycemic rats

In an attempt to repeat the findings of Oscar and Hawkins (26), Preston et al. (28) found no increase in the uptake of 14C-mannitol in anaesthetised rats after 2450MHz CW exposure for 30 min at power densities of 0.1 to 30mW/cm^2. Preston et al. further concluded that the increased BBB permeability, which had been observed by Oscar and Hawkins (26) in cerebellum and medulla, possibly had been misinterpreted and was not due to the EMF exposure. Rather, changes in blood flow and water influx or egress were supposed to be responsible for the BBB permeability in these caudal parts of the brain. Also, further attempts, made by Merritt et al. (1978) (29), to replicate the findings of Oscar and Hawkins from 1977, resulted in the conclusion that no repetition of the initial findings could be made. Merritt et al. (29) tried to replicate also the findings of Frey et al. (25), but reported that no changes were seen.

However, Frey commented upon this in an article in 1998, where he pointed out that, in fact, statistical analysis by the editor and reviewer of the data from the study by Merritt et al. provided a confirmation of the findings of Frey et al. (25) (30).

No alteration of BBB permeation of 14C-sucrose and 3H-inulin was found by Ward et al. (31)after exposure of anaesthetised rats to CW at 2450MHz for 30 min at power densities of 0, 10, 20, or 30 mW/cm^2 after correction for thermal effects. Similarly, Ward and Ali (32) observed no permeation after 1.7GHz exposure at SAR of 0.1 W/kg, using the same exposure duration and injected tracers as Ward et al. (31). Absence of EMF induced BBB permeability was also reported by Gruenau et al. (33), after injection of 14C-sucrose in conscious rats and exposure 30 min pulsed energy (2.8GHz at 0, 1, 5, 10, or 15mW/cm^2) or continuous wave (2.8 GHz, 0, 10, or 40 mW/cm^2).

Proof of EMF-induced BBB permeability was put forward by Albert and Kerns (34), who exposed un-anaesthetised Chinese hamsters to 2,450MHz CWs for 2 h at SARs of 2.5 W/kg. In one-third of the exposed animals there was an increased permeability of the BBB to horseradish peroxidase (HRP) and the endothelial cells of these irradiated animals had a 2–3-fold higher number of pinocytotic vesicles with HRP than the sham animals. The mechanism of BBB permeability seemed to be reversible, since animals allowed to recover for 1 or 2 h after the EMF exposure had almost no HRP permeation. A total number of 80 animals were included in this study.

Temperature Dependence

In further studies, more attention was directed towards the effects of hyperthermia, resulting from exposure at high SAR-levels, on BBB permeability.

A study correlating changes of BBB permeability with the quantity of absorbed microwave energy by Lin and Lin (35), using Evans blue and sodium fluorescein as indicators of BBB permeation, showed that 20 min of 2,450MHz exposure of anaesthetised Wistar rats caused no alteration of BBB permeability even at SAR values of 80 W/kg. Notably, the same lack of alteration was observed also at lower SAR-values, down to 0.04 W/kg. In further studies by the same group (36), no permeation of Evans blue could be observed after exposure to 2,450MHzB RFs for 5–20 min when the SAR-values ranged from 0.04–200 W/kg. Not until a SAR-value of 240 W/kg, with ensuing rise in brain temperature to 43ºC, was applied, the BBB permeability increased. These observations of demonstrable increases of BBB permeability associated with intense, microwave-induced hyperthermia were supported by another study by the same group (37).

In a series of EMF exposures at 2,450MHz CW, Williams et al. (38-40) concluded that increase of BBB permeability might not be explained by microwave exposure, but rather temperature increases and technically derived artefacts such as increase of the cerebral blood volume and a reduction in renal excretion of the tracer. Significantly elevated levels of sodium fluorescein (38) were found only in the brains of conscious rats made considerably hyperthermic by exposure to ambient heat for 90 min or 2,450MHz CW microwave energy for 30 or 90 min, but this was at high SAR values, 13 W/kg—far beyond the ICNIRP limit of 2 W/kg (41) —and not comparable to the experiments performed by, among others, our group, as described below.

With more research into the area of EMF induced BBB permeability, it became evident that with high-intensity EMF exposure resulting in tissue heating, the BBB permeability is temperature dependent (42). Thus, the importance of differentiating between thermal and non-thermal effects on the integrity of the BBB was realized. This is the reason why studies with increases of BBB permeability due to exposure to SAR-values well above recommended

exposure levels (43-46) need to be considered from another point of view, as compared to those focusing on the non-thermal effects of EMFs.

Continued Studies—MRI and BBB Permeability

Following the increasing use of magnetic resonance imaging (MRI), the effects of MRI radiation upon BBB permeability were investigated more thoroughly. MRI entails the concurrent exposure of subjects to a high-intensity static field, a radiofrequency field, and time-varying magnetic field. Shivers et al. (47) observed that exposure to a short (23 min) standard (of those days) clinical MRI procedure at 0.15 Tesla (T) temporarily increased the permeability of the BBB to horseradish peroxidase (HRP) in anaesthetised rats. This was revealed by electron microscopy (EM), to be due to an amplified vesicle-mediated transport of HRP across the microvessel endothelium, to the ablumenal basal lamina and extracellular compartment of the brain parenchyma. This vesicle-mediated transport also included transendothelial channels. However, no passage of the tracer through disrupted interendothelial tight junctions was present.

During the next few years, more groups studied the effects of MRI exposure on the BBB permeability by injection of radioactive tracers into rats. One supported (48)while others contradicted (49, 50) the initial findings made by Shivers et al. (47). Garber et al. exposed rats to MRI procedures at 1.5, 0.5, and 0.3 T with RFs of 13, 21, and 64 MHz, respectively (48). Brain mannitol concentration was significantly increased at 0.3 T and 0.5 T but not at 1.5 T. No decrease in plasma mannitol concentration of MRI exposed animals was found and thus the authors concluded that effects of MRI associated energies on mannitol transport do not occur measurably in the body, and might be more specific to brain vasculature. Preston et al. (50) found no significant permeation of blood-borne 14C-sucrose into brain parenchyma in anesthetized rats subjected to 23 min of MRI at 4.7 T and RFs at 12.5 kHz. However, the authors pointed out that if the MRI effect was focal and excess tracer counts were found only in restricted sites, there could have been MRI induced extravasation of sucrose that was not detected, due to the preponderance of normal tissue counts. When Preston et al. (50) compared the lack of BBB leakage in their study to the MRI induced leakage which had been observed by Shivers et al. (47), they also concluded that certain characteristics of electric and

magnetic fields, which were present in the study by Shivers et al. but not in their own work, could have been critical to the observed effects.

In 1990, further studies by the Shivers-Prato group were presented (51) and the group could now quantitatively support its initial findings, in a series of 43 Sprague-Dawley rats. The BBB permeability to diethylenetriaminepentaacetic acid (DTPA) increased in rats after two sequential 23 min MRI exposures at 0.15 T. It was suggested that the increased BBB permeability could result from a time-varying magnetic field mediated stimulation of endocytosis. Also, the increased BBB permeability could be explained by exposure-induced increases of intracellular Ca^{2+} in the vascular endothelial cells. Since the Ca^{2+} is an intracellular mediator, increases of BBB permeability could possibly be initiated in this way. A few years later, in a series of 50 rats, the Shivers - Prato group also found that the BBB permeability in rats is also altered by exposure to MRI at 1.5T for 23 min in 2 subsequent exposure sessions (52).

Studies by the Lund Group

Two of us found these observations highly interesting:

- the neurosurgeon (LGS) in the hope to utilize possible applications of EMF to make the blood-brain barrier (BBB) more penetrable to chemotherapy, in order to treat brain cancers more effectively. An intact BBB keeps out chemotherapy agents, allowing cancer cells to hide behind the BBB.

- the radiophysicist (BRRP) interested in possible adverse effects of the MRI technique.

After a visit to Shivers' group in London Ontario in 1988, we started work in Lund in 1988, studying the effects of MRI on rat brain and we found, by the use of Evans Blue, the same increased permeability over BBB for albumin (53).

This work was continued by separating the constituents of the MRI field: RF, undulant magnetic field, and static magnetic field. Since RF turned out to be the most efficient component of the MRI, the following studies focused mainly on the RF effects. Striving for

investigating the actual real-life situation, endogenous substances, which naturally circulate in the vessels of the animals, were used. In line with this, albumin and also fibrinogen leakage over the BBB were followed after identification of albumin with rabbit antibodies (see Figure 2 and 3) and rabbit anti-human fibrinogen.

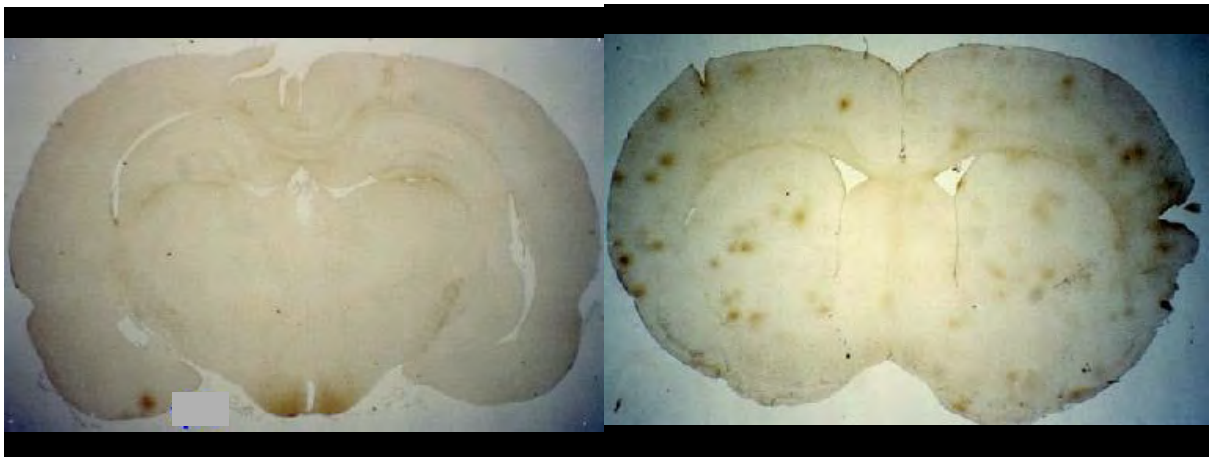

Figure 2. Albumin extravasation in rat brain (material from Persson et al. 1997)(54).
Left: control brain with albumin staining in hypothalamus, which serves as an inbuilt-control of the staining method, since the hypothalamus lacks BBB, and one occasional staining.
Right: Brain of EMF exposed rat, with multiple albumin positive foci.

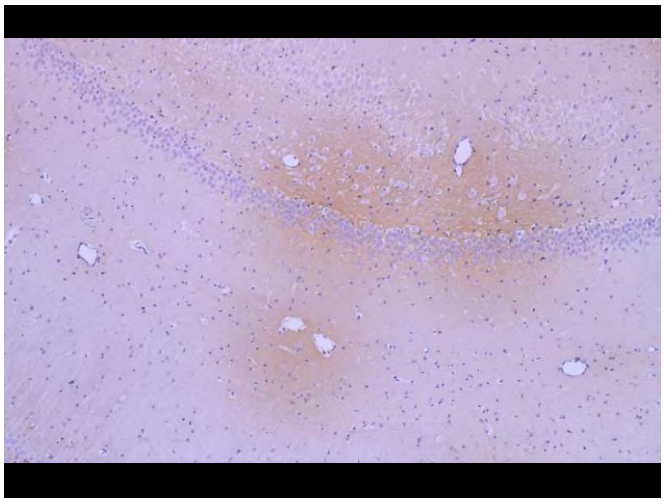

Figure 3. Albumin extravasation around vessels in the brain of an EMF exposed rat.

The work by Blackman et al. (55, 56) ~~made the ground~~ laid the groundwork for studies on the frequency modulation 16 Hz and its ~~harmonies~~ harmonics 4 and 8 Hz. A carrier wave of 915 MHz was used. At the suggestion of Östen Mäkitalo (Telia), a pioneer in mobile phone

development, who introduced 50 Hz (DUX) and 217 Hz (GSM) modulation in new digital wireless communication systems, we also included theses frequencies. This paralleled the first BBB study results that were published in 1992-1994 (57-59).

The result of our continued work, comprising more than 1000 animals, with exposure to both CWs and pulsed modulated waves, in the most cases lasting for 2 h, showed that there was a significant difference between the amount of albumin extravasation in the exposed animals as compared to the controls. In the exposed group 35–50% of the animals had a disrupted BBB as seen by the amount of albumin leakage, while the corresponding leakage in the sham exposed animals was only 17% (for results see Figure 4) (54).

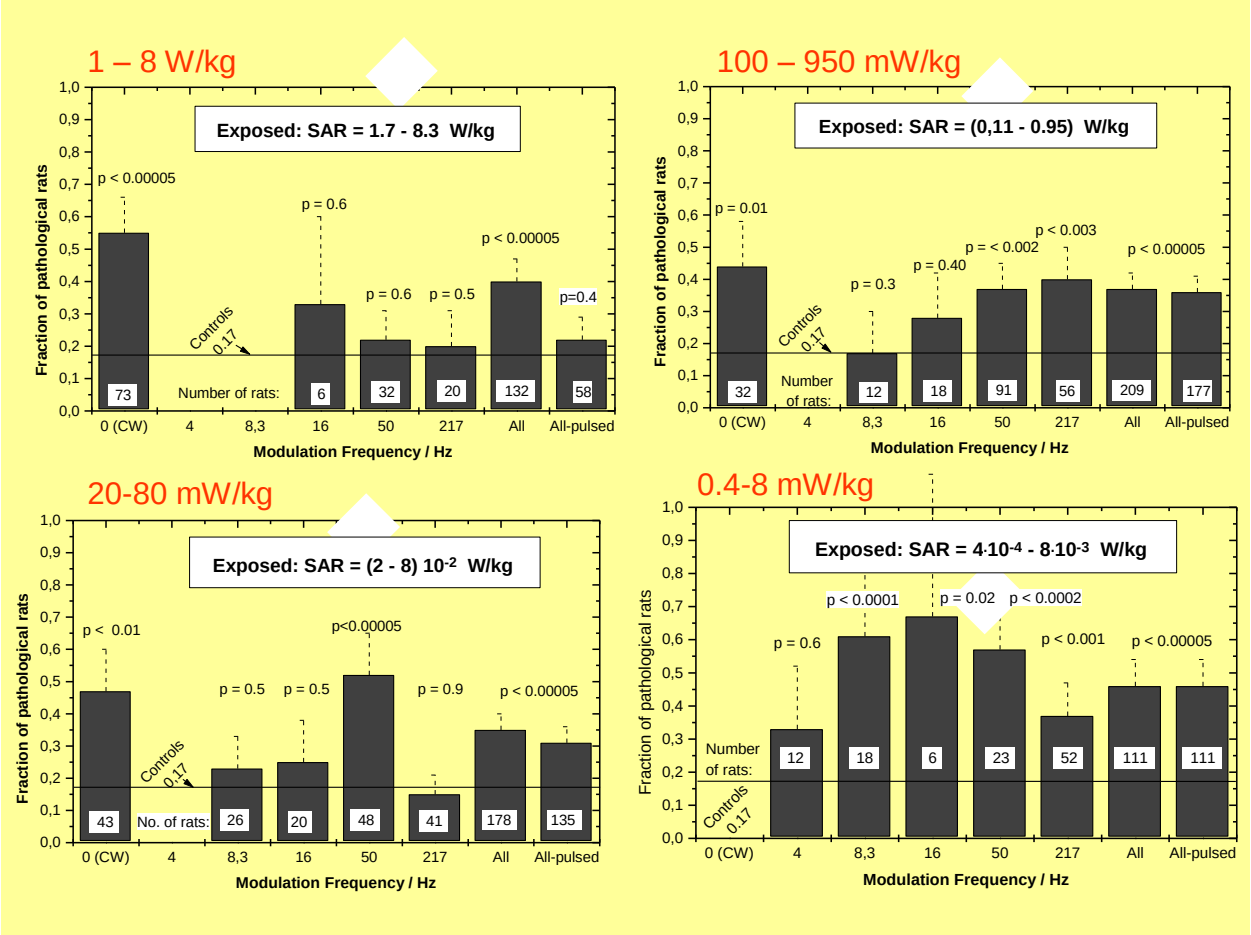

Figure 4. Albumin extravasation score as a result of EMF exposure (results from the study by Persson et al. (54)).

The fact that sham-exposed control animals also show some amount of albumin extravasation (see Figure 4), is most likely due to our very sensitive methods for immune histological examination. However, it is hard to explain the fact that although all animals in the 1997 series were inbred Fischer 344 rats, only every second animal, at the most, showed albumin leakage after EMF exposure. The question, what might protect the remaining 50% of the exposed animals from BBB disruption, is highly intriguing. It should be noted that in our large series, only in one single animal fibrinogen leakage has been observed (54).

Another conclusion from the 1997 study is that the number of pathological leakages in exposed animals is more frequent, and also more severe, per animal compared to the controls. This is an interesting observation as the prevailing opinion is that pulse modulated electromagnetic fields are more potent in causing biological effects.

In a statistical re-evaluation of our material published in 1997, where only exposed rats with a matched unexposed control rat are included, we found for the most interesting modulation frequency 217 Hz, i.e. that of GSM, that at SAR-values of 0.2 to 4 mW/kg 48 exposed rats had a significantly increased albumin leakage ($p < 0.001$) as compared their 48 matched controls. On the other hand, SAR-values of 25-50 mW/kg, gave no significant difference between 22 exposed rats *vs* their matched controls (Wilcoxon´s Rank Test, 2-sided p-value) (60).

In all our earlier studies we showed albumin extravasation immediately after exposure as described above. In later years we have performed a series of experiments where the animals were allowed to survive for 7 days (61), 14 days, 28 days (62) or 50 days (63) after one single 2-hour exposure to the radiation from a GSM mobile phone. All were exposed in TEM-cells to a 915 MHz carrier wave as described below. The peak power output from the GSM mobile phone fed into the TEM-cells was 1 mW, 10 mW, 100 mW and 1000 mW per cell respectively for the 7-14-28-days survival animals, resulting in average whole-body SAR of 0.12 mW/kg, 1.2 mW/kg, 12 mW/kg and 120 mW/kg for four different exposure groups SAR-values of 2, 20 and 200 mW/kg mW/kg for 2 hours for the 50-days survival animals.

Albumin extravasation over the BBB after GSM exposure seemed to be time-dependent, with significantly increased albumin in the brain parenchyma of the rats, which had survived for 7 and 14 days, but not for those surviving 28 days. After 50 days, albumin extravasation was

significantly increased again, with albumin-positive foci around the finer blood vessels in white and gray matter of the exposed animals.

In connection to the albumin passage over the BBB, albumin also spread in the surrounding brain tissue. A significantly increased uptake of albumin in the cytoplasm of neurons could be seen in the GSM exposed animals surviving 7 and 14 days after exposure, but not in those surviving 28 or 50 days.

Neuronal uptake

Extravasated albumin rapidly diffused down to, and beyond, concentrations possible to demonstrate accurately immunohistologically. However, the initial albumin leakage into the brain tissue (seen within hours in ~40% of exposed animals in our previous studies) most likely started a vicious circle of further BBB opening.

It has been postulated that albumin is the most likely neurotoxin in serum (64). Hassel et al. (65) have demonstrated that injection of albumin into the brain parenchyma of rats gives rise to neuronal damage. When 25 µl of rat albumin is infused into rat neostriatum, 10 and 30, but not 3 mg/ml albumin causes neuronal cell death and axonal severe damage. It also causes leakage of endogenous albumin in and around the area of neuronal damage. Albumin in the dose 10 mg/ml is approximately equivalent to 25% of the serum concentration.
It is less likely that the albumin leakage demonstrated in our experiments locally reaches such concentrations. However, we have seen that in the animals surviving 28 and 50 days after 2 hours of GSM exposure, there was a significantly increased incidence of neuronal damage as compared to the sham controls. In the 7-days and 14-days survival animals, on the other hand, no such increase of neuronal damage was seen.

In the 50-days post-exposure survival study, a 2 h exposure to GSM at SAR values 200, 20, and 2 mW/kg resulted in a significant ($p = 0.002$) neuronal damage in rat brains of the exposed animals as compared to the controls 50 days after the exposure occasion (Salford et al., 2003)(63). We have followed up this observation, as mentioned above, in a study where 96 animals were sacrificed 14 and 28 days respectively after an exposure for 2 h to GSM mobile phone electromagnetic fields at SAR values 0 (controls), 0.12, 1.2, 12 and 120 mW/kg. Significant neuronal damage is seen after 28 days and albumin leakage after 14. Our

findings may support the hypothesis that albumin leakage into the brain is the cause for the neuronal damage observed after 28 and 50 days (62).

The damaged neurons in the above mentioned studies took the shape of so-called dark neurons. Three main characteristics of the damaged dark neurons have been proposed (66): (i) irregular cellular outlines, (ii) increased chromatin density in the nucleus and cytoplasm and (iii) intensely and homogenously stained nucleus. The damaged dark neurons found in the 50 days-survival animals were investigated regarding signs of apoptotic markers, but we found no positive staining for Caspase-3, a marker for apoptosis (Bexell et al. unpublished results). However, the albumin leakage out in the neuropil in connection to EMF exposure might start other deleterious processes, leading to the formation of the dark neurons.

A group in Turkey performed similar experiments. However, also the presumed protective effects of the antioxidant Ginko biloba (Gb) were examined by Ilhan et al. (67). About 22 female Wistar rats were exposed to a 900 MHz electromagnetic GSM near-field signal for 1 h a day for 7 days. In the GSM only group, the pathological examination revealed scattered and grouped dark neurons in all locations, but especially in the cortex, hippocampus and basal ganglia, mixed in among normal neurons. A combined non-parametric test for the four groups revealed that the distributions of scores differed significantly between the control and the GSM only exposure group ($p < 0.01$).

Long-term study, including studies of memory and behaviour

In a recent long-term study from our laboratory, rats were exposed to GSM radiation 2 hours weekly during 55 weeks (two different exposure groups with 0.6 mW/kg and 60 mW/kg at the initiation of the exposure period). After this protracted exposure, behaviour and memory of the exposed animals were tested. Whereas the behaviour of the animals was not affected, the GSM exposed rats had significantly impaired episodic memory as compared to the sham controls (68). After the finalization of these tests, that is 5-7 weeks after the last exposure, the animals were sacrificed by perfusion fixation. Albumin extravasation, an indicator of BBB leakage, was increased in about 1 animal in each group of low GSM exposed, high GSM exposed, sham exposed and cage control rats. About 40 % of the animals had neuronal damage. GFAP staining, as an indicator of glial reaction, revealed positive results in 31-69 % of the animals for different groups and the aggregation product lipofuscin was increased in

44-71 % of the animals for different groups. With the Gallyas staining (aiming at cytoskeletal structures), no changes were seen. When comparing the results between the different groups, it turned out that there was no statistically significant difference for any of these parameters due to GSM exposure (69). When comparing these findings to those from animals which had been exposed only once for 2 hours, it seems likely that during the 55 weeks of repeated exposure, albumin leakage at an initial stage of the experimental period might have been absorbed after some time, and that at a certain, but unknown, time point during this protracted, more than 1 year long-exposure period, some adaptation process might have been activated. However, this could not compensate for cognitive alterations, demonstrated by the episodic memory tests.

TEM-cells

In the majority of our studies, EMF exposure of the animals has been performed in transverse electromagnetic transmission line chambers (TEM-cells, see Figure 5) (53, 54, 59, 61-63, 68-71). These TEM-cells are known to generate uniform electromagnetic fields for standard measurements. Each TEM-cell has two compartments, one above and one below the center septum. Thus, two animals can be exposed at a time. The animals are un-anaesthetized during the whole exposure. Since they can move and turn in the TEM-cells as they like, the component of stress-induced immobilization (described by Stagg et al. (72)) is effectively minimized. Through our studies, we have concluded that the amount of albumin leakage is neither affected by the sex of the animals, nor their placement in the upper or lower compartments of the TEM-cells.

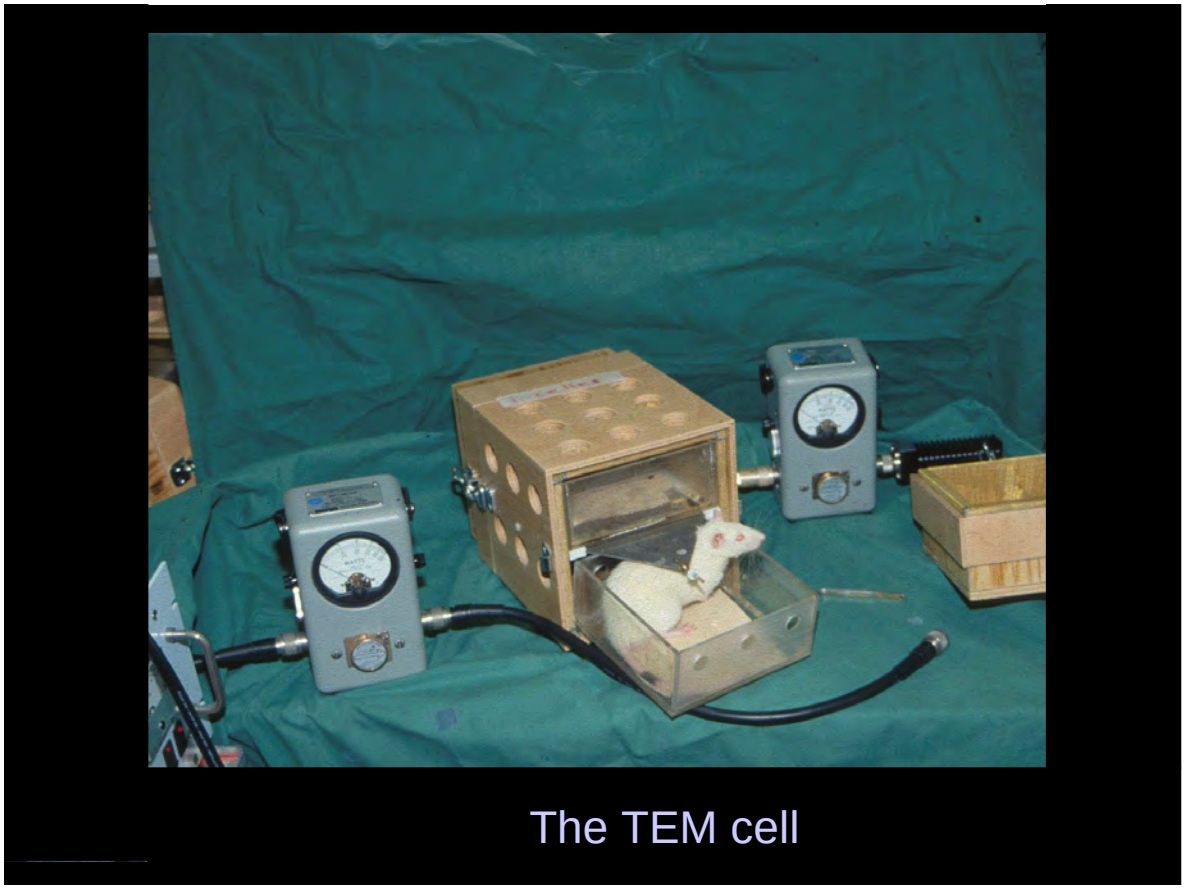

Figure 5. TEM-cells for EMF exposure.

GSM-1800 modulated and CW microwaves in an anechoic chamber

In Lund we have also utilized an anechoic chamber for studies on microwaves from a real GSM-1800 mobile telephone, which were amplified and transferred to a dipole antenna in the anechoic chamber. The output power was varied to study the effect of various SAR values.
In a series of 65 rats exposed for 2 h with 1800-GSM at SAR: 0.027 mW/kg, and 12 rats exposed for 2 h with continuous wave, we found significantly increased albumin leakage (see figure 6) as compared to 103 control rats ($p<0,03$ and $p<0,02$, respectively). (Unpublished results).

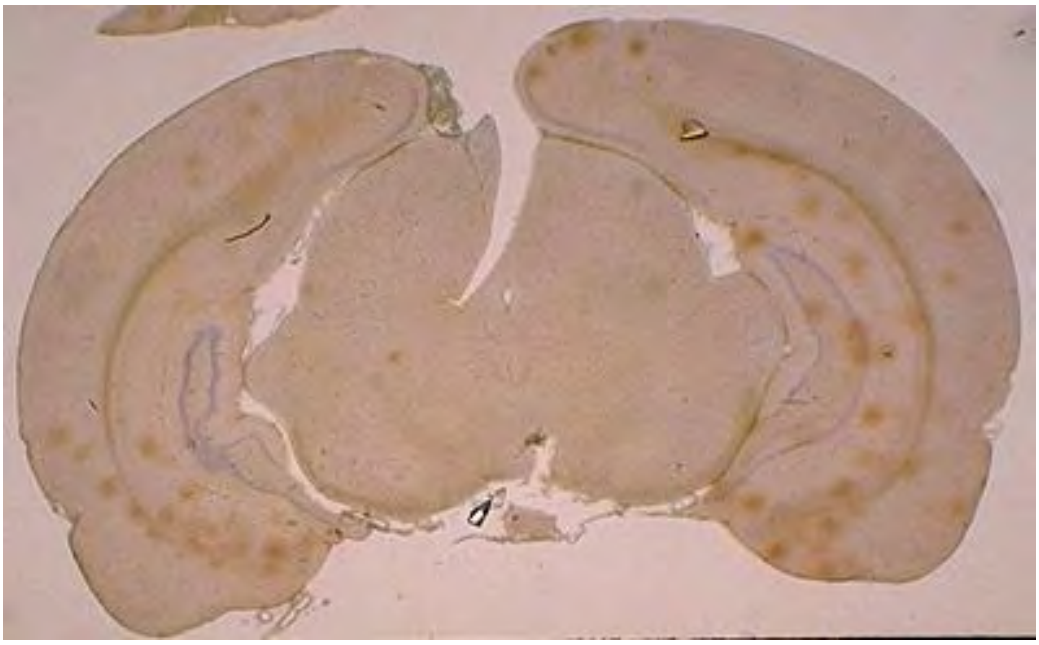

Figure 6.
Pathological leakage around vessels demonstrated by immunostaining against albumin. Fischer 344 rat exposed for 2 h with 1800-GSM at SAR: 0.027 mW/kg

Other Studies on BBB Permeability, Focusing on the Effects of RF EMFs of the Type Emitted by Mobile Phones

With the increasing use of mobile phones, much attention has been directed towards the possible effects on BBB permeability, after exposure to the type of RF EMFs emitted by the different sorts of mobile phones.

Repetitions of our initial findings of albumin leakage have been made by Fritze et al. (73), with 900 MHz exposure of rats for 4 h at brain power densities ranging from 0.3–7.5 W/kg. Albumin extravasation into the brain tissue was seen, with significant difference between controls and rats exposed reported for 7.5 W/kg, which is a thermal level. However, Fisher exact probability test (two-tailed) performed on the reported results, reveals significant ($p < 0.01$, Fisher exact probability test) difference for the subthermal level group (SAR 0.3 W/kg plus 1.3 W/kg, compared to sham exposed and cage control animals) where in total 10 out of 20 animals showed one or more extravasations direct after exposure (Salford et al. (20)).

Another group, working in Bordeaux, and led by Prof Pierre Aubineau, has also demonstrated evidence of albumin leakage in rats exposed for 2 h to 900 MHz at non thermal SAR-values, using fluorescein-labeled proteins. The results were presented at two meetings by Töre et al. (74, 75). The findings are very similar to those of our group, described above.
At the BEMS meeting in 2002 in Quebec City in Canada, the Aubineau-Töre group presented results from exposure GSM-900 EMFs at SAR values of 0.12, 0.5, and 2.0 W/kg. Seventy Sprague-Dawley rats were included in the study. In addition to normal sham and normal GSM exposed rats, also rats subjected to chronic dura mater neurogenic inflammation, induced by bilateral sympathetic superior cervical ganglionectomy, were included. Arterial blood pressure was measured during the exposure, and Töre et al. (74, 75) concluded that the pressure variations (100–130mm Hg) were well below those limits, which are considered to be compatible with an opening of the BBB of rats. In order to induce opening of the BBB in rats, arterial blood pressure needs to reach values of 170 mmHg, according to Töre et al. (74, 75). At SAR of 2 W/kg a marked BBB permeabilization was observed, but also at the lower SAR-value of 0.5 W/kg, permeabilization, although somewhat more discrete, was present around intracranial blood vessels, both those of the meninges and of the brain parenchyma. Comparing the animals, which had been subjected to ganglionectomy, to the other animals, Töre et al. made an interesting observation: as expected, albumin extravasation was more prominent in the sympathectomised sham-exposed rats as compared to normal exposed rats. This was due to the fact that the sympathectomised rats were in a chronic inflammation-prone state with hyper-development of pro-inflammatory structures, such as the parasympathetic and sensory inputs as well as mast cells, and changes in the structure of the blood vessels. Such an inflammation-prone state has a well-known effect on the BBB leakage. However, when comparing sham-exposed sympathectomised rats to GSM-exposed sympathectomised rats, a remarkable increase in albumin leakage was present in the GSM exposed sympathectomised rats compared to the sham rats. In the GSM-exposed sympathectomised rats, both brain areas and the dura mater showed levels of albumin leakage resembling those observed in positive controls after osmotic shock. Indeed, more attention should be paid to this finding, since it implicates that the sensitivity to EMF-induced BBB permeability depends not only on power densities and exposure modulations, but also on the initial state of health of the exposed subject.

In rats, uptake of a systemically administered rhodamine-ferritin complex through the BBB also has been observed, after exposure to pulsed 2.45GHz EMFs at average power densities of

2 W/kg by Neubauer et al. (76). The authors observed that the magnitude of BBB permeability depended on power density and duration of exposure. Exposure to a lower power density (1 W/kg) and shorter duration of the exposure (15 min) did not alter the BBB permeability, as compared to higher power densities (SAR 2 W/kg) and longer duration of exposure (30–120 min). The microtubules seemed to play a vital role in the observed BBB permeability, since treatment with colchicine, which inhibits microtubular function, resulted in near-complete blockade of rhodamine-ferritin uptake. The mechanism underlying the observed leakage was presumed to be correlated to pinocytotic-like transport.

In other studies, no effect of EMF exposure has been observed on the BBB integrity. With exposure to 1,439MHz EMFs, 1 h daily during 2 or 4 weeks (average whole-body energy doses of 0.25 W/kg) no extravazation of serum albumin trough the BBB was observed in a series of 36 animals by Tsurita et al.(77). However, in this small material only 12 animals in total were EMF exposed (6 rats exposed for 2 weeks and 6 rats exposed for 4 weeks). Also, lack of interference with the BBB function of rats was found after 1,439MHz exposure for 90 min/d for 1–2 weeks at average brain power densities of either 2 or 6W/kg by Kuribayashi et al.(78). A total number of 40 animals were included in the study.

Finnie et al. (79) came to the conclusion that no increase in albumin leakage over the BBB resulted from EMF exposure in a series of 60 mice. With whole body exposure of mice to GSM-900 EMFs for 1 h at a SAR of 4 W/kg or sham exposure, no difference in albumin extravazation was observed between the different groups. Also, free-moving cage controls were included in the study, and interestingly, there was no significant difference between these non-restrained mice as compared to the sham and EMF-exposed animals. Thus, the authors concluded that there were no stress-related exposure module confinement effects on the BBB permeability.

Finnie et al. (80) continued to investigate more long-lasting exposure effects. In a series of experiments, a total of 207 mice were exposed 60 min daily, 5 days per week for 104 weeks at average whole body SARs of 0.25, 1.0, 2.0, and 4.0W/kg. This led to a minor disruption of the BBB, as seen by the use of endogenous albumin as a vascular tracer. However, it should be added that the authors performed no statistical analyses to evaluate the albumin leakage through the small vessels in the brain. In an answer to correspondence in the same journal (81), the authors presented the original data from the long-term study in one table, from which

one can conclude that non-leptomeningeal albumin leaking vessels were seen in few sham-exposed animals, and in one-third of the animals in the 0.25 W/kg group and to a lesser extent in the higher SAR groups.

The fact that some research groups observe albumin leakage/transport over the BBB after EMF exposure and others do not, has led to a rather intense debate between the researchers but also in society, which is puzzled by the divergent findings. A major concentration of the involved research groups took place at Schloss Reisensburg in Germany in 2003, where the technical approaches in the studies of BBB effects were discussed. Two world-renowned researchers in the BBB field, Dr. David Begley of Kings College, London, and Prof. Olaf Poulsen of Copenhagen, Denmark, chaired the FGF/COST 281 Reisensburg, November 2–6 meeting. They made the final statement as a summary of the meeting: ‘‘It seems clear that RF fields can have some effects on tissues’’. The statement was made to a large extent on the basis of the concordant findings of the Bordeaux group, represented by Prof. Aubineau, and the Lund group, represented by Prof. Salford and Prof. Persson.

The histopathological examinations of the brains are not uncomplicated. Some laboratories that have tried to replicate our studies have not been able to demonstrate the albumin leakage. We have recently had problems with the albumin staining due to change of suppliers of avidin, biotin, serum and antibodies. The lateral hypothalamic nuclei in the immediate vicinity of the third ventricle are well known for their normally insufficient BBB. This has served as an inbuilt control of adequate albumin staining in all our experiments since 1990. In our study on combined effects of RF- and ELF-EMF, for the first time, we could not demonstrate albumin extravasation in basal hypothalamus. Not until our third attempt with new staining material, we got our positive control and could also demonstrate albumin leakage in the exposed brains (61).

The biological effects of RF exposure depend on many parameters, such as mean power level and the time variations of the power (82) and whether in vivo or in vitro experiments are performed. In the in vivo situation, different kinds of animals, and also the same kind of animals but of different breeds, might react differently. It might not necessarily be the strongest RF fields that give rise to the most obvious biological effects (54, 63). In many cases, the weak and precisely tuned EMFs have the most important biological function; two examples of this are cellular communication and protein folding. It seems quite likely that in

different experimental set-ups, and in different living organisms, the signal has to be tuned to different properties in order to cause any effect. This could perhaps in some part explain why, in some cases, there are quite obvious effects of RF exposure, whereas in others, no such effects can be seen.

Other Studies on BBB permeability and neuronal damage

As has been mentioned above (p. 26) Ilhan et al. (67), in 2004 reported neuronal damage in female Wistar rats, which had been exposed to a 900 MHz electromagnetic GSM near-field signal for 1 h. a day for 7 days. They found scattered and grouped dark neurons in the cortex, hippocampus and basal ganglia, mixed in among normal neurons. A combined non-parametric test for the four groups revealed that the distributions of scores differed significantly between the control and the GSM only exposure group ($p < 0.01$).

Later, Masuda et al. (83) tried to replicate the findings by our group of albumin extravasation and dark neurons. F344 rats (n=64) were exposed to 915 MHz signals for 2 hours (SAR of 0, 0.02, 0.2 and 2 W/kg), and albumin extravasation and dark neurons were investigated 14 and 50 days after the exposure. No albumin extravasation was seen, neither in control or exposed rats, and no difference in the occurrence of dark neurons could be found due to EMF exposure. An interesting difference as compared to the studies by Salford et al. mentioned above, was that animals, after perfusion fixation, were left in a 4ºC storage for 18 hours before the brains were removed. The question is whether this might have led to dilution of the very sensitive albumin extravasation, which is often more pronounced in the circumventrical organs as compared to the brain extravasates (personal communications with our neuropathologist Arne Brun). This might explain the fact, that no albumin extravasation could be seen in neither the cage control animals, the shams or the GSM exposed animals.

Another study by Mason and his group at Brooks Airforce Resarch Laboratory, San Antonio, also tried to confirm our findings of albumin extravastion by using the same type of TEM-cells for EMF Exposure (84), although the exposure parameters where somewhat different with only 30-min exposure, including only male rats of the Fischer 344 CD-VAF strain and utilizing only the upper compartment of the TEM cells. Exposure was at whole-body SAR values of 0.002 to 20 W/kg. Regarding extracellular albumin accumulation, the results were

not formally analyzed, as motivated by too low scores of albumin. Regarding intracellular albumin uptake, no significant difference between the different groups was reported. However, as presented in the paper by McQuade et al.(84), at the lowest SAR of 1.8 mW/kg at 16 Hz, of 33 exposed rats, 11 had 2 or 3 positivities (33% of the animals) and 22 had none or 1 positivity. In the sham animals, 18% were positive and among the cage controls only 12%. These results are reminiscent of prior work by the Lund group reporting that 17% of the sham animals had some albumin leakage, while only at the most 50% of the identical and equally handled, but RF exposed animals displayed albumin extravasation (60).

In a third study aiming to replicate the Lund findings of dark neurons, a group in Bordeaux (85) exposed 14 weeks old Fischer 344 rats (which, however, were restrained in a rocket-type exposure setup), to the GSM-900 signal for 2 h at various brain-averaged SARs (0, 0.14 and 2.0 W/kg). Eight rats were included in each of these groups.
Albumin leakage and neuronal degeneration was evaluated 14 and 50 days after exposure.
It was reported that no statistically significant albumin leakage was observed and that neuronal degeneration assessed using cresyl-violet or the more specific marker Fluoro-Jade B, was not significantly different among the tested groups. Here we want to point out that the Bordeaux group makes a major deviation from the way we have evaluated the occurrence of dark neurons in the tissue slices. While we counted the overall number of dark neurons, de Gannes et al. (85) chose to subdivide the slices into 12 different small regions, which were compared individually to each other (fig 3 in the publication). This gave the effect that a clear overall difference in number of observed dark neurons between animals 50 days after exposure to 2 W/kg for two hours versus sham exposed, disappeared in the statistics. On the contrary, if all the numerical values for the bars representing the scored dark neurons observed in each brain zone and region 50 days after exposure to 2 W/kg are compared to all those of the sham animals, a highly significant difference (Kruskall-Wallis) between animals exposed to 2 W/kg and sham is demonstrated (Mann-Whitney) $p = 0.003$! This is in concordance with the Lund experience!

Indirect studies and studies on the blood cerebrospinal fluid barrier

The integrity of the BBB has also been investigated indirectly. Cosquer et al. (86) treated rats with the muscarinic antagonist scopolamine methylbromide, which is known to induce

memory impairments, followed by EMF exposure at 2.45GHz for 45 min at average whole body SARs of 2W/kg. Opening of the BBB after EMF exposure was hypothesised to affect the performance in a radial arm maze. However, no such alterations were observed and the authors concluded that no BBB opening seemed to have occurred. In agreement with this, no albumin extravasation was noticed.

Ushiyama et al. (87) investigated the effects on the blood cerebrospinal fluid barrier after RF-EMF exposure. With a microperfusion method, cerebrospinal fluid from rat brain was collected in vivo. Fluorescent intensity of FITC-albumin in perfusate was measured. Rats exposed to 1.5GHz RFs during 30 min at SAR-values of 0.5, 2.0, 9.5W/kg for adult rats and 0.6, 2.2, 10.4W/kg for juvenile rats, respectively, were compared to sham-exposed controls. Under these conditions, no increase in FITC-albumin was seen in the cerebrospinal fluid of exposed rats as compared to sham exposed controls. It was concluded that no effect on the function of the blood cerebrospinal fluid barrier was seen.

In a recent study, the permeability of the human BBB after mobile phone exposure was assessed measuring blood levels of S100B and transthyretin in human volunteers by Söderqvist et al. (88). S100B is a calcium-binding protein, and it has been shown to be increased in serum after damage to the BBB. Transthyretin, also known as pre-albumin, is synthesised both in the liver and the choroid plexus. 30 min of GSM-900-like exposure at SAR-values of 1 W/kg was used. No difference was seen regarding S100, but transthyretin was increased 60 min after the termination of exposure as compared to the control situation. The concentrations of S100B and transthyretin were also analysed 30 min prior to provocation and after 30 min rest, showing a decrease after 30 min rest, which was suggested, might be due to less stress after the 30 min rest. Thus, it is interesting that despite this decline, which might be due to relaxation, still an increase in thransthyretin could be measured 30 min after exposure. It was also put forward, that it could not be excluded that the thransthyretin rise might be a compensation to the previous decrease, and that new studies including more participants and also a sham group would be needed.

We have in the past investigated whether MW exposure, CW and at different SAR levels might enhance S-100 protein levels in the blood of a large proportion of our rats. We could conclude that no significant differences were seen (see Figure 7 below) (to be published).

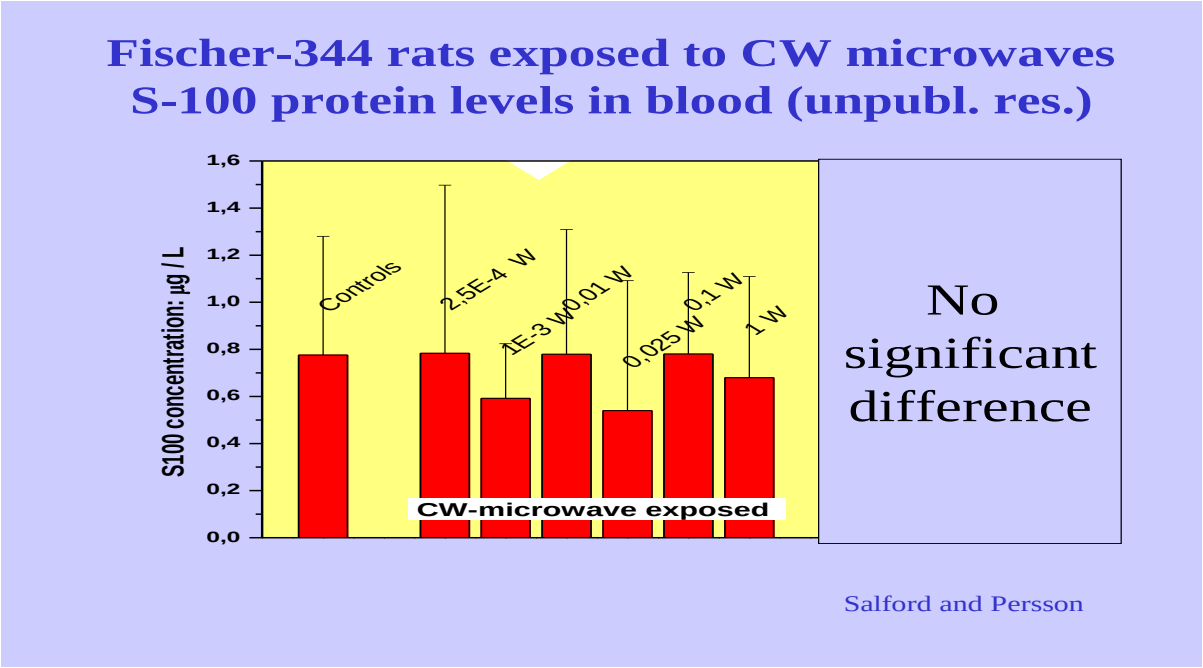

Figure 7. S-100 in the blood of rats after EMF exposure (to be published in Acta Scientiarum Lundensia).

In another study, by Sirav and Seyhan (89), exposure to CW EMFs at 900 and 1,800 MHz for 20 min, increased the BBB permeability of male but not female rats. Evans blue dye, which binds to serum albumin after injection, was used to quantitatively measure BBB permeability. A strength of this study, was the ability to objectively quantify the Evans blue uptake in the brain. The finding that only male, and not female rats, are affected, is however not fully addressed.

In Vitro Models

In recent years, there has been an increasing use of in vitro models in the search for BBB effects of EMF exposure. In vitro models of the BBB have been studied, as by Schirmacher et al. (90), with co-cultures consisting of rat astrocytes and porcine brain capillary cells. Exposure to GSM-1800 for 4 d with average SAR of 0.3 W/kg increased the permeability of 14C-sucrose significantly compared to unexposed samples in the studied BBB model. These findings were not repeated in experiments performed later by the same group, after modifications of their in vitro BBB model (91). The modified BBB model had a higher general tightness. It was speculated that at a higher original BBB permeability, which was

present in the first study by Schirmacher et al. (90), the cultures were more susceptible to the RF EMFs. Using porcine brain microvascular-endothelial cell cultures as an in vitro model of the BBB, no effects on barrier-tightness, transport behavior, and integrity of tight junction proteins were observed-after exposure to UMTS EMFs at 1.966 GHz for 1–3 d at different field strengths at 3.4–34 V/m, generating a maximum SAR of 1.8 W/kg (92).

In the search after the mechanism underlying non thermal EMF effects, Leszczynski et al. (93) observed human endothelial cells, with the interesting finding that GSM-900 exposure for 1 h with SAR-values of 2 W/kg resulted in changes in the phosphorylation status of many proteins. Among the affected pathways, the hsp27/p38MAPK stress response pathway was found, with a transient phosphorylation of hsp27 as a result of the mobile phone exposure. This generated the hypothesis that the mobile-phone induced hsp27-activation might stabilize stress fibers and in this way cause an increase in the BBB permeability. Furthermore, it was also suggested that several brain-damaging factors might all contribute to the mobile phone-induced effects observed in the brain and other structures as well.

Further perspectives of the importance of the BBB including the human situation

BBB in the Context of Alzheimer's Disease and the findings by the Zlokovic Group

The BBB, as mentioned previously, is of essential role for maintaining an accurate brain function. As described by Zlokovic (94), in a review regarding BBB in correlation to neurodegenerative disorders, BBB breakdown can be due to tight junction disruption, alterations of angiogenesis or vessel regression, hypoperfusion, inflammatory response and alterations of the transport of molecules across the BBB (94). Further, as Zlokovic hypothesises, this might contribute to neurodegenerative disorders, such as Alzheimer's disease (AD), Parkinson's disease, multiple sclerosis and amyotrophic lateral sclerosis.

In the review by Zlokovic (94), a neurovascular disease pathway is presented, regarding possible genesis of AD, where it is suggested that changes in vascular genes and receptors in brain capillaries and small arteries might disrupt BBB functions, leading to an accumulation

of amyloid beta (Aβ), a neuroinflammatory response and BBB breakdown and further on accumulation of Aβ, loss of the BBB to clear Aβ (due to affected synaptic transmission, neuronal injury and recruitment of microglia) and secretion of proinflammatory cytokines. Ultimately, this is suggested to lead to disappearance of the capillary unit, increasing Aβ deposits and synaptic and neuronal loss (94).

This observation might explain how vascular disease contributes to Alzheimer's disease (AD) risk; the heterogeneity of AD; and supports the idea that exclusively focusing on amyloid is likely to be disappointing.

Neuronal injury resulting from vascular defects that are not related to amyloid-beta but is related to damage results from a breakdown of the blood-brain barrier and a reduction in blood flow (94). Although Amyloid beta definitely has an important role in Alzheimer's disease it's very important to investigate other leads, perhaps where amyloid-beta isn't as centrally involved.

Human apolipoprotein E has three isoforms: APOE2, APOE3 and APOE4. APOE4 is a major genetic risk factor for Alzheimer's disease and is associated with Down's syndrome dementia and poor neurological outcome after traumatic brain injury and haemorrhage. Neurovascular dysfunction is present in normal APOE4 carriers and individuals with APOE4-associated disorders. In mice, lack of APOE leads to blood-brain barrier (BBB) breakdown, whereas APOE4 increases BBB susceptibility to injury. How APOE genotype affects brain microcirculation remains elusive. Using different APOE transgenic mice, including mice with ablation and/or inhibition of cyclophilin A (CypA), it has been shown show that expression of APOE4 and lack of murine APOE, but not APOE2 and APOE3, leads to BBB breakdown by activating a proinflammatory CypA-nuclear factor-kappa B-matrix-metalloproteinase-9 pathway in pericytes. These findings suggest that CypA is a key target for treating APOE4-mediated neurovascular injury and the resulting neuronal dysfunction and degeneration. The data reviewed above support an essential role of neurovascular and BBB mechanisms in contributing to both, onset and progression of AD (95, 96).

BBB in the context of Alzheimer's Disease – Importance of EMF Exposure

In this context, the findings of Arendash et al., that long-term EMF reduced brain Aβ deposition through Aβ anti-aggregation actions in AD mice, are highly interesting (97). It was also found, by Mori and Arendash et al., that long-term exposure to high frequency EMF treatment prevented cognitive impairment in AD transgenic (Tg) mice and improved memory in normal mice and that an increase in neuronal activity could be observed in the EMF exposed groups (98). Furthermore, it was found by the group that EMF treatment enhances brain mitochondrial functions in AD Tg as well as normal mice and that no increase in brain temperature could be found in connection to the EMF exposure (99). An interesting aspect in this context, is the role of mitochondria for many cellular functions, including reactive oxygen species generation, apoptosis, and $Ca2^{+}$ homeostasis as was mentioned by Dragicevic et al. and reviewed by Nicholls (99, 100).

In the first mentioned study by Arendash et al. (97), mice were EMF exposed with start at young age or at adult age. In the young-age group, 24 mice were divided into 4 subgroups: n=6 were Tg controls, n=6 were Tg animals treated with EMF, n=6 were non-transgenic (NT) controls and n=6 were NT animals treated with EMF. 2.5, 4-5 and 6-7 months after daily GSM-900 EMF exposure (two 1-hour sessions daily, at SAR 0.25 W/kg), the animals were evaluated by cognitive tests. At the end of the study, Aβ in the brains was evaluated by immunohistochemistry. No effect on cognitive functions was observed after 2 months of exposure. However, for the Tg+EMF mice with start of EMF exposure at young age, the cognitive function was maintained after 6-7 months of exposure, while it deteriorated in the Tg group. In a final task for NT mice after 7 months of EMF, the EMF actually improved the mnemonic function. In the adult-age group, Tg animals had impaired cognitive functions at the age of 4 months. 28 Tg and NT mice were included. After long-term EMF exposure (2, 5 and 8 months) the memory was tested. While 2 months of EMF exposure had no effect, 5 months of exposure had positive effects only on NT mice, and 8 months of exposure had beneficial effects for the Tg mice, with better results in the Tg+EMF group as compared to the Tg controls. Also the NT+EMF mice had an improved function as compared to NT controls after 8 months. Staining for Aβ revealed lower values on both hippocampus and the entorhinal cortex in the Tg+EMF group as compared to the Tg control group. Hippocampal

tissue from Tg mice were then exposed to EMF for 4 days, after which it was shown that the Aβ amount had decreased as compared to non-exposed control tissue. It was also reported that a ~1° temperature increase was observed in EMF exposed animals during exposure, but not in between exposure sessions (97).

In the study by Mori and Arendash (98), n=6 mice were Tg controls, carrying the mutant APPK670N , n=10 mice were Tg treated with EMF, n=4 mice were NT controls and n=5 mice were NT treated with EMF. EMF exposed animals were placed in a Faraday cage, receiving two 2-hour periods of EMF treatment at GSM-900 frequencies, pulse modulated at SAR 0.25-1.05 W/kg. The neuronal expression of c-Fos was taken as an indicator of neuronal activity. With immunohistochemistry, it was found that c-Fos was increased in both the NT+EMF group, as well as in the Tg+EMF group in the entorhinal cortex. However, only this one brain region was analyzed, since c-Fos expression was too low in other regions, which the authors hypothesised might be due to that c-Fos in an early response gene, and that at a certain time after stimulation, when the animals were sacrificed, the expression had already declined in other regions, such as hippocampus. In a cognitive test (Y-maze), it was found that EMF improved the performance in both NT and Tg group as compared to untreated controls. It should also be noted, that despite the very interesting findings, the number of included animals is quite small (98).

EMF and ^{18}FDG Uptake – Recent Studies

The question whether EMF exposure from mobile phones has neuronal effects in the human situation was recently addressed by an American research group led by Volkow et al., conducting a PET study on ^{18}F-fluorodeoxyglucose (^{18}FDG) uptake (101). Though PET-studies on humans in correlation to EMF exposure have also been previously made, the purpose of this study was to extend the study material and use the more direct measure of brain glucose metabolism by the uptake of ^{18}FDG instead of the previously used CBF (cerebral blood flow) measure, which might be a more indirect sign of neuronal activity and also reflect short-term alterations (60s) as compared to the more long-lasting ones observed with ^{18}FDG (suggested to be in the range of 30 min). ^{18}FDG is actively transported across the BBB into the cells, where it is phosphorylated, and is, among others, used as a prognostic value for following low-grade brain tumours, where an increased uptake in previously low-

grade tumours is an indicator of anaplastic transformation (for review into the topic of ^{18}FDG and brain tumours (102).

(space)

In the study by Volkow et al. (101), in total, 47 persons were involved, and effects upon brain glucose metabolism of EMF exposure were evaluated using PET with injection of ^{18}FDG. PET scans were performed both with and without EMF exposure (50 min of GSM-900 with maximum SAR of 0.901 W/kg), and the participants were blinded to the exposure situation. Whereas whole-brain metabolism was not affected, there were regional differences, in the right orbitofrontal cortex and the lower part of the right superior temporal gyrus (that is, the same side as the mobile phone was placed at) with increased metabolism in the exposure situation of about 7% as compared to control. There was a positive correlation between the strength of the E-field from the phones and the brain activation. Interestingly, it was hypothesized that RF-EMF exposure might increase the excitability of brain neurons.

Following the study by Volkow et al. (101), Kwon et al. (103) also investigated effects of GSM-900 exposure upon brain ^{18}FDG uptake. Thirteen persons were exposed to GSM-900 for 30 minutes to the right side of the head, and all subjects were also sham-exposed, and blinded to the exposure situation (SAR-values of maximum 0.74 W/kg in the head and 0.23 W/kg in the brain tissue). Contrary to the findings of Volkow et al. (101), the study by Kwon et al. (103) demonstrated a decrease in brain ^{18}FDG uptake after GSM-900 exposure, with decreased uptake values in the temporoparietal junction. A volume-of-interest analysis focused upon the right temporal lobe, showed a decreased ^{18}FDG uptake in the anterior inferior temporal cortex. No effects on task performance were found, and no correlation between temperature or ^{18}FDG uptake (a temperature increase of <0.21°C was found on the skin on the exposed side of the head) (103).

In the animal situation, Frilot et al. investigated the effect of ELF magnetic field exposure (2.5 G at 60 Hz) upon ^{18}FDG uptake in rats, comparing uptake with and without EMF exposure. An increased glucose uptake was found in the hindbrain when the field was orthogonally to the sagittal plane, but not when the angle varied randomly between the field and sagittal plane. These effects were hypothesized to be coupled to induction of electric field on the gate of ion channels (104).

Possible connection between BBB leakage and nerve cell injury

It has been suggested that BBB leakage is the major reason for nerve cell injury, such as that seen in dark neurons in stroke-prone spontaneously hypertensive rats (105). Much speaks in favour of this possibility. The parallel findings in the Lund material of neuronal uptake of albumin and dark neurons may support the hypothesis that albumin leakage into the brain is the cause for the neuronal damage observed after 28 and 50 d. It should, however, be pointed out that the connection is not yet proven (Figure 8).

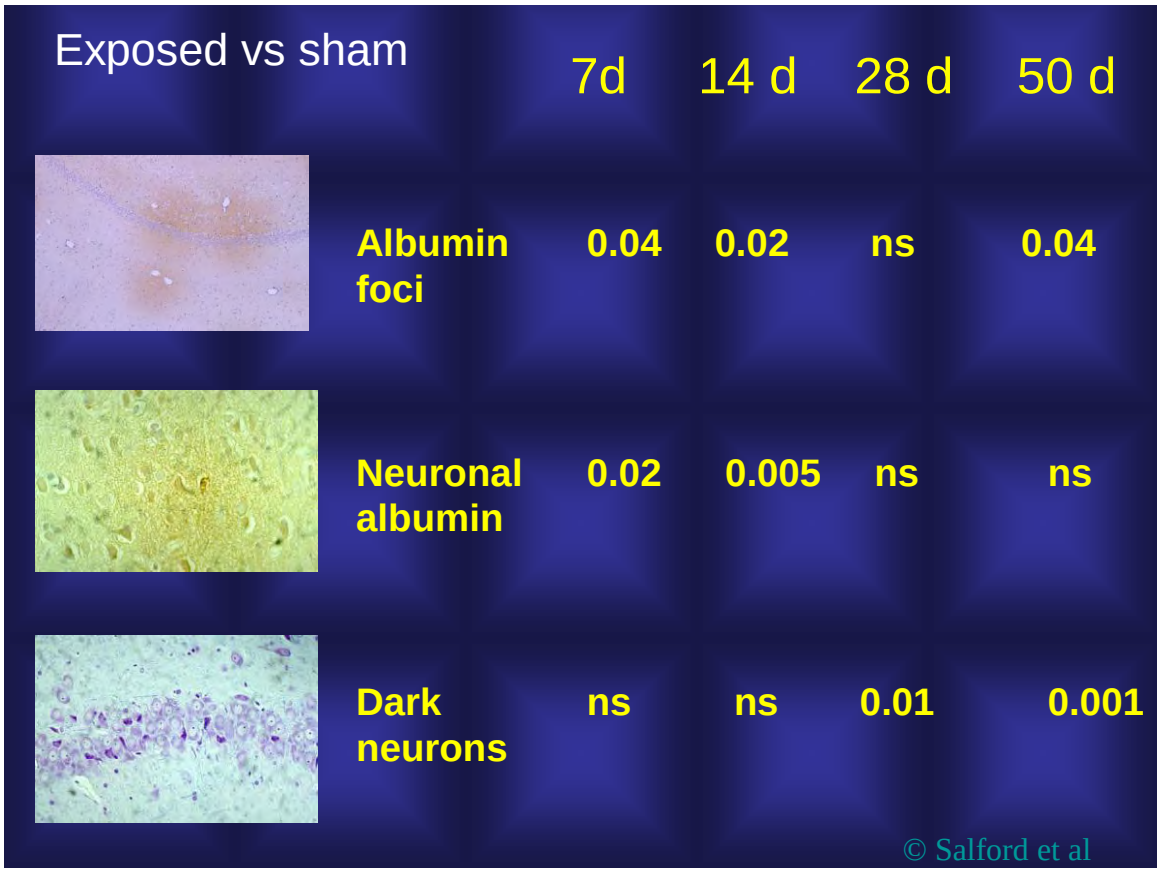

Figure 8. Results from the Lund group (61-63)

Also, other unwanted and toxic molecules in the blood may leak into the brain tissue in parallel with the albumin, and concentrate in and damage the neurons and glial cells of the brain. In favour of a causal connection between albumin and neuronal damage is a series of experiments performed in rats by another group at Lund University; albumin leaks into the brain and neuronal degeneration is seen in areas with BBB disruption in several circumstances: after intracarotid infusion of hyperosmolar solutions in rats (106) in the stroke

prone hypertensive rat (105); and in acute hypertension by aortic compression in rats (22). Furthermore, it has been shown in other laboratories that epileptic seizures cause extravasation of plasma into brain parenchyma (21), and in the clinical situation the cerebellar Purkinje cells are heavily exposed to plasma constituents and degenerate in epileptic patients . There are indications that an already disrupted BBB is more sensitive to the RF fields than an intact BBB (74, 91). It has been stated by other researchers that albumin is the most likely neurotoxin in serum (64). It has been demonstrated that injection of albumin into the brain parenchyma of rats gives rise to neuronal damage. When 25 micro-litres of rat albumin is infused into rat neostriatum, 10 and 30, but not 3 mg/ ml albumin causes neuronal cell death and axonal severe damage (65). It also causes leakage of endogenous albumin in and around the area of neuronal damage. However, it is still unclear whether the albumin leakage demonstrated in our experiments locally reaches such concentrations.

Possible mechanisms

Microarray analysis of the expression of all the rats' genes in cortex and hippocampus, after exposure to GSM RFs or sham exposure for 6 h, has shown interesting differences between exposed animals and controls as described by Nittby et al. (107). Genes of interest for membrane transport show highly significant differences. This may be of importance in conjunction with our earlier findings of albumin leakage into neurons around capillaries in exposed animals. It can be noted here that among the significantly altered genes from these evaluations, two variants of the gene RGS4 are up-regulated in hippocampal tissue from exposed rats as compared to the sham-exposed rats (unpublished results). RGS is a regulator of G protein signalling, and it has been proposed that RGS4 might regulate BBB permeability in mammals, in a way corresponding to the role of its Loco homolog G protein coupled receptor (GPCR) in developing and maintaining the BBB permeability of Drosophila (7).

It has also been suggested in other connections that manifestations of BBB disruption might also be mediated by the formation of free radicals, such as O_2^-, H_2O_2, and hydroxyl radical, which are supposed to oxidize cell membrane lipids by virtue of the high concentration of polyunsaturated fatty acids in these membrane constituents (108). As an example of this, it was reported by Chan et al.(109), that treatment of the brain of rats with a free-radical

generating system resulted in lipid-peroxidation, and an increased permeation of Evans blue due to barrier breakdown.

Recently, a detailed molecular mechanism, by means of which mobile phone radiation might exert its effects, has been proposed (110). By using Rat1 and HeLa cells, it was shown that EMF exposure resulted in rapid activation of ERK/ MAPKs (mitogen-activated protein kinase). The activation of these ERKs was mediated by reactive oxygen species (ROS), resulting in a signalling cascade ultimately affecting transcription, by the central key role of ERKs in signalling pathways.

In the continued search for the mechanisms behind EMF mediated effects, their interaction with calcium-45 transport in bio-membranes has been studied (111) and $Ca2^{+}$-efflux over plasma membranes has been observed in plasma vesicles from spinach exposed to ELF magnetic fields (112). With this model, quantum mechanical theoretical models for the interaction between magnetic fields and biological systems are tested. The model proposed by Blanchard and Blackman (113), in which it is assumed that biologically active ions can be bound to a channel protein and in this way alter the opening state of that channel, could in this way be quantitatively confirmed. Thus, the membrane is one site of interaction between the magnetic fields and the cell, and more specifically, the $Ca2^{+}$-channels, are one of the targets. More recently, new models for the interaction between magnetic fields and hydrogen nuclei also have been proposed.

EMF-induced $Ca2^{+}$-efflux over plasma membranes, understandably, can have many different effects on the target cells. Some agents that increase the BBB permeability act through a contractile mechanism that widens the intercellular junctions of the capillary endothelium. An increase of free $Ca2^{+}$ should mediate these changes, thereby resulting in measurable alterations of intracellular $Ca2^{+}$-levels in brain capillary cells after exposure to BBB-disrupting agents (108).

Another hypothesis is that EMF-induced intracellular $Ca2^{+}$-alterations might affect Ets genes, which are transcription factors expressed in different tissues (114). In this context, we could add that in our gene expression material from GSM-exposed rats vs., sham-exposed rats, one Ets variant gene is actually significantly up-regulated in hippocampus and one Ets1 gene is significantly up-regulated in cortex of the exposed animals.

EMF induced BBB permeability – with the aim of medical use

In the attempt to further try to understand the underlying mechanisms of the RF effects, we recently undertook a study upon snail nociception, with 1-hour GSM-1800 exposure of the land snail *H. pomatia*. This revealed, that the exposure induced analgesia in the snail model, with a significantly increased latency of reaction when placed on a hot plate, as compared to when only sham exposed. The vast knowledge about the physiology of the snail, its neurotransmission systems and it simplicity as compared the mammals may provide a tool for successful continued search for the mechanisms behind the effects of the GSM EMF upon biology (115).

In a recent study by Kuo et al (116), it was described how EMFs might be utilized to facilitate transport across the BBB. In an *in vitro* model, human micro-vascular endothelial cells were co-cultured with human astrocytes. Effects of EMF upon P-glycoprotein (P-gp) and multi-drug resistance -associated proteins (MRP) were tested in connection to treatment with anti-retroviral drugs, where the MRPs and P-gp are known to play an important role in multidrug resistance, which is encountered in carcinomas and therapies for acquired immune-deficiency (Kuo et al. 2012). With increasing EMF frequencies up to 900 MHz (both 715MHz and 900 MHz), the endocytotic uptake of calcein was increased (5mW, square wave with amplitude modulation at 20 MHz for 4 hours). Treatment with EMF could also inhibit expression of MRP and P-gp after treatment with anti-retroviral drugs, indicating that it might be useful in order to deliver antiretroviral proteins into the brain, by decreasing the efflux of the drugs due to the MRPs and P-gl.

Kuo et al. (117) also showed that EMF exposure (915 MHz EMFs at 5 mW with 20 MHz amplitude modulation for 4 hours) in combination with cationic solid lipid nanoparticles (CSLNs) could increase the transport of the antiretroviral drug Saquinavir 22-fold across human brain-microvascular endothelial cells (as compared to a 17-fold increase when only CSLNs were used).

Conclusions

In this review, we have reported the results of our group's research during the last 24 years, and the results of similar, but seldom identical, experiments of several other groups around the world. When summing up what we have described here, we are convinced that RF electromagnetic fields have effects upon biology, and we believe that it is more probable than unlikely, that non-thermal electromagnetic fields from mobile phones and base stations do have effects also upon the human brain. However, in this context, it is also important to point out, that the studies from our laboratory, as well as most studies presented above and available in literature, have been performed using animals and not humans. Thus no definitive conclusions can be drawn regarding effects of mobile phone use upon the human BBB.

However, studies in humans utilizing radiopharmaceuticals have been performed by Volkow et al. (101) upon brain glucose metabolism, and as was described by Saha et al. (118) already in 1994, studies with PET or SPECT and radiopharmaceuticals are used in brain imaging.

Further, a tool to directly study the human BBB has recently been described (119). It is based upon a non-radioactive methodology for *in vivo* non-invasive, real-time imaging of BBB permeability for conventional drugs, using nitroxyl radicals as spin-labels and MRI. In this connection, it should be mentioned though, that MRI has the drawback of possibly itself influence upon the results.

Based upon what has been presented here, we feel that the WHO IARC classification of RFR at the level 2B is adequate at present.

The question whether existing FCC/IEE and/or ICNIRP public safety limits and reference levels are adequate to protect the public is not easily answered. The reported studies on EMF induced BBB disruption have shown partially contradictory results from different laboratories. However, the fact that an abundance of studies do show effects is an important warning. This is true even if it can be summarized that the effects most often are weak and are seen in about 40% of the exposed animals.

However, we have stressed the following opinion in several publications during the past years: - "*The intense use of mobile phones, not least by youngsters, is a serious memento. A neuronal damage may not have immediately demonstrable consequences, even if repeated. It may, however, in the long run, result in reduced brain reserve capacity that might be unveiled by other later neuronal disease or even the wear and tear of ageing. We can not exclude that after some decades of (often), daily use, a whole generation of users, may suffer negative effects such as autoimmune and neuro-degenerative diseases maybe already in their middle age*".

One remarkable observation, which we have made in our studies throughout the years, is that exposure with whole-body average power densities below 10 mW/kg gives rise to a more pronounced albumin leakage than higher power densities, all at non-thermal levels. These very low SAR-values, such as 1 mW/kg, exist at a distance of more than one meter away from the mobile phone antenna and at a distance of about 150–200 m from a base station. Further, when a mobile phone operating at 915 MHz (and its antenna) is held 1.4 cm from the human head, the very low SAR levels of 10 mW/kg exist in deep-lying parts of the human brain such as the basal ganglia, and the power density of 1 mW/kg and less is absorbed in thalamus bilaterally.

With this information as a background, it is difficult to recommend safety limits as the function of existing mobile systems might not allow for limits that produce SAR levels below 1 or 0,1 mW/kg in the human brain, which are reported to cause a pathological leakage of the BBB and to neuronal damage.

Demonstrated effects on the BBB, as well as a series of other effects upon biology (120) have given rise to scientific concern and to public anxiety. It is up to the society and our politicians and also the providers of the radiofrequency-emitting technologies to support continued research in order to understand the nature of the effects, thereby neutralizing or at least reducing them. Also, it should be kept in mind that proven effects on biology also means that positive potentials might be revealed. This might be useful in medical applications, for example a controlled opening of the BBB would enable previously excluded pharmaceuticals to reach their targets within the brain tissue.

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Attachment 9

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Review

Neuroanatomic observations of the brain in autism: a review and future directions

Margaret L. Bauman *, Thomas L. Kemper

Children's Neurology Service, Massachusetts General Hospital, 15 Parkman Street, Boston, MA 02114, USA

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Abstract

Infantile autism is a behaviorally defined disorder associated with characteristic cognitive, language and behavioral features. Several postmortem studies have highlighted areas of anatomic abnormality in the autistic brain. Consistent findings have been observed in the limbic system, cerebellum and related inferior olive. In the limbic system, the hippocampus, amygdala and entorhinal cortex have shown small cell size and increased cell packing density at all ages, suggesting a pattern consistent with development curtailment. Findings in the cerebellum have included significantly reduced numbers of Purkinje cells, primarily in the posterior inferior regions of the hemispheres. A different pattern of change has been noted in the vertical limb of the diagonal band of broca, cerebellar nuclei and inferior olive with plentiful and abnormally enlarged neurons in the brains of young autistic subjects, and in adult autistic brains, small, pale neurons that are reduced in number. These findings combined with reported age-related changes in brain weight and volume, have raised the possibility that the neuropathology of autism may represent an on-going process.
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Keywords: Brain; Neuroanatomy; Limbic system; Cerebellum; Large brain; Neurochemistry

Contents

It has been just over 60 years since Kanner (1943) first described an intriguing disorder in which children exhibit a significant disturbance in cognition and behavior in the absence of obvious physical or brain dysmorphology. For a period of time, parenting and environmental factors were believed to be responsible for the impaired language, social aloofness, perseverative and stereotypic behaviors and obsessive need for sameness that characterize this disorder. Gradually, however, with the observation that many of those affected had abnormalities on their electroencephalograms (Small, 1975), and a higher than expected incidence of seizures (Deykin and MacMahon, 1979), evidence for a neurological basis for the disorder began to unfold.

Given the complexity and variety of symptoms with which autistic individuals present, it has been difficult to conceptualize a defining cohesive neurological mechanism that might underlie the core features of this disorder. Some of the earliest efforts to address this question utilized neurophysiologic technology, with resulting studies reporting abnormalities of auditory-nerve and brainstem-evoked responses (Student and Schmer, 1978; Tanguay et al., 1982) and rapid eye movement sleep patterns (Tanguay et al., 1976). Although more recent investigations have failed to confirm these original observations (Rumsey et al., 1984; Courchesne et al., 1985), they nonetheless played an

* Corresponding author. Tel.: +1 617 414 7012.
E-mail address: drb@ladders.org (M.L. Bauman).

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important role in initiating the path toward further neurobiologic research in autism.

Among biological abnormalities so far described in autism, neuroanatomic observations provide some of the strongest evidence. Results from postmortem and imaging studies have implicated the involvement of many major structures of the brain including the limbic system, cerebellum, corpus callosum, basal ganglia and brainstem. However, despite a growing body of data implicating the involvement of multiple sites and differing types of abnormalities, questions remain about many of these findings. Moreover, there is little direct information regarding the autistic brain during early development since most of the postmortem studies have been limited to investigations involving older children and adults. Nonetheless, available data provides evidence for a prenatal onset of at least some of the neuroanatomic abnormalities reported in the autistic brain.

Identification of the underlying neuroanatomic substrate in the autistic brain remains an ongoing challenge, compromised largely by the dearth of human pathological material of reasonable quality for study, technical limitations and the lack of an animal model. One of the first neuropathologic studies of an autistic brain was published by Aarkrog (1968) who described "slight thickening of the arterioles, slight connective tissue increase in the leptomeninges, and cell increase" in a right frontal lobe biopsy. Some years later, in 1976, Darby published a review of 33 cases of childhood psychosis in which he suggested a relationship between limbic system lesions and the affective features of autism, but no specific pathology was described (Darby, 1976). Subsequently, in 1980, Williams et al. examined autopsy material obtained from four individuals with autistic features, looking primarily for cell loss and gliosis (Williams et al., 1980). No consistent abnormalities were observed.

In 1985, observations of the brain of a 29-year-old well-documented man with autism was reported, studied in comparison with an identically processed age and sex-matched control, using the technique of whole brain serial section (Bauman and Kemper, 1985). Both brains were examined by means of a comparison microscope, multiple sections being studied side by side in the same field of view. The most significant findings were confined to regions of the limbic system and cerebellar circuits. No abnormalities were found in any regions of the cortex, an observation further supported by a detailed analysis of cortical neuronal and glial cell counts in another autistic brain by Coleman et al. (1985) and by repeated surveys of the original and subsequent whole brain serial section material (Kemper, personal communication). It should be noted, however, that Bailey et al. (1998) have noted neocortical malformations to be a prominent feature in their autopsy material. In four out of their six cases, they found evidence of thickened cortices, areas of increased neuronal density, irregular laminar patterns, increased number of neurons on layer I, and abnormally oriented pyramidal cells. In addition, more recently, Casanova et al. (2002) have reported that, in comparison to controls, the cerebral cortex in the autistic brain demonstrates more numerous minicolumns, and that these minicolumns were found to be smaller and more compact in configuration in the three cortical areas studied. Thus, at this point, the presence, consistency and significance of cerebral cortical abnormalities in the autistic brain remains uncertain. How these observational differences relate to the clinical heterogeneity of the subjects studied and to the disorder in general will be an important focus of future research.

Since the 1985 report, eight additional clinically well-documented cases have been similarly studied using the same methodology (Kemper and Bauman, 1996). None of these cases have shown any gross abnormalities. Patterns of myelination have appeared to be comparable to that of controls in all cases. Examination of the cortex has likewise been unremarkable when compared with controls with the exception of small neuronal cell size and increased cell packing density in the anterior cingulate gyrus in all brains, an observation not appreciated in the original case. Only two microscopic cortical malformations have been noted in this series of cases. A small heterotopic lesion on the infra-orbital region in one hemisphere was found in a child with a history of a severe seizure disorder, and multiple heterotopic cells were observed in the cerebellar molecular layer in a second autistic child with significant developmental delay (Kemper and Bauman, 1998). Systematic examination of the forebrain, hypothalamus, and basal ganglia in these cases have failed to show any differences from controls.

Areas of the forebrain that have been found to be abnormal have included the hippocampus, subiculum, entorhinal cortex, amygdala, mammillary body, anterior cingulate gyrus and septum, structures which comprise a major portion of the limbic system. In comparison with controls, these areas showed reduced neuronal cell size and increased cell packing density (increased numbers of neurons per unit volume) bilaterally (Bauman and Kemper, 1994). Golgi analysis of CA1 and CA4 pyramidal neurons has shown decreased complexity and extent of dendritic arbors in these cells (Raymond et al., 1989). In the amygdala, the most significant increase in cell packing density was noted in the most medially placed nuclei. With the exception of a single child of normal intelligence, the lateral nucleus has appeared to be uninvolved.

Small neuronal size and increased cell packing density was also observed in the medial septal nucleus (MSN). However, in the nucleus of the diagonal band of Broca (NDB) of the septum, a different pattern of abnormality was noted. In this nucleus, the neurons were adequate in number but were unusually large in the brains of all of the autistic children less than 13 years of age when compared to controls. In contrast, the cells of the NDB in all of the autistic brains older than 21 years of age were small and pale and markedly decreased in number (Kemper and Bauman, 1998). Unfortunately, no whole brain serial sections from autistic adolescents have been available for study and thus, it

is unknown when and how rapidly these changes might occur over this period of time.

Outside of the limbic system, the most apparent and consistent abnormalities have been confined to the cerebellum and related inferior olive. All the autistic brains reported to date, regardless of age, sex or cognitive abilities have shown a significant decrease in the number of Purkinje cells, primarily effecting the posterolateral neocerebellar cortex and adjacent archicerebellar cortex of the cerebellar hemispheres (Arin et al., 1991). Similar anatomic findings have been reported by Ritvo et al. (1986) and more recently by Bailey et al. (1998), thus making the presence of reduced numbers of Purkinje cells the most reproducible pathological observation in the autopsied autistic brain. Despite reports of hypo- and hyperplasia of the vermis with magnetic resonance imaging (MRI) (Courchesne et al., 1994), we have found no change in Purkinje size or cell number in this cerebellar region (Bauman and Kemper, 1996). Reduced numbers of Purkinje cells in the cerebellar hemispheres have been observed in both childhood and adult cases, in individuals with and without a history of seizures or medication usage and appear to be unrelated to cognitive function. With few exceptions, there has been an absence of glial hyperplasia (Bauman and Kemper, 1996; Bailey et al., 1998) suggesting that the cerebellar lesions have been acquired early in development. Animal studies have shown a progressively decreasing glial response after cerebellar lesions occurring at increasingly earlier ages (Brodal, 1940).

In addition to the presence of reduced number of Purkinje cells, abnormalities have also been observed in the fastigeal, globose and emboliform nuclei in the roof of the cerebellum, which like the NDB, appear to alter with age. In these three nuclear groups, all the adult brains have shown small pale neurons that are significantly decreased in number. In contrast, in all the childhood brains (ages 5–13 years), the neurons in these same nuclear groups, in addition to those of the dentate nucleus, have been found to be enlarged and plentiful in number (Bauman and Kemper, 1994).

A similar pattern of change in cell size has also been observed in the inferior olive of the brainstem but the number of neurons has been found to be preserved. Given the known close relationship of the olivary climbing fiber axons to the Purkinje cell dendrites (Holmes and Stewart, 1908), the preservation of the olivary neurons in the face of a significant reduction in Purkinje cell number strongly supports a prenatal origin for the cerebellar abnormalities. Studies in the fetal monkey indicate that the olivary climbing fiber axons synapse with the Purkinje cells dendrites in a transitory zone beneath the Purkinje cells called the lamina dessicans, thus forming a single unit (Rakic, 1971). In the human fetus, this zone is no longer present after 28–30 weeks gestation (Rakic and Sidman, 1970). Thus, given the resulting tight bond between the olivary neurons and the Purkinje cells after this time, loss or damage to the cerebellar Purkinje cells results in an obligatory retrograde loss of olivary neurons (Holmes and Stewart, 1908; Norman, 1940; Greenfield, 1954). Since, in the autistic brain, the number of the olivary neurons is preserved, it is likely that whatever event resulted in the reduction of the Purkinje cells in these cases has to have occurred before this tight bond has been established, and thus before 28–30 weeks gestation.

Abnormalities in the brainstem have also been observed in a small number of cases. Rodier et al. (1996) have described dysgenesis of the facial motor nucleus and agenesis of the superior olivary nuclei in a single case with autism and Moebius syndrome. The nature and location of these findings would suggest an onset during the first 4 weeks post-conception, during the time of neural tube closure. Bailey et al. (1998) have described the presence of ectopic neurons lateral to the olives bilaterally in one case and malformation of the olive in three cases, thus providing further pathological evidence for a prenatal onset of this disorder. A review of our own material has yielded olivary nuclear malformations, similar to those reported by Bailey et al. (1998) in some cases. A more systematic analysis of serially sectioned brainstem material from autistic subjects and age and sex-matched controls is now in progress.

1. Directions for future research

Based on the findings in the cerebellum, combined with brainstem and cerebral cortical abnormalities in some cases (Rodier et al., 1996; Bailey et al., 1998), there appears to be reasonable evidence to suggest that least some of the brain abnormalities observed in the autistic brain are of prenatal origin. In addition, however, there is a growing body of data that indicates that the underlying neurobiological processes involved in autism may be on-going and that postnatal factors may also be important. It has been observed, for example, that overall brain weight in children with autism is statistically heavier than that of age and sex-matched controls, while the weight of the autistic adult brain tends to be lighter than that of controls (Bauman and Kemper, 1997). More recently, imaging studies have indicated increased brain volume in autism, most prominent between the ages of 2 and 4.5 years of age, a feature which later appears to plateau during adolescence (Courchesne et al., 2001). In addition, microscopic observations of enlarged cells in some brain regions in autistic children and small pale cells that are reduced in number in these same areas in adults strongly indicate changes with age. Clinically and pathologically, this process does not appear to a degenerative one and may reflect the brain's attempt to compensate for its atypical circuitry over time. Future research will need to address the timing and pathogenesis of these changes and to consider how the resulting findings may impact on the clinical features of the disorder.

The observation of postnatal brain enlargement is intriguing and a number of hypotheses have been posed to explain its origins. Clinically, the head circumference of the autistic child has been said to be either normal or slightly small at birth but later increases in size during early

to mid-childhood (Lainhart et al., 1997; Courchesne et al., 2003). A number of possible neurobiological mechanisms have been proposed to explain this apparent early brain "overgrowth" including increased neurogenesis, decreased neuronal cell death, increased production of non-neuronal brain tissues (i.e. glial cells), decreased synaptic pruning and abnormalities of myelin. At this point, there is no firm pathological evidence to support any of these suggested hypotheses. Finding the answer to these questions may go a long way toward advancing our understanding of the underlying pathogenesis of this disorder.

Immunohistochemical studies utilizing autopsy brain material obtained from autistic subjects have been increasingly reported in the literature in recent years. Blatt et al. (2001) noted reduced binding of GABAa receptors in the hippocampus in the brains of four autistic adults studied in comparison to controls, but found no significant changes in kainate, cholinergic or serotonergic receptors. Perry et al. (2001) found a decrease in nicotinic receptors in tissue obtained from autistic frontal and parietal cortex, with a decrease in M1 receptors only in the parietal cortex. Although the basal forebrain failed to show similar findings, this region showed a marked increase in brain-derived neurotrophic factor (BDNF). More recently, this same research group has noted a decrease in three of the four nicotinic receptors in the cerebellum but no abnormalities of either M1 or M2 receptors or in choline acetyltransferase activity in this same area (Lee et al., 2002). Studies such as these are just beginning to expand our knowledge base about the neurobiology of autism and future investigations should continue to explore the underlying neurochemistry of the autistic brain with a particular focus on those regions known to be anatomically abnormal.

2. Conclusion

Our understanding of the neurobiology of autism has advanced substantially over the past 20 years but there is still much to be learned. There is an urgent need for an animal model to address numerous questions, which, because of the limited availability of suitable human autopsy material and the technical limitations involved in the study of the human brain, cannot be adequately addressed at this time. It is hoped that advancements in technology coupled with a better definition of the genetic, neurochemical and neuroanatomic profile of autism and its broader phenotype, will result in a more detailed understanding of the pathogenesis and neurobiology of the disorder, and ultimately to earlier identification and more effective interventions and treatment.

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Attachment 10

Autism spectrum disorders and underlying brain pathology in CHARGE association

Maria Johansson* MD;
Maria Råstam MD PhD;
Eva Billstedt BA;
Susanna Danielsson MD, Department of Child and Adolescent Psychiatry, Göteborg University, Göteborg;
Kerstin Strömland MD PhD, Department of Ophthalmology, Göteborg University, Göteborg, Sweden.
Marilyn Miller MD PhD, Department of Ophthalmology and Visual Sciences, Eye and Ear Infirmary, University of Illinois, Chicago, IL, USA.
Christopher Gillberg MD PhD, Department of Child and Adolescent Psychiatry, Göteborg University, Göteborg, Sweden.

**Correspondence to first author at* Department of Child and Adolescent Psychiatry, Göteborg University, Kungsgatan 12, SE 411 19 Göteborg, Sweden.
E-mail: maria.johansson@pediat.gu.se

The rate of autism spectrum disorders (ASDs) and brain abnormalities was analyzed in 31 individuals (15 males, 16 females; age range 1mo to 31y, mean age 8y 11mo) with CHARGE association, as part of a multidisciplinary study. A meticulous neuropsychiatric examination was performed, including standardized autism diagnostic interviews. Judgement regarding ASDs was impossible in three infants and three patients who were deaf and blind. Five individuals met diagnostic criteria for autism, five for an autistic-like condition, and seven for autistic traits. Brain abnormalities were indicated in almost three-quarters of examined individuals, and midline abnormalities of the forebrain in one-third. Awareness of the coexistence of CHARGE and ASDs is important in habilitation care in CHARGE. Moreover, the results indicate that a subgroup of ASDs may be associated with errors in early embryonic brain development.

CHARGE (coloboma of the eye, heart defect, atresia of the choanae, retarded growth and/or development, genital hypoplasia, and ear anomalies and/or hearing impairment) association (Pagon et al. 1981) is a condition with multiple congenital malformations, probably arising during early embryological development. Blake et al. (1998) proposed major and minor criteria, and that four major (coloboma, choanal atresia, characteristic ear abnormalities, and cranial nerve dysfunction) or three major and three out of seven minor criteria (genital hypoplasia, developmental delay, cardiovascular malformations, growth deficiency, orofacial cleft, tracheoesophageal fistula, and distinctive face) would qualify for a diagnosis of CHARGE. They further stated that this condition should be considered in infants meeting one or two major and several minor criteria. Severe impairment of vision and hearing is common in CHARGE.

The estimated prevalence of CHARGE is 1:10 000 to 1:15 000. A teratogenic cause has not been substantiated and most cases are sporadic. A recognizable syndrome within CHARGE has been suggested (Graham 2001). Until recently, reported chromosome anomalies have been inconsistent. Vissers et al. (2004) revealed mutations in a member of the chromodomain gene family (*CHD7*) in 10 out of 17 individuals characterized as having CHARGE syndrome.

Other congenital conditions with craniofacial malformations, indicating a disturbance during the first trimester, have been linked to autism spectrum disorder (ASD), namely Goldenhar syndrome (Landgren et al. 1992), chromosome 22q.11 deletion (Niklasson et al. 2002), fetal alcohol syndrome (Aronsson et al. 1997), Möbius sequence (Johansson et al. 2001), and thalidomide embryopathy (Strömland et al. 1994), of which the first three have been hypothesized to be caused by abnormal migration of neural crest cells. Ozonoff et al. (1999) described ASDs in Joubert syndrome, an autosomal-recessive disorder with cerebellar and brainstem malformations, coloboma, retarded growth/development, and abnormal eye movements.

Previously published reports of ASDs in CHARGE have, to our knowledge, been case reports or unexpected findings in studies not designed to examine the association of ASDs and CHARGE (Table I).

Learning disability* (LD) was previously considered to be almost universal in CHARGE. More recent reports have described extremely variable mental functioning (Raqbi et al. 2003).

Lin et al. (1990) reviewed 144 cases and reported 'structural central nervous system (CNS) defects, excluding generalized cortical atrophy, hydrocephalus, and ventriculomegaly' in 55% of them. Forebrain and midline anomalies were most striking (absence/hypoplasia of the olfactory bulbs and tracts, dysgenesis/hypoplasia of the frontal lobe and optic nerve, holoprosencephaly, and agenesis of corpus callosum and septum pellucidum). Hindbrain anomalies also occur in CHARGE (focal cerebellar heterotopias, agenesis of the seventh cranial nerve nuclei and cerebellar vermis, brainstem hypotrophy, cerebellar hypoplasia, and Dandy–Walker malformation), as well as general cerebral atrophy, microcephaly, lissencephaly, gyral abnormalities, haemorrhagic/ischaemic lesions, CNS asymmetry, ventricular dilatation, and craniostenosis (Lin et al. 1990, Tellier et al. 1998, Källén et al. 1999).

See end of paper for list of abbreviations.

*US usage: mental retardation.

The main aim of the present study was to use current standardized autism diagnostic instruments to analyze the relation between CHARGE and ASDs in a group of patients with CHARGE. An increased rate of ASDs would provide indirect evidence for an association between the birth defects in CHARGE and ASDs. We also wished to try to identify CNS and chromosomal abnormalities that might contribute to the pathogenesis of ASDs in CHARGE, prenatal and perinatal risk factors, and the period of development during which these might have operated.

Methods

The study was performed as part of a prospective, multidisciplinary survey of CHARGE (Strömland et al. 2005). Cardiology, child neuropsychiatry, neuropsychology, child neurology, paediatric ophthalmology, oto-rhinolaryngology, odontology, and speech pathology were represented. Approval was obtained from the local ethics committee and informed consent from all patients/principal caregivers.

PARTICIPANTS

A call for patients was announced in the *Journal of The Swedish Medical Association* and through organizations of specialty for Swedish physicians. The aim of the study outlined in the call was to survey the clinical picture in CHARGE. ASDs were not mentioned. Inclusion criteria were four or more of the six acronym characteristics or three of these plus additional characteristics.

Thirty-one patients (15 males, 16 females; age range 1mo to 31y, mean age 8y 11mo at neuropsychiatric examination) met the inclusion criteria. Twenty-eight patients were aged 2 years or older and assessed for ASDs. Three patients who were deaf and blind could not be reliably assessed for ASD. Two patients were siblings (sister and brother). Another female was reported by Fernell et al. (1999). The patients were recruited from all over Sweden and examined at the Queen Silvia Children's Hospital in Göteborg.

NEUROPSYCHIATRIC ASSESSMENT

Autism and other ASDs are defined by specific abnormalities in reciprocal social interaction and communication, and unusual interests and behaviours. The focus of the neuropsychiatric assessment was to identify combinations of disturbances in these domains and to assess the level of intellectual functioning.

Level of intellectual functioning

The cognitive level was assessed with The Wechsler Intelligence Scale for Children (Wechsler 1992) or the Vineland Adaptive Behaviour Scales (Sparrow et al. 1984).

Symptoms and diagnoses of ASD

Autistic symptoms were scrutinized by one investigator with the use of the Autistic Behaviour Checklist (ABC; Krug et al. 1980), the Childhood Autism Rating Scale (CARS; Schopler et al. 1980) and the Diagnostic and Statistical Manual of

Table I: Previous published reports of autism spectrum disorder in CHARGE

Reference	*Report*	*Diagnosis/characteristics of CHARGE and autistic symptoms described in report*
Rapin and Ruben (1976)	Case reports of 16 patients characterized by 'anomalies with malformed ears', 'selected because the complexity of their anomalies was thought to be instructive'	One patient not diagnosed with CHARGE or autism in this report, described to have coloboma, low-set malformed unrolled pinnas, hearing loss, malformations of the middle ear and bony labyrinth, vestibular dysfunction, facial palsy, weakness of the lateral rectus muscles and the muscles innervated by the oculomotor nerve, autistic features, severely aberrant behaviour, and severe mental retardation (IQ 20–49)
Davenport et al. (1986)	Report of 15 patients with CHARGE sought to describe 'the spectrum of clinical features in CHARGE syndrome'	One patient diagnosed with CHARGE, described as 'autistic appearing' and profoundly retarded
Wiznitzer et al. (1987)	Report of 100 patients with 'complex ear anomalies with or without hearing loss' sought to 'describe neurologic findings in children with ear anomalies'	One patient diagnosed with CHARGE and autism. No information about diagnostic criteria for autism
Jure et al. (1991)	Report aimed to 'describe the clinical features of 46 hearing impaired autistic children'	Two patients diagnosed with CHARGE and autism. Diagnostic criterion: DSM-III-R
Simon Harvey et al. (1991)	Report of 17 patients, of whom seven survived infancy, which are described concerning behaviour, sought to 'determine the developmental outcome of patients with CHARGE in whom the diagnosis could be made in early infancy'	Two patients diagnosed with CHARGE, described as having 'autistic–like behaviour'
Fernell et al. (1999)	Case report sought to 'report three children with CHARGE and concomitant autistic disorder'	Three patients diagnosed with CHARGE and autism. Diagnostic criteria/instruments: DSM-IV, ADI-R, CARS

Authors, year of publication, aim of report concerned, autistic symptoms, other neuropsychiatric impairments, diagnosis and characteristics of CHARGE in described cases are shown. ADI-R, Autism Diagnostic Interview – Revised; CARS, Childhood Autism Rating Scale.

Table II: Brain abnormalities, skull abnormalities, head circumference, length, and radiological imaging of the brain of each patient

N[a]	*Sex*	*Age (y:m)*	*Criteria*	*IQ*	*Structural cerebral anomalies/abnormalities of white/grey matter*
1–24	M	5:4	CA, AD	PLD	Bilat. hypoplasia: N opticus, optic chiasm, optic tract (MRI). Marked hypoplasia: infundibilum hypothalami (MRI). Susp. bilateral aplasia: olfactory tract (MRI). Bilat incomplete rotation of hippocampus (MRI). Abnormal gyri: basal medial frontal lobes (MRI). Small: cerebrum, pedunculus cerebri bilat., pons (MRI)
2–9	F	6:2	CA, AD	PLD	
3–4	F	7:8	CA, AD	PLD	Corpus callosum hypogenesis (MRI, CT). Septum pellucidum agenesis (CT, US). Susp. septo-optisk dysplasia (US). Parieto-occipital gyri abnormal, sparse white matter (MRI). Focal lesion lateral to right lateral ventricle (MRI)
4–13	M	13:4	CA, AD	PLD	Abnormal configuration: sella turcica shallow, infundibulum hypothalami long (MRI)
5–6	F	16:8	CA, AD	MLD	
6–21	M	4:4	ALC	MLD	Corpus callosum hypoplasia (MRI)
7–14	F	6:7	ALC	PLD	Hypoplasia: frontal lobes (CT). Thickening of left N opticus close by optic bulb (CT). Sylvian fissures widened (CT). Widened interhemispheric fissure (US). Susp. focal medial occipital lesion (US)
8–16	M	14:5	ALC	NA	Cerebral asymmetry (CT). Frontal lobe fissures, Sylvian fissures widened (CT)
9–12	F	17:2	ALC	SLD	All brain fissures widened: general atrophy or hypoplasia? (MRI, CT). Susp. focal left periventricular lesion (MRI)
10–15	F	17:11	ALC	PLD	Corpus callosum hypoplasia (MRI). Focal left periventricular lesion (MRI)
11–29	M	2:6	AT	MLD	Focal frontal subcortical lesion (MRI)
12–30	F	3:.3	AT	MLD	
13–31	F	9:1	AT	MLD	Susp. partial corpus callosum agenesis (CT)
14–25	M	9:6	AT	MLD	
15–26	F	7:4	AT	MLD	
16–11	F	9:5	AT	SLD	Asymmetry: cerebral (left side prominent), cerebellar (right side prominent; MRI). Small pituitary gland (MRI)
17–23	F	11:3	AT	SLD	Hypoplasia: left N opticus, adenohypophysis, cisternal hernia in sella turcica (MRI)
18–17	F	6:1	AT?	MLD	[f]
19–2	M	10:7	AT?	NA	
20–10	F	13:1	AT?	NA	Susp. vermis agenesis (CT). Frontal lobe fissures widened (CT)
21–20	M	2:4	–	MLD	
22–18	M	16:3	–	A	[f]
23–19	M	10:9	–	NA	[f]
24–8	F	3:7	–	MLD	
25–7	M	3:9	–	NA	[f]
26–5	F	0:1	*	*	Corpus callosum thin and pushed forward (MRI). Reduction of periventricular white matter (MRI)
27–27	F	0:3	*	*	
28–28	M	0:6	*	*	
29–3	M	6:9	*	PLD	Hypoplasia: N opticus, optic chiasm (MRI, CT)
30–1	M	9:5	*	PLD	
31–22	M	31:7	*	PLD	

Patients are listed according to severity of autism spectrum disorder (ASD). Entries in italics indicate clinical judgement or suspicion.
[a]Participant numbers in forthcoming multidisciplinary report (Strömland et al. 2005) after hyphen; [b]Previously diagnosed with microcephaly; [c]Microcephaly previously suspected. [d]Information from notes in medical records, formal radiological report not available; [e]Current measure of head circumference/height not available; [f]No information; radiological examinations not performed/not available; [g]Radiological examination mainly focused on N opticus, optic chiasm, and optic tract; [h]Examination performed after diagnostic study.

Table II: continued

Abnormalities of ventricles/subarachnoid cerebrospinal fluid space	*Skull abnormalities*	*Occipital frontal head circumference, cm*	*Height cm*	*Brain imaging*		
				MRI	*CT*	*US*
Enlargement: lateral ventricles (abnormal angle of collateral trigones), 4th ventricle widened (sagittal plane; MRI)		44.5, below –2SD[b]	101, –3SD	+	+	
	Skull flattened form	47.5, –2SD[c]	102,just below –3SD		+	+
Hydrocephalus: lateral (CT, US), 3rd (CT, US), 4th ventricles (MRI, CT), prepontine cistern (MRI)		51.5, –1.5SD	112, –2SD	+	+	+
Enlargement: 4th ventricle, posterior fossa cisterns, suprasellar cisterns (MRI)		51, below –2SD	129, below –2SD	+	+	
	5–6mo: susp craniosynostosis	50, below –2SD	161, –1SD		+[d]	+[d]
		48, –2SD	94.5, –3SD	+	+	
Enlargement: lateral, 3rd, 4th ventricles, subarachnoid cisterns (CT). Asymmetry: left lateral ventricle, subarachnoid cisterns more prominent (CT)	Craniosynostosis. Small posterior fossa. *Left half of face markedly smaller*	51.3, just below 0SD	110, –2SD		+	+
Susp. enlargement: intracerebral ventricles (MRI, CT)	Marked skull asymmetry	55.5, just below 0SD	152, –2SD		+	+
Hydrocephalus; intracerebral ventricles, basal, and subarachnoid cisterns (MRI, CT)	Craniostenosis Small posterior fossa	54, just below 0SD	158, –1SD	+	+	+
Enlargement: intracerebral ventricles (MRI). Asymmetry: left lateral ventricle prominent (MRI)		55, just below 0SD	157, just below –1SD	+	+	
		49, –0.5SD	83, –2.5SD	+	+	
		52, 0.5SD	88, –3SD	+		+
Widened subarachnoid cisterns (CT)		52, –0.5SD[c]	124, –2SD		+	
		[e]	[e]		+	
		51, –0.5SD	110, below –2SD		+	
Enlargement, asymmetry: subarachnoid cistern surrounding left cerebellar hemisphere (MRI), left lateral ventricle (MRI). Enlargement: cerebellomedullary cistern, cistern of chiasma (CT)	Asymmetric skull bracycephalic form. Craniosynostosis	51, just below –1SD	128, –2SD	+	+	
		54, 0.5SD	136, –1SD	+	+	
[f]	[f]	50.5, –0.5SD	105, below –2SD			
Enlargement: subarachnoid cisterns (CT) Asymmetry: posterior horns of lateral ventricles (CT)		54, just below 0SD	136, –1SD		+[d]	+
Widening: foramen of Magendie (CT)		50, below –2SD	133, below –2SD	+	+	
		49.5, –0.5SD	78cm, below –3SD		+	
[f]	[f]	[e]	165, –0.5SD			
[f]	[f]	55, just above 1SD	145, just above 0SD			
Enlargement: supracerebral subarachnoid cisterns (MRI)		48.5, –1.5SD	90, –3SD	+[g]		
[f]	[f]	49, just below –2SD	92.5, –3SD			+
Enlargement: intracerebral ventricles, supracerebral subarachnoid cisterns (MRI, CT)	Asymmetric skull, occipital areas of defective bone tissue	40.5 (9mo), below –3SD[h]	60.5 (9mo), below –3SD	+[h]	+[h]	+
		38, –1.5SD	58, –1SD	+[h,i]	+[h,i]	+
Asymmetry: lateral ventricles (US)	Frontal fossa (lamina cribrosa) low position (MRI, CT). Susp. area of defective bone tissue in frontal base of the skull	45, 1SD	66, –1SD	+[h,i]	+[h,i]	+
		47.5, below –2SD	100, below –2SD	+[d]	+[d]	
Enlargement: intracerebral ventricles, cerebellomedullary cistern(CT)		50. (8y), below –2SD	117 (8y), below –2SD		+	+
Enlargement: 4th ventricle (MRI, CT), foramen of Magendie (CT)		55.5, just below –0.5SD	177, 0SD	+[d]	+	

[i]Radiological examination mainly focused on posterior fossa and temporal bones. –, not present or not performed; ? possible; [*]patient too young or too sensorily disabled; CA, childhood autism; AD, autistic disorder; PLD, profound learning disability (IQ<20); MRI, magnetic resonance imaging; CT, computed tomography; US, ultrasound scan; MLD, mild learning disability (IQ 50 to 69); ALC, autistic-like condition; NA, near average intelligence (IQ 70 to 85); SLD, severe learning disability (IQ 20 to 49); AT, autistic traits; A, average intelligence (IQ>85).

Mental Disorders 4th edition (DSM-IV; American Psychiatric Association 1994) checklist for autistic disorder. The Autism Diagnostic Interview – Revised (ADI-R; Lord et al. 1994) and the Diagnostic and Statistical Manual of Mental Disorders 3rd edition revised (DSM-III-R; American Psychiatric Association 1987) checklist for autistic disorder were completed independently by another investigator. Childhood autism (CA)/autistic disorder (AD) was diagnosed in participants who clinically met the DSM-III-R and the DSM-IV criteria for AD and also the ADI-R algorithm criteria for CA. Autistic-like condition (ALC)/atypical autism was diagnosed in patients showing severe impairments in social interaction and restricted communication and/or behaviour, meeting six or seven of the DSM-III-R symptom criteria and five of the DSM-IV symptom criteria for AD. Autistic traits (AT) were diagnosed in patients showing severe impairments in social interaction and restricted communication and/or behaviour, meeting three to five of the DSM-III-R symptom criteria and three or four of the DSM-IV symptom criteria for AD. The concepts of 'pervasive developmental disorder, not otherwise specified' and Asperger syndrome overlap to some degree with the terms AT, ALC, and atypical autism.

RADIOLOGICAL IMAGING AND LABORATORY TESTS

Magnetic resonance imaging (MRI)/computed tomography (CT)/ultrasound scan or laboratory tests were not undertaken during the diagnostic study but had been performed previously in most patients. Results from radiological imaging, performed after the study, were obtained in three infants (Table II). Results from radiological imaging of the temporal bone/inner ears were available in nine patients (information concerning the facial nerve canal in six of these). Results from chromosomal analysis were collected in 25 patients and from DNA analysis (fluorescence *in situ* hybridization [FISH]) in six individuals.

HEAD CIRCUMFERENCE/HEIGHT

Swedish normative values for height are available from birth to 16 years of age (Karlberg et al. 1976), and for head circumference up to 2 years of age (Karlberg et al. 1988). For older children, normative values for Norwegian children (Knudtzon et al. 1988) were used, and centile values were transformed into SD values. Values of less than 2.5 centiles were approximated to 'just below' or 'below' or –2 SD.

PHYSICAL TESTS

Two paediatric ophthalmologists examined anterior and posterior eye-segments, visual function, and eye movements. An otologist performed a rotation/caloric test and diagnosed ear malformations. Audiometry had been performed before the study.

ANALYSIS OF PRENATAL NON-OPTIMAL CONDITIONS

Information was obtained from parental questionnaires and maternal health care/delivery unit records on prenatal conditions of participants.

STATISTICAL ANALYSIS

Pitman non-parametric tests (Good 2000) were applied in analyzing the correlation between several background factors and the severity of ASD/level of LD (see Table IV for clarification). Two-tailed tests were used.

Results

AUTISM SPECTRUM DISORDERS/ASDS

Seventeen individuals (of those 25 evaluated regarding ASD) had ASD (Table IV).

CA/AD

Five patients of the 25 met the study criteria for CA/AD. They all had sufficient hearing and/or vision to enable some social interaction. At the time of the diagnostic study the clinical picture of one 5-year-old male resembled Kanner autism. Three of the patients with CA/AD were, during the first years of life, very aloof and avoided contact with people. During later preschool years they seemed to appreciate carers but still gave very limited contact and only tried to get other people's attention to get help. One 16-year-old female had mild LD (MLD; IQ 50 to 69) and occasionally engaged in social interaction. Four individuals with CA/AD had profound LD (PLD; IQ<20).

ALC

Five out of 25 patients had ALC. One male had near average intelligence (NA; IQ 70 to 85), severe bilateral hearing deficit, and no verbal communication. He fulfilled the ADI-R algorithm criteria for CA/AD, as well as five DSM-IV and five DSM-III-R symptom criteria for AD. In his case the DSM-III-R criteria concerning abnormalities in intonation and volume were omitted. Except for one female with PLD, whose vision had deteriorated during the year before the study, all patients with ALC had sufficient vision and/or hearing to enable social interaction.

AT

AT was diagnosed in 7 out of 25 patients. None of them had severe impairment of both hearing and vision. One female had previously been diagnosed with attention-deficit disorder and AT; another had previously been diagnosed with attention-deficit–hyperactivity disorder and AT (Table V).

OTHER NEUROPSYCHIATRIC PROBLEMS

Three individuals exhibited impairments in social interaction, communication, or behaviour consistent with impairments within the autism spectrum, although too few and too mild at the time of the study for a diagnosis of ASD (Table IV). Two of these had NA and frequent vocal and/or motor tics.

BEHAVIOURAL DISTURBANCES

Self-injury was correlated to severity of ASD ($p<0.05$) and level of LD ($p<0.05$; Table V). In two patients who were deaf and blind and four females with marked impairment of vision and/or hearing and ASD, the self-destructive behaviour had caused serious physical damage. Hyperactivity was significantly correlated with level of LD ($p<0.05$) but not with severity of ASD. Attention deficit, deficits of impulse control, sleeping problems, and aggressive behaviour were common but were not significantly correlated with severity of ASD/level of LD.

UNUSUAL RESPONSES TO STIMULATION OF SENSORY/AUTONOMIC SYSTEM

Indifference to pain was correlated with both severity of ASD ($p<0.05$) and level of LD ($p<0.05$; Table V). One female with CA/AD and severe hearing impairment had previously shown oversensitivity to noises, accompanied by clear behavioural problems. One female with AT and one male with some autistic features but no diagnosis of ASD were described to have

extremely good skills of visual recognition. Profuse perspiration was reported in three patients.

LD

Twenty-two out of the 28 individuals evaluated had LD

Table III: Occurrence of cranial nerve dysfunction, facial nerve palsy, course of facial nerve canal in temporal bone, middle and inner ear anomalies, balance disorder, and vestibular anomaly in diagnostic groups

Characteristic	*CA/AD n=5*	*ALC n=5*	*AT n=7*	*AT? n=3*	*No ASD n=5*	*DB n=3*	*<2y n=3*	*All patients examined*
Cranial nerve dysfunction	3	3	4	2	2	3	–	17/31
Facial palsy[a]	2	2	2[b]	1	2[b]	3	–	12/31
Anomalous course of facial nerve canal	–	–	2	–	–	1	2	5/6
Middle ear anomaly	2	1	2	1	1	–	–	7/31
Inner ear anomaly	5	5	2	2	2	3	2	21/31
Balance disorder	4	3	6	3	4	1	*	21/24
Vestibular anomaly	2 (2RC)	4 (2RI, 2RC)	4 (2RI, 2RC)	1 (RC)	1 (RC)	3 (2RI, 1RC)	2 (2RI)	17/31 (8RI, 9RC)

[a]Unilateral in all patients; [b]One additional patient was previously diagnosed with facial palsy but did not show this at the time of the study; –, not present; ?, possible; *, too young; CA, childhood autism; AD, autistic disorder; ALC, autistic-like condition; AT, autistic traits; ASD, autism spectrum disorder; DB, deaf–blind; RC, indicated by rotation/caloric test; RI, indicated by radiological examination.

Table IV: Distribution of ADI-R subdomain, CARS, Autistic Behaviour Checklist, DSM-IV, and DSM-III-R scores in diagnostic groups

Factor	*CA/AD n=5*	*ALC n=5*	*AT n=7*	*AT? n=3*	*No ASD n=5*	*DB n=3*	*<2y n=3*	*All patients n=31*
ADI-R[a]							*	
Social								
<10	–	1	5	3	5	3		17
≥10	5	4	2	–	–	–		11
Communication								
<8V, <7NV	–	2	6	3	5	3		19
≥8V, ≥7NV	5	3	1	–	–	–		9
Behaviour								
<3	–	–	1	3	5	–		9
≥3	5	5	6	–	–	3		19
Onset <3yrs								
<1		1	1	2	4	–		8
≥1	5	4	6	1	1	3		20
CARS[b]							*	
<30	–	3	7	3	5	3		21
30–36	1	2	–	–	–	–		3
≥37	4	–	–	–	–	–		4
ABC[c]							*	
<45	–	1	1	3	5	1		11
45–66	2	2	5	–	–	2		11
≥67	3	2	1	–	–	–		6
DSM-IV[d]							*	
<3	–	–	–	3	5	–		8
3–4	–	–	7	–	–	3		10
5	–	4	–	–	–	–		4
≥6	5	1	–	–	–	–		6
DSM-III-R[e]							*	
<3	–	–	–	1	5	–		6
3–5	–	1	6	2	–	2		11
6–7	–	4	1	–	–	1		6
≥8	5	–	–	–	–	–		5

[a]Autism Diagnostic Interview-Revised (ADI-R) cut-off scores: social, 10; communication verbal (V), 8; communication non-verbal (NV), 7; behaviour, 3; onset, 1. Autism, according to ADI-R-algorithm, is diagnosed when cut-off score is exceeded in all four subdomains; [b]Childhood Autism Rating Scale (CARS) cut-off scores: 30 to 36, mild autism; ≥37, severe autism; [c]Autistic Behaviour Checklist (ABC) cut-off score in original study (Krug et al. 1980): 53 to 66, questionable autism; ≥67 autism with high probability. Nordin and Gillberg (1996) proposed a cut-off score of ≥45 in individuals with cognitive and/or physical disabilities; [d]Diagnostic and Statistical Manual of Mental Disorders 4th edition (DSM-IV) study criteria: 3 to 4, AT; 5, ALC; ≥6, CA/AD. For statistical analyses the DSM-IV diagnostic classification system was used for subdividing autism spectrum disorder (ASD) into four levels of severity: 0 = no criteria met; 1=autistic traits (AT) and AT?; 2=autistic-like condition (ALC); 3=childhood autism/autistic disorder (CA/AD); [e]DSM-III-R study criteria: 3 to 5, AT; 6 to 7, ALC; ≥8, CA/AD.–, not present; ?, possible; *, too young; DB, deaf–blind.

(Table VI). Nine patients had PLD, three severe LD (SLD; IQ 20 to 49) and 10 MLD. Six patients had intelligence in the normal range; five of these had NA.

RELATIONSHIP BETWEEN INTELLECTUAL LEVEL AND DIAGNOSIS OF ASD

Level of LD and degree of ASD were highly correlated ($p<0.001$). Apart from a male with ALC and NA, all the patients with severe autistic symptoms also had LD. Sixteen out of the 19 patients with LD who could be assessed for ASD had ASD.

CEREBRAL ABNORMALITIES

Structural brain abnormalities were indicated in 15 out of the 27 patients examined (Table II). Forebrain midline abnormalities were most common; agenesis/hypogenesis/abnormal configuration of corpus callosum ($n=5$), septum pellucidum ($n=1$; CT with ultrasound scan), the hypophysis-sella/hypothalamus region ($n=4$), and the optic chiasm ($n=2$). Neither severity of ASD nor level of LD was significantly correlated with 'white/grey matter abnormalities collapsed'/forebrain midline abnormalities. However, both 'white/grey matter abnormalities collapsed' and forebrain midline abnormalities occurred more frequently in those with CA/AD/ALC than in the those with AT/AT?/no autistic symptoms: in 8/10 with CA/AD/ALC versus 5/11 with AT/AT?/no autistic symptoms (ns); in 5/10 with CA/AD/ALC versus 3/11 with AT/AT?/no autistic symptoms (ns). Hindbrain abnormalities ($n=3$) were not correlated with severity of ASD/level of LD/forebrain midline abnormalities. Agenesis of the cerebellar vermis was indicated by CT in a female with normal MRI and a markedly

Table V: Occurrence of behavioural and sensory disturbances in diagnostic groups

Characteristic	*CA/AD n=5*	*ALC n=5*	*AT n=7*	*AT? n=3*	*No ASD n=5*	*DB n=3*	*<2y n=3*	*All patients examined n=28*
Attention deficit	1/5	2/5	5/7[a]	2/7	–	–	*	10/28
Impulsive behaviour	–	2/5	–	–	1/5	–	*	3/28
Hyperactivity	4/5	5/5	5/7	1/3	1/5	2/3	*	18/28
+	2	1	1	–	1	1		6
++	1	3[b(2)]	3	–	–	1[b]		8
+++	1	1[b]	1	1	–	–		4
Self-destructivity	4/5	3/5	5/7	–	–	3/3	*	15/28
+	1	2	3[b(1)]			1		7
++	1	–	1			–		2
+++	2	1	1			2		6
Rituals/routines	4/5	5/5	7/7	1/3	–	2/3	*	19/28
+	1	2	3	–		1		7
++	1	–	4	1		1		7
+++	2	3[b(1)]	–	–		–		5
Stereotyped body movements	5/5	3/5	3/7	–	–	3/3	*	14/28
+	–	1[b]	–			–		1
++	3	2	3[b(1)]			1		9
+++	2[b(2)]	–	–			2		4
Aggressive behaviour	5/5	4/5	6/7	1/3	–	3/3	*	19/28
+	4	2	3	–		2		11
++	1	–	3	1		1[b]		6
+++	–	2	–	–		–		2
Sleep disturbances[c]	2/5	3/5	–	–	1/5	2/3	*	8/28
Tics	–	–	–	2/3[d]	–	–	*	2/28
Unusual interest in auditory stimuli	3/5	1/5	–	–	–	–	*	4/28
Oversensitivity to noise[e]	1/5	1/5	3/7	–	–	–	*	5/28
+	–	–	1					1
++	1[b]	1	2[b(1)]					4
Unusual interest in visual stimuli	4/5	4/5	3/7	–	–	–	*	11/28
Extremely good visual recognition	–	–	1/7	1/3	–	–	*	2/28
Unusual interest in smell, taste	4/5	2/5	2/7	–	–	2/3	*	10/28
Insensitivity to pain	4/5	4/5	4/7	1/3	–	2/3	*	15/28
Profuse perspiration	1/5	1/5	–	–	1/5	-	*	3/28

Classification of severity of behavioural and sensory impairments is based on Autism Diagnostic Interview-Revised (ADI-R), Childhood Autism Rating Scale (CARS) and Autistic Behaviour Checklist (ABC) results, other parental information and information in medical records. +, ADI-R score 1 and/or CARS 1 or 2/and/or present according to ABC; ++, ADI-R score 2 and/or CARS score 3 and/or present according to ABC- to a considerable degree according to parental information/medical records; +++, ADI-R score 3 and/or CARS score 4 and/or present according to ABC - to an extreme degree according to parental information/medical records. [a]One patient in 'autistic traits (AT)-group' previously diagnosed with attention-deficit disorder, another with attention-deficit–hyperactivity disorder; [b]Severity of previously occurring behaviour given because more pronounced than current behaviour; number in parentheses indicates number of patients in which severity of previous behaviour is given; [c]Requiring medical treatment or medical consultation; [d]According to parental information meeting the A, B, D and E, but not the C criteria for Tourette syndrome/chronic motor tics; [e]Patients who did not use a hearing aid. –, not present; ?, possible; *, too young; CA, childhood autism; AD, autistic disorder; ALC, autistic-like condition; ASD, autism spectrum disorder; DB, deaf–blind.

asymmetrical electroencephalogram. Widened cerebral fissures ($n=4$)/small cerebral hemispheres ($n=1$)/reduced white matter ($n=2$) indicating hypoplasia/atrophy, cerebral ($n=2$)/cerebellar asymmetry ($n=1$) and enlarged cerebrospinal fluid spaces ($n=15$) were also recorded. Head circumference was less than or equal to –2SD in 10 patients, and occurred more frequently in those with CA/AD/ALC, than in those with AT/AT?/no ASD (5/10 compared with 2/13; ns). Height as measured in SDs was significantly correlated with head circumference as measured in SDs ($p<0.005$).

Table VI: Autism spectrum disorder and different background factors in diagnostic groups

Parameter	*CA/AD n=5*	*ALC n=5*	*AT n=7*	*AT? n=3*	*No ASD n=5*	*DB n=3*	*<2y n=3*	*All patients n=31*
Age (y:m)								
Range	5:4/16:8	4:4/17:11	2:6/11:3	6:1/13:1	2:4/16:3	6:9/31:7	0:1/0:6	0:1/31:7
Mean	9:10	12:8	7:6	9:11	7:4	15:11	0:3	8:11
Sex ratio (M:F)	2:3	2:3	2:5	1:2	4:1	3:0	1:2	15:16
Cognitive level								
A	–	–	–	–	1	–	*	1
NA	–	1	–	2	2	–	*	5
MLD	1	1	5	1	2	–	*	10
SLD	–	1	2	–	–	–	*	3
PLD	4	2	–	–	–	3	*	9
Visual impairment	5/5	3/5	3/7	1/3	2/5	3/3	2/3	19/31
VI	1	–	1	–	–	–	–	2
PSVI	1	2	1	–	2	–	2	8
SVI	3	1	1	1	–	3	–	9
Hearing impairment, mean (SD; dB)	72 (16.4)	74 (25.1)	63 (33.0)	53 (32.1)	45 (16.6)	98 (2.9)	67 (41.6)	63 (29.0)

–, not present; ?, possible; * too young; CA, childhood autism; AD, autistic disorder; ALC, autistic-like condition; AT, autistic traits; ASD, autism spectrum disorder; DB, deaf–blind; M, male; F, female; A, average intelligence (IQ>85); NA, near average intelligence (IQ 70 to 85); MLD, mild learning disability (IQ 50 to 69); SLD, severe learning disability (IQ 20 to 49); PLD, profound learning disability (IQ<20); VI, visual impairment, visual acuity≤0.3 (20/60 vision); PSVI, probably severe visual impairment (vision difficult to assess because of other major impairments); SVI, severe visual impairment, visual acuity≤0.1 (20/200 vision); dB, decibel.

Table VII: Occurrence of non-optimal prenatal conditions related to clinical data in diagnostic groups

Condition	*CA/AD n=5*	*ALC n=5*	*AT n=7*	*AT? n=3*	*No ASD n=5*	*DB n=3*	*<2y n=3*	*All patients n=31*
First-trimester vaginal bleeding	–	2/5[a(1)]	3/7	–	–	–	1/3[a]	6/31
Previous spontaneous abortions	1/5	3/5	2/7	2/3	–	1/3	1/3	10/31
1	1	1	1	2	–	1	1	7
2	–	1	1	–	–	–	–	2
3	–	1	–	–	–	–	–	1
Previous extrauterine pregnancy	1/5	–	–	–	–	–	–	1/31
Assisted reproduction	–	–	–	–	1/5	–	2/3	3/31
ICSI	–	–	–	–	–	–	2	2
Pregnancy preceded by treatment with ovulation-stimulating hormones	–	–	–	–	1	–	–	1
Duplex pregnancy with spontaneous abortion of one twin	–	–	–	–	–	–	1/3	1/31
Fetal diagnostics	–	–	1/5	–	–	–	1/5	2/31
Amniocentesis	–	–	1	–	–	–	–	1
Several utrasonographies	–	–	–	–	–	–	1	1
Category C drugs[b]	–	1/5[c]	1/7[d]	–	1/5[c]	1/3[e]	1/3[f]	5/31
Smoking throughout pregnancy (5 to 15 cigarettes per day)	2/5	2/5	4/7	–	–	1/3	–	9/31
Maternal disorders	4/5	3/5	7/7	2/3	3/5	3/3	2/3	24/31
Diabetes mellitus	1	–	1	–	–	–	–	2
Gestational diabetes	–	–	–	1	–	–	–	1
Other maternal disorders[g]	3	3	6	1	3	3	2	21

[a]Progesterone treatment; number in parentheses indicates number of patients in which this occurred; [b]Drugs that have caused or are suspected to be risk factors for foetus and/or newborn infant without directly causing malformations; [c]Acetylsalisylic acid; [d]Ipren; [e]Sulfonamid; [f]Cetobemidone, Hydromorphone; [g]Respiratory tract infection, urinary tract infection, gastrointestinary infection, fever of unknown cause, hypertension, renal calculus, asthma. –, not present or not performed; ?, possible; AD, autistic disorder; ALC, autistic-like condition; ASD, autism spectrum disorder; AT, autistic traits; CA, childhood autism; DB, deaf–blind; ICSI, intracytoplasmic sperm injection.

Severity of ASD and level of LD were not significantly correlated with either head circumference or height as measured in SDs. Head circumference was 1SD or more below the SD for height in two patients with CA/AD.

CRANIAL NERVE AND VESTIBULAR DYSFUNCTION, VISUAL, AND HEARING DEFICITS

Neither the severity of ASD nor the level of LD was correlated with cranial nerve dysfunction (cranial nerve [N] VII, $n=10$; N VIII, $n=17$; N X, $n=1$) or isolated N VII palsy (Table III). CT/MRI examination showed an abnormal or unidentifiable course of the facial nerve canal in five out of six patients. One of these had facial nerve palsy, two had no facial palsy at the time of the study, of whom one had previously been diagnosed with this, and two were infants. One female with facial nerve palsy had a normal facial nerve canal on radiological examination. Twenty-one patients had disturbance of balance, including atactic gait. Seventeen individuals were judged to have vestibular anomaly. Visual impairment was independently correlated with severity of ASD ($p<0.05$) and level of LD ($p<0.001$). Hearing impairment was independently and significantly correlated with severity of ASD ($p<0.05$), but not with level of LD (Table VI).

ROUTINE CHROMOSOMAL ANALYSIS AND FISH TEST

Routine chromosomal analysis ($n=25$) and FISH test ($n=6$) were normal in all patients for whom data were available.

ADVERSE PREGNANCY EVENTS

See Table VII; further details are given in Strömland et al. (2005). About two-thirds of the mothers reported ingestion of two or more drugs and/or infections. First-trimester vaginal bleeding ($n=6$), several previous spontaneous abortions ($n=3$), ingestion of drugs that are, or are suspected to be, risk factors for the fetus and/or the newborn without directly causing malformations ($n=5$), poorly controlled diabetes mellitus/gestational diabetes ($n=3$), and smoking throughout pregnancy ($n=9$) were recorded. Two infants were born after *in vitro* fertilization by intracytoplasmic sperm injection and one child was born after treatment of the mother with ovulation-stimulating hormones. One mother reported heavy alcohol consumption during the sixth gestational week.

Discussion

The present study is not population-based and the sample size was relatively small. The mode of recruitment might have contributed to severe cases being over-represented. Given this, our sample might not be representative. Nevertheless, our cases are typical of other CHARGE cases in the literature, in both systemic and brain malformations. To our knowledge this is possibly the most comprehensive report in the field so far, particularly with regard to the neuropsychiatric work-up.

Our data suggest that ASD is much more common in CHARGE than in the general population. More than one-third of all patients examined met criteria for CA/AD/ALC, and half of them were typical cases of CA/AD. In the general population the rate of CA/AD is about 0.1 to 0.2% and the rate of all ASDs 0.5 to 1.0% (Gillberg and Wing 1999). Hence, in our CHARGE group, the rate of autism/ASD was at least 50-fold greater than in the general population. These findings suggest a strong association between ASD and CHARGE. LD is strongly associated with CA/AD. However, only about 25% of those with an IQ of less than 50 have ASD (Nordin and Gillberg 1996). In the present study, all patients with an IQ of less than 50 who were evaluated regarding autism had ASD.

Besides LD, sensory impairments and nerve palsies aggravate the identification of ASD in CHARGE. In this series the severity of ASD was correlated with both visual and hearing deficits. There are theories that both blindness and profound hearing impairment can cause autistic-like features. Children who are blind and deaf, raised in a spoken-language environment, perform less well in 'autism sensitive' tests measuring 'theory of mind' (Minter et al. 1998, Peterson and Siegal 1999). 'Blindisms' (eye-poking, finger flicking, motor stereotypies) in severely visually impaired children have been interpreted as being due to a loss of sensory input and obscured expressive ability. However, there is evidence that both blindness and hearing impairment delay rather than prevent the development of a theory of mind (Hobson 1993, Peterson and Siegal 1999). According to Hindley (2000), social aloofness, lack of interest, delay in development of play, rituals, and stereotypies distinguish children with hearing impairment and autism from other children who have hearing impairment only. In retinopathy of prematurity, Ek et al. (1998) found autism to be strongly associated with cerebral damage rather than blindness. Autism, reported in children with congenital rubella embryopathy, a combination of severe visual and hearing impairment and LD, is likely to be caused by brain damage. Hypothetically, both visual and hearing impairment (as well as ASD) might be markers of brain damage in CHARGE.

Diagnostic difficulties pertaining to other disabilities were taken into account. In some patients with severe disabilities other than ASD, some ADI-R, CARS, and ABC items were difficult to score and were, therefore, omitted. The sensitivity of the ADI-R, the ABC, and the CARS is lower in individuals with higher IQ and milder ASD. Our results, therefore, might reflect an underestimation of autistic symptoms in the more able individuals and an overestimation in the low-functioning patients, who also had the most severe sensory deficits.

Six out of the 10 patients with CA/AD/ALC had PLD and one SLD. At the lower end of the intelligence range, the prevalence of the most severe ASDs increases. Fundamental social skills have been considered as the key in the diagnosis of autism, especially in individuals with underlying medical conditions, because communication deficits and repetitive behaviours occur in a variety of developmental disabilities. All our patients with CA/AD/ALC showed very limited social interaction and considerable behavioural disturbances (Table V), in which the role of sensory deficits is uncertain. However, behaviour problems in individuals with PLD without severe social impairment are mostly mild, whereas they are often severe in aloof persons with PLD (Wing 1981).

One-quarter of the patients were functioning in the low average/normal range, which supports recent reports of extremely variable cognitive functioning in CHARGE. Significant correlation of LD with visual impairment but not with hearing deficit in CHARGE was also described by Raqbi et al. (2003).

No or limited information from radiological examinations was available in some patients (Table II). The recorded rate of brain malformations is, therefore, probably underestimated. In most previous reports of microcephaly in CHARGE, it is not clear whether growth retardation was controlled for. However, Oley et al. (1988) reported microcephaly, defined in relation to height and weight, in five out of 20 patients.

Considering the retardation of growth and development in CHARGE and that head circumference, compared with the overall body size, decreases with age during childhood, perhaps the 'normal' head circumference in these individuals would be 'macrocephalic'.

As brain abnormalities occurred in the vast majority of our patients, and more frequently in those with ASD, it does not seem likely that autism or LD could have been accounted for purely by sensory impairments. This, coupled with the fact that most of those with LD had ASD, indicates a specific link between CHARGE and ASD.

THEORETICAL SPECULATIONS

Neural crest cells are considered to be derived from cells in the midline of the early neural tube and pass positional values from the hindbrain to peripheral structures. There is radiological evidence for hindbrain abnormalities and brainstem dysfunction, namely of cranial nerves, in CHARGE, Möbius sequence, and Joubert syndrome (Menenzes and Coker 1990, Byerly and Pauli 1993, Johansson et al. 2001). Cranial nerve dysfunction has been associated with teratogens during early gestation in thalidomide embryopathy and Möbius sequence (Strömland et al. 1994, Bandim et al. 2003). These circumstances suggest embryologic maldevelopment of hindbrain structures in ASD in CHARGE.

Radiological imaging of the brainstem had not been performed in our patients. A peripheral cause of cranial nerve dysfunctions was indicated by aplastic or hypoplastic semicircular canals and an anomalous course of, or difficulty in identifying, the facial nerve canal in five out of six patients examined (four cochlear implantation candidates and two infants, thus possibly a subgroup of those with the most severe hearing loss). However, five patients with balance disorder (inner ears not examined with MRI/CT) had no hearing or vestibular impairment. Future radiological imaging of the brainstem should explore the role of brainstem anomalies in CHARGE.

Fewer individuals were evaluated for ASD than for LD; there were, therefore, smaller numbers in the statistical analyses of the former. Numbers in subgroups were too small for multivariate statistics. Given the finding of frequent forebrain midline anomalies in autism, the pineal–hypothalamic–pituitary–adrenal axis, previously discussed in the pathogenesis of autism (Chamberlain and Herman 1990), might be considered in the pathogeneses of ASD in CHARGE. These regions control emotions, behaviour, growth, feeding, sleep, warmth, fluid regulation, and gastrointestinal function. There is evidence for a disturbed neural network in autism, involving the temporoparietal/prefrontal cortex, the limbic system (anatomically or physiologically closely related to the hypothalamus), cerebellum, and corpus callosum.

No common aetiological factor was found. However, some possible risk factors during pregnancy, possibly indicating some kind of vulnerability (maternal, paternal or fetal, possibly inherited), were identified. Vaginal bleeding has been associated with autism (Gillberg and Coleman 2000) and Möbius sequence (Johansson et al. 2001). More than one previous spontaneous abortion was observed in 10% of mothers, which is higher than the rate (3%) found in a control series (Hagberg et al. 1988). Poorly controlled maternal diabetes has been suggested to interfere with cephalic neural crest migration. Grix et al. (1982) reported infants who fulfilled diagnostic CHARGE criteria born to diabetic mothers. Maternal smoking is also a recognized teratogen. The increase in anomalies after *in vitro* fertilization, described by some authors, has been explained by parental factors causing the infertility.

There is evidence for midline vulnerability during the embryonic stage. Alcohol exposure compromises the development of the neural tube midline, midline neurons (such as the raphe), septal nuclei, and the crossing of commissural fibres in mice (Zhou et al. 2003). Special facial characteristics of fetal alcohol syndrome and midline anomalies of the cardiac septum and brain structures may be consequences of embryological midline vulnerability. 'Organizing centres' in the developing neural tube midline produce signalling factors involved in the specification or differentiation of neuronal cell types, Homeobox genes (*Hox, Gbx, Otx,* and *Pax*) functioning in early brain development and coding 'downstream targets', and early neural regulatory molecules, such as retinoic acid, may be targets of teratogenic agents, leading to the disruption of neural tube development. Alleles of the *Hoxa1* and *Gbx2* genes have been shown to occur more often in individuals with ASDs (Rodier 2004). The association of a mutation in a chromodomain gene with CHARGE (Vissers et al. 2004) does not validate our conclusions. However, not all CHARGE cases are likely to be genetic, and even if important genetic risk factors are present, environmental and 'cascading' factors might be important.

Systemic as well as brain anomalies in this series seem to be developed during the fourth to sixth gestational week. Cerebral midline structures (the commissural plate, and the adenohypophyseal and chiasmatic primordiums) and the oropharyngeal membrane appear during the fourth to fifth embryonic week in humans (Müller and O'Rahilly 2003). In autism, autopsy and MRI studies have indicated abnormalities in structures in which neurogenesis occurs during approximately the fifth gestational week (the facial nerve nucleus, the brainstem, the median septum, and the amygdaloid nucleus of the limbic system; Gillberg and Coleman 2000).

Conclusions

The occurrence of ASD in more than half of the patients indicates that ASD is common in CHARGE. Awareness of this associated symptomatology is important in the habilitation of children with CHARGE. The brain and systemic anomalies in these patients with ASD suggest that autism might in some cases be associated with errors in embryonic brain development.

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List of abbreviations

ABC	Autistic Behaviour Checklist
AD	Autistic disorder
ADI-R	Autism Diagnostic Interview – Revised
ALC	Autistic-like condition
ASD	Autism spectrum disorder
AT	Autistic traits
CA	Childhood autism
CARS	Childhood Autism Rating Scale
FISH	Fluorescence *in situ* hybridization
MLD	Mild learning disability; IQ 50–69
NA	Near average intelligence; IQ 70–85
PLD	Profound learning disability; IQ < 20
SLD	Severe learning disability; IQ 20–49

Attachment 11

Melatonin in autism spectrum disorders: a systematic review and meta-analysis

DANIEL A ROSSIGNOL[1] | RICHARD E FRYE[2]

1 International Child Development Resource Center, Melbourne, FL, USA. **2** Division of Child and Adolescent Neurology and Children's Learning Institute, Department of Pediatrics, University of Texas Health Science Center at Houston, Houston, TX, USA.

Correspondence to Dr Daniel Rossignol at 3800 West Eau Gallie Blvd, Melbourne, FL, 32934, USA. Email: rossignolmd@gmail.com

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ABBREVIATIONS
ASD Autism spectrum disorders
ASMT Acetylserotonin methyltransferase
6-SM 6-Sulphatoxymelatonin

AIM The aim of this study was to investigate melatonin-related findings in autism spectrum disorders (ASD), including autistic disorder, Asperger syndrome, Rett syndrome, and pervasive developmental disorders, not otherwise specified.

METHOD Comprehensive searches were conducted in the PubMed, Google Scholar, CINAHL, EMBASE, Scopus, and ERIC databases from their inception to October 2010. Two reviewers independently assessed 35 studies that met the inclusion criteria. Of these, meta-analysis was performed on five randomized double-blind, placebo-controlled studies, and the quality of these trials was assessed using the Downs and Black checklist.

RESULTS Nine studies measured melatonin or melatonin metabolites in ASD and all reported at least one abnormality, including an abnormal melatonin circadian rhythm in four studies, below average physiological levels of melatonin and/or melatonin derivates in seven studies, and a positive correlation between these levels and autistic behaviors in four studies. Five studies reported gene abnormalities that could contribute to decreased melatonin production or adversely affect melatonin receptor function in a small percentage of children with ASD. Six studies reported improved daytime behavior with melatonin use. Eighteen studies on melatonin treatment in ASD were identified; these studies reported improvements in sleep duration, sleep onset latency, and night-time awakenings. Five of these studies were randomized double-blind, placebo-controlled crossover studies; two of the studies contained blended samples of children with ASD and other developmental disorders, but only data for children with ASD were used in the meta-analysis. The meta-analysis found significant improvements with large effect sizes in sleep duration (73min compared with baseline, Hedge's g 1.97 [95% confidence interval {CI} CI 1.10–2.84], Glass's Δ 1.54 [95% CI 0.64–2.44]; 44min compared with placebo, Hedge's g 1.07 [95% CI 0.49–1.65], Glass's Δ 0.93 [95% CI 0.33–1.53]) and sleep onset latency (66min compared with baseline, Hedge's g −2.42 [95% CI −1.67 to −3.17], Glass's Δ −2.18 [95% CI −1.58 to −2.76]; 39min compared with placebo, Hedge's g −2.46 [95% CI −1.96 to −2.98], Glass's Δ −1.28 [95% CI −0.67 to −1.89]) but not in night-time awakenings. The effect size varied significantly across studies but funnel plots did not indicate publication bias. The reported side effects of melatonin were minimal to none. Some studies were affected by limitations, including small sample sizes and variability in the protocols that measured changes in sleep parameters.

INTERPRETATION Melatonin administration in ASD is associated with improved sleep parameters, better daytime behavior, and minimal side effects. Additional studies of melatonin would be helpful to confirm and expand on these findings.

Autism spectrum disorders (ASD) are a heterogeneous group of neurodevelopmental disorders that share a common behavioral definition. ASD is defined by impairments in communication and social interaction accompanied by restrictive and repetitive behaviors.[1] An estimated one out of 110 individuals in the United States is currently affected with ASD.[2] Some studies have reported that children with ASD, as a group, have a higher prevalence of sleep abnormalities than typically developing children,[3–11] with a prevalence ranging from 40% to 86%.[7,8,12–14] These abnormalities include taking longer to fall asleep (longer 'sleep onset latency'),[15–17] frequent night-time awakenings,[16,17] and reduced sleep duration.[13,18] Sleep problems have been associated with disruptive daytime behavior or medical abnormalities in some children with ASD. For example, sleep disorders were significantly associated with both mood disorders and gastrointestinal abnormalities in one study of 160 children with ASD.[19] Sleep problems in individuals with ASD have also been associated with poor social

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interaction,[17,20,21] increased stereotypy,[21] problems in communication,[17,22] and overall autistic behavior.[15,21] Some studies have also reported an association between sleep problems and developmental regression in ASD,[3,5,23–25] although this association has not been found in every study.[19,26] Furthermore, abnormalities in genes associated with circadian rhythms have been reported in ASD.[27,28] About two decades ago, Chamberlain and Herman hypothesized that abnormalities in melatonin secretion may play a role in the development of ASD.[29] Interestingly, individuals with Smith–Magenis syndrome can manifest autistic behaviors and have an abnormal melatonin circadian rhythm;[30] this syndrome has responded to melatonin treatment.[31] Additionally, individuals with Angelman syndrome can manifest both autistic behaviors[32] and sleep abnormalities,[33] and melatonin has been shown to improve insomnia in this syndrome,[34,35] although the melatonin dose must sometimes be kept low as these individuals can have slow melatonin metabolism owing to decreased CYP1A2 enzyme activity.[34]

Melatonin is an endogenous neurohormone produced predominantly in the pineal gland.[36] In individuals with normal vision, the secretion of melatonin increases shortly after darkness, peaks in the middle of the night, and falls slowly during the early morning hours. Melatonin is synthesized from L-tryptophan through several metabolic intermediates, most notably serotonin. As depicted in Figure S1, (supporting information published online only) L-tryptophan is converted into 5-hydroxytryptophan (5-HTP) and then into serotonin (5-hydroxytryptamine) in two metabolic steps. Serotonin is then converted into *N*-acetylserotonin and finally into melatonin in two additional metabolic steps that involve two enzymes: arylalkylamine *N*-acetyltransferase and acetylserotonin methyltransferase (ASMT).[37] A major metabolite of melatonin is 6-sulphatoxymelatonin (6-SM). Although melatonin is best known for its regulatory role of the circadian rhythm,[38,39] it is also a potent antioxidant,[40] has anti-inflammatory properties,[41] is involved in the immune response,[42–44] and helps regulate synaptic plasticity.[45,46] Abnormalities in melatonin pathways have been reported in circadian rhythm[47,48] and non-circadian rhythm disorders such as diabetes[49,50] and ASD.[51]

Melatonin is commonly used for insomnia in children, has a favorable side-effect profile, is inexpensive and readily available, and is often efficacious for sleep abnormalities.[52] Interestingly, a small number of individuals with sleep problems and intellectual disability have been reported to have an initial positive response to melatonin that wanes over time;[53] in these individuals this phenomenon may be related to slow melatonin metabolism, possibly due to decreased CYP1A2 enzyme activity.[53] Compared with placebo, melatonin has been shown to improve sleep in children with insomnia with and without attention-deficit–hyperactivity disorder.[54,55] Several controlled studies have reported that melatonin improves sleep in children with ASD.[56–58] However, a systematic, comprehensive review of this recently evolving literature has not been published. Furthermore, no meta-analysis has been published on the effects of melatonin in ASD. In this article, we systematically review melatonin in ASD and examine studies that report on the following: concentrations or physiology of melatonin or melatonin-related metabolites in ASD; melatonin-related genes in ASD; correlation of the concentration of melatonin and melatonin metabolites with ASD behaviors; the prevalence of melatonin usage in ASD; physician recommendations concerning the use of melatonin in ASD; melatonin treatment studies in ASD and meta-analytic statistics of placebo-controlled studies to examine the effect size of treatment; and the potential side effects of melatonin in ASD.

What this paper adds

- Physiological levels of melatonin and/or melatonin derivates are commonly below average in ASD and correlate with autistic behavior.
- Abnormalities in melatonin-related genes may be a cause of low melatonin levels in ASD.
- This study indicates that treatment with melatonin significantly improves sleep duration and sleep onset latency in ASD.

METHOD

Search strategy

For this review, we included individuals with autistic disorder, Asperger syndrome, Rett syndrome, and pervasive developmental disorders, not otherwise specified. We performed a computer-aided search of PubMed, Google Scholar, CINAHL, EMBASE, Scopus, and ERIC databases from their inception to October 2010 using the search terms 'autism', 'autistic', 'ASD', 'Asperger', 'Rett', 'pervasive', and 'PDD' in all combinations with 'melatonin.' The references cited in identified publications were also searched to locate additional studies. Figure S2 (supporting information published online only) depicts the publications identified during the search process.

Study selection

Both reviewers screened titles and abstracts of all potentially relevant publications. Studies were initially included if they (1) involved individuals with ASD and (2) reported unique data on melatonin or melatonin-related factors. After screening all records, 35 publications were identified that met these inclusion criteria.[5,8,51,56–87] Table SI (supporting information published online only) reports the characteristics of these 35 publications. Five of these studies reported on a blended sample of individuals with ASD and individuals with other neurologic abnormalities.[57,60,73,74,86] Two[57,86] of these five studies were placebo controlled and the authors were contacted to obtain the data on the individuals with ASD in order to include them in the meta-analysis: one study[86] contained 51 children with a neurodevelopmental disability, including 16 children with ASD, and the authors of the study provided information on the ASD subgroup,[88] while the other study[57] comprised 12 children with ASD and/or fragile X syndrome. The data were obtained from graphs reported in the publication on the five children with ASD only (not those with fragile X syndrome) as the data were not accessible to the original study authors. In all of

the studies where melatonin was administered as a treatment, the study participants had a sleep abnormality.

Data extraction and statistical analysis

For treatment studies, two types of values were derived: prevalence values and comparisons of changes in sleep parameters with melatonin treatment. A mean prevalence value was computed by dividing the total number of participants with a certain characteristic pooled for all studies by the number of participants evaluated for all studies. A 95% confidence interval (CI) was then calculated assuming a Bernoulli distribution.[89] We followed PRISMA 2009 guidelines.[90]

For the meta-analysis, outcome variables included total sleep duration, number of night-time awakenings, and sleep onset latency. We performed meta-analysis only on (1) studies that used a randomized double-blind, placebo-controlled design, (2) individuals with ASD and not other developmental disorders, and (3) studies that reported quantitative data on total sleep time, sleep onset latency, and/or number of night-time awakenings. Five studies met these criteria for meta-analysis.[56–58,78,86] The methodological quality of these five studies was independently assessed by each reviewer using the Downs and Black checklist.[91] The intraclass correlation coefficient (using average measures with a two-way random effect model and an absolute agreement definition) between the two reviewers was calculated using the Statistical Package for the Social Sciences (version 19.0, SPSS Inc., Chicago, IL, USA). The following statistics were computed for the comparisons between sleep parameters with melatonin treatment and baseline sleep parameters and sleep parameters with placebo treatment: (1) the pooled means with confidence intervals, (2) the effect size using two different methods, and (3) the homogeneity statistic Q in order to assess the between study variation.[92] Two different approaches were used to calculate the effect size: Glass's Δ, which is based only on the control standard deviation,[93] and Hedge's g, which uses the mean sample stan-

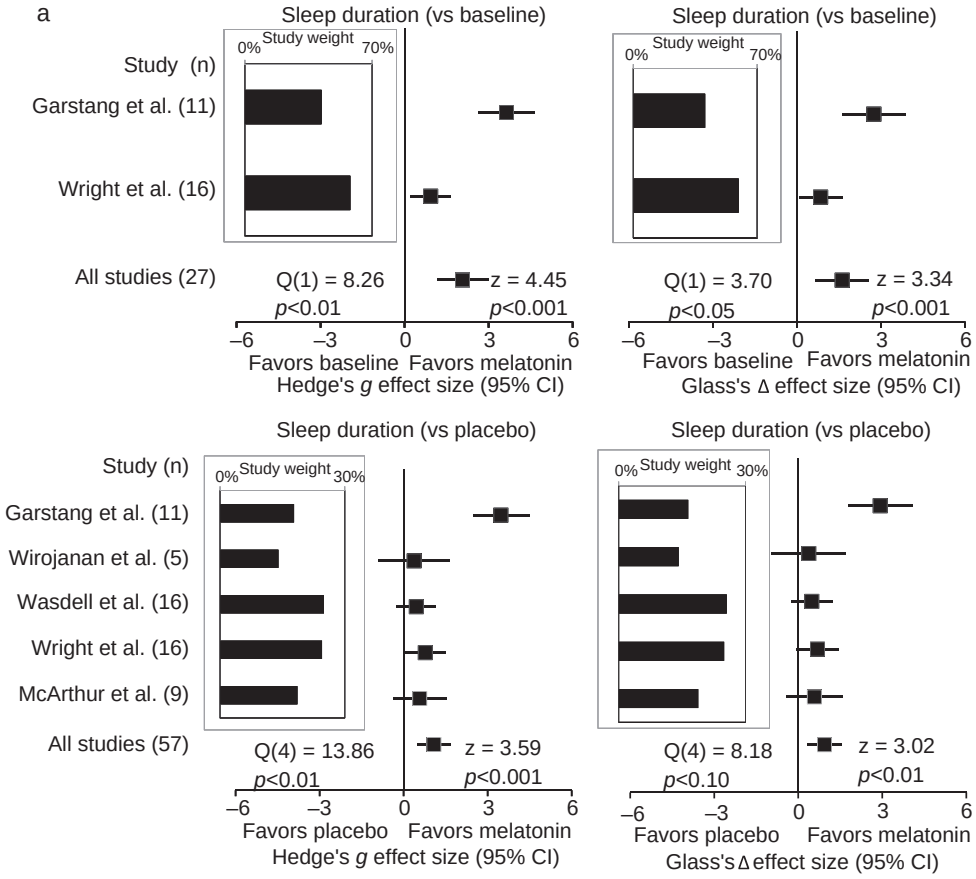

Figure 1: Forest plots showing the effect of melatonin on sleep parameters in autism spectrum disorders (ASD) compared with baseline and placebo. The effect size derived from studies that compared melatonin with baseline (pre-melatonin treatment) measurements and placebo treatment are presented separately in each panel. The effect size using both Hedge's g and Glass's Δ are presented. Each graph includes the homogeneity statistic Q in the lower left corner and a measure of statistical significance of the effect size, the z-score, in the lower right corner. A separate bar chart within each graph depicts the weight to which each study contributed to the final effect size calculation. (a) Melatonin demonstrates a significant effect on sleep duration compared with both baseline and placebo using both effect size calculations. (b) Melatonin demonstrates a significant effect on sleep onset latency compared with both baseline and placebo using both effect size calculations. (c) Melatonin demonstrates no significant effect on night-time awakenings as compared with both baseline and placebo using both effect size calculations. CI, confidence interval.

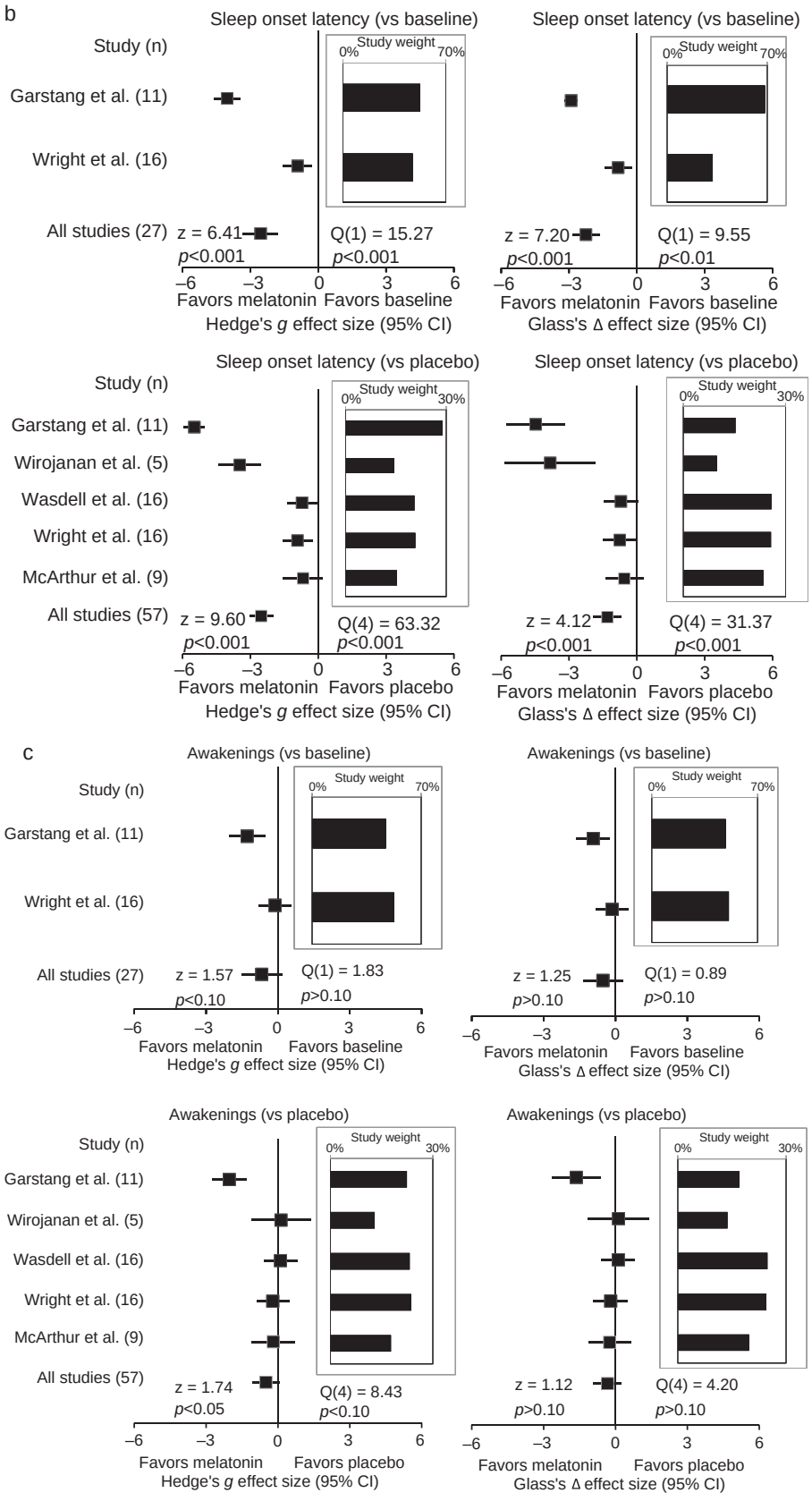

Figure 1: *Continued.*

dard deviation of the two conditions being compared.[94] The data generated from this analysis are depicted as forest plots in Figure 1. Lastly, to assess for potential publication bias, we inspected funnel plots, a scatter plot of treatment effect (i.e. the mean difference) against the study precision (i.e. inverse of the standard error). Funnel plots were constructed using meta-analysis with interactive explanations (MIX: version 2.0; BiostatXL, Yamato-shi, Japan;[95] see Fig. S3, supporting information published online only). A linear regression conducted on the funnel plot values was used to assess quantitatively the presence of significant funnel plot asymmetries. The statistical threshold for all studies was set at an alpha of 0.05.

RESULTS

Included studies

The computerized search initially identified 68 unique publications after duplicates were removed. An additional five publications were identified by searching references. After screening and assessment, 35 references met the inclusion criteria as depicted in Figure S2. The selected studies were separated into two main themes: studies examining the biochemistry or physiology of melatonin in ASD and studies examining melatonin as a treatment in ASD. These studies are discussed separately.

Studies of the biochemistry or physiology of melatonin in ASD

Studies measuring concentrations of melatonin or melatonin derivatives in ASD

Table SII (supporting information published online only) lists specific details of the nine studies[51,59,77,79–81,84,85,87] reporting melatonin or melatonin metabolite concentrations in ASD, with all reporting at least one abnormality. Seven studies[51,59,77,79,81,85,87] reported lower melatonin or melatonin metabolite concentrations in individuals with ASD than in healthy individuals or laboratory reference ranges. Two of these studies reported a significantly lower mean melatonin concentration measured between 12 am and 4 am in individuals with ASD compared with healthy individuals.[77,81] One controlled study reported a plasma melatonin level at or below 50% of the mean control melatonin concentration in 65% of individuals with ASD (significance not reported).[51] Another study reported that 63% of individuals with ASD had a 6-SM level at or below 50% of the mean 6-SM level found in healthy individuals (significance not reported).[85] The remaining three studies[59,79,87] reported that melatonin or 6-SM levels were lower than laboratory reference values (significance not reported in these studies).

Two studies reported that daytime melatonin levels were significantly higher in the group with ASD than in healthy individuals.[81,84] However, no study reported an elevated melatonin or melatonin derivative at night in the group with ASD compared with healthy individuals. Four studies[51,77,79,81] reported an abnormal melatonin circadian rhythm in individuals with ASD compared with healthy individuals. Two studies[51,80] measured serotonin levels. In one of these studies, an inverse relationship between platelet serotonin and urinary 6-SM was found,[80] while the other study reported concomitantly elevated plasma serotonin and depressed plasma melatonin and ASMT activity in children with ASD compared with healthy individuals.[51]

Studies examining polymorphisms in melatonin-related genes in ASD

Five studies[51,61,63,67,76] examined genes that code for melatonin receptors (MTNR1A, MTNR1B, and GPR50) or enzymes involved in melatonin synthesis (alkylamine *N*-acetyltransferase and ASMT). Four of these studies[51,63,67,76] examined the *ASMT* gene, which codes for the last enzyme involved in melatonin synthesis. *ASMT* abnormalities in the group with ASD were reported in all four studies. One study reported a partial duplication of *ASMT* in 6 to 7% of individuals with ASD compared with 2% of healthy individuals (p=0.003).[67] In another study, several single-nucleotide polymorphisms in *ASMT* were reported in 2.8% of individuals with ASD compared with 0.5% of healthy individuals.[76] One study reported several single-nucleotide polymorphisms, including a novel R319X stop mutation, in *ASMT* in 2.6% of individuals with ASD compared with 1.8% of healthy individuals.[63] Finally, one study reported that two single-nucleotide polymorphisms in *ASMT* (rs4446909 and rs5989681) were significantly more frequent in individuals with ASD than in healthy individuals (p<0.001) and were associated with a significant decrease in ASMT enzymatic activity and lower plasma melatonin levels.[51] Furthermore, in this study, decreased ASMT activity was significantly correlated with lower plasma melatonin levels in individuals with ASD but not in healthy individuals.[51] The one study that examined the arylalkylamine *N*-acetyltransferase gene did not find any abnormalities.[76] Variants in genes (*MTNR1A* and *MTNR1B*) that code for melatonin receptors 1A and 1B were reported in two studies,[61,76] with one study reporting a variant in 2.8% of individuals with ASD compared with 0% of healthy individuals [76] and the other study reporting that variants were not significantly different compared with healthy individuals.[61] Finally, two variants in G protein-coupled receptor 50 (GPR50), which codes for a melatonin-related receptor, were found to be associated with ASD in one study, but this finding did not hold up after correction for multiple comparisons,[61] and a second study found no significant association.[76] Overall, these findings suggest that genetic abnormalities in enzymes involved in melatonin metabolism and/or melatonin receptor function could contribute to lower melatonin concentrations or an altered response to melatonin in a small percentage of individuals with ASD.

Correlations between melatonin metabolites and ASD findings

Four studies reported a correlation between levels of melatonin or melatonin metabolites and ASD symptoms or clinical findings.[51,59,81,85] For example, night-time urinary excretion of 6-SM was reported to be inversely correlated with the severity of impairments in verbal communication and play[85] as well as daytime sleepiness in individuals with ASD.[59] In one

study, a lower mean serum melatonin level was associated with an abnormal electroencephalogram in individuals with ASD.[81] Finally, lower ASMT activity was associated with hyperactivity but was not found to be related to Autism Diagnostic Interview – Revised scores in another study.[51] These studies suggest that melatonin metabolism is directly or indirectly related to certain autistic behavior.

Studies examining melatonin as a treatment in ASD

Studies reporting the prevalence of melatonin usage in ASD

Three survey studies,[8,65,70] involving a total of 1071 individuals, reported a prevalence of melatonin use in individuals with ASD. The prevalence ranged from 2.9[8] to 10.8%[70] with an mean prevalence of 7.2% (95% CI 5.6–8.7%).

Studies reporting prevalence of physician recommendations of melatonin in ASD

Three survey studies[60,69,82] reported the prevalence of recommendations for melatonin usage in ASD among 2483 physicians although two studies also included several pediatric conditions other than ASD.[60,82] The overall prevalence of physicians recommending the use of melatonin from these studies was 32.4% (95% CI 30.6–34.2%) with a range of 24.9[82] to 39%.[60] In one study, 22% of physicians did not feel 'knowledgeable' enough about melatonin to recommend its use, 14% discouraged melatonin use, and 39% were accepting of its use if the child was already taking melatonin.[69] These studies indicate that, even though melatonin is not approved as a treatment for insomnia in ASD by the US Food and Drug Administration, a relatively large percentage of physicians recommends melatonin treatment for individuals with ASD.

Studies of the effects of melatonin treatment on sleep in ASD

A total of 18 studies[5,56–58,64,66,68,71–75,77–79,83,86,87] reported the effects of melatonin treatment on sleep parameters in individuals with ASD (Table SI). One study reported on melatonin usage in individuals with ASD who were over 18 years of age[68] and another reported the effects of melatonin in individuals with ASD aged between 3 and 28 years.[73] The remaining 16 studies[5,56–58,64,66,71,72,74,75,77–79,83,86,87] involved individuals aged between 2 and 18 years, but none of these studies reported treatment effects by age group (e.g. age up to 6y, 6–12y, or 12–18y). The dosage of melatonin in these studies ranged from a low of 0.75mg[66] to a high of 15mg[86] with rare use of 25mg.[74] The length of melatonin usage in these studies ranged from 14 days[83] to over 4 years.[74] In order to identify sleep abnormalities, studies used a variety of subjective (parent-report questionnaires and sleep diaries) and objective (actigraphy) measures. Actigraphy involves a sensor (typically worn on the wrist) that measures movement as a surrogate for wakefulness and is felt to be more accurate and objective than other measures,[10,78] especially when used in conjunction with sleep diaries.[52]

All studies

Out of these 18 studies, 12,[64,66,68,72–75,77,79,83,86,87] totaling 349 individuals, reported the percentage of individuals with ASD who experienced an improvement in sleep with melatonin. This percentage ranged from 67%[77] to 100%,[64,68,72,75,79,87] with an overall improvement rate of 84.2% (95% CI 81.4–88.9%). The two studies that used actigraphy reported sleep improvements with melatonin in 92%[86] to 93%[83] of individuals, although one[86] of these studies contained a blended sample of individuals with and without ASD. In order to reduce the effects of individuals without ASD, we examined the four non-case series[66,68,77,83] that contained only individuals with ASD (142 individuals) and found an overall sleep improvement rate of 84.7% (95% CI 78.7–90.6%). One additional article reported the results of a parental online survey by the Autism Research Institute; although this was not a formal study and, therefore, was not included in the aforementioned analysis, this survey found that out of 1105 children with ASD, 65% had an improvement with melatonin usage and 27% had no change after melatonin usage.[62]

Uncontrolled studies

Of the 18 studies examining melatonin treatment, 13 were uncontrolled[5,64,66,68,71–75,77,79,83,87] and ranged in size from one child[64,72,75,87] to 107 children.[66] Because these studies were uncontrolled, they were not included in the meta-analysis. All 13 studies reported some type of improvement in sleep with melatonin use. Improvements were described in total sleep duration,[5,68,71,72] number of night-time awakenings,[5,64,68,75] and sleep onset latency.[5,64,71,79,83,87] Four studies reported general improvements in overall sleep without a further description.[66,73,74,77] One study reported a significant decline in night terrors within 2 days of starting melatonin[75] and two studies reported a decrease in screaming in the middle of the night.[79,87] Four studies reported worsening of sleep when melatonin was stopped or the dose was lowered,[5,64,79,83] and one study reported that improvements with melatonin were maintained at both 12- and 24-month follow-ups.[5] Four studies reported improvements with melatonin when other sleep medications had previously failed.[64,68,79,87]

Controlled studies

The remaining five studies[56–58,78,86] were randomized, double-blind, placebo–controlled crossover studies, the details of which are reported in the supplementary material (Table SIII, supporting information published online only). Four studies[56–58,86] included children with non-syndromic ASD, whereas one study[78] contained nine children with syndromic ASD (Rett syndrome). The mean score on the Downs and Black checklist as performed by both reviewers for these studies was 19.9 of 31 (SD=1.66; range 17–22) and the intraclass correlation coefficient between the two reviewers was 0.870 (p=0.033). Three studies compared both a baseline and a placebo with melatonin treatment[56,58,86] while two studies compared only melatonin treatment with placebo.[57,78] Furthermore, for one of these studies,[86] the data provided by the study authors[88] (described in the 'Method' section) for children with ASD only did not include baseline measurements. Four[56,58,78,86] of the five studies had a washout period between the two treatment arms that varied from 3 to 5 days[86] to 1 week[56,78] to 1 month,[58] while one

study[57] did not contain a washout period. A shorter washout period (or no washout period) can result in a smaller effect size. However, the length of the washout period was not related to the effect size of the study.

Compared with placebo, these studies reported significant improvements in total sleep duration,[56–58,78,86] number of night-time awakenings,[56] and sleep onset latency.[56–58,78,86] Figure 1 depicts the effect sizes (both Glass's Δ and Hedge's *g*) for the changes in sleep parameters with melatonin treatment, for each study and all studies combined, compared with placebo and baseline. The inclusion or exclusion of the one study containing syndromic ASD[78] did not significantly change the results of the meta-analysis. The overall effect sizes of the differences between melatonin treatment and both baseline and placebo were significant and large for sleep duration and sleep onset latency, although the effect size was about 50% larger for sleep onset latency. The overall effect size for the number of night-time awakenings was significant when compared with placebo only when using the Hedge's *g* effect size calculation with an alpha probability of 0.04. Overall, sleep duration was 73 and 44 minutes longer during melatonin treatment than with baseline and placebo respectively, and sleep onset latency was 66 and 39 minutes shorter during melatonin treatment than during baseline and placebo respectively.

The *Q* statistic indicated that the effect size varied significantly across clinical studies. This was predominantly due to the consistently large effect size for all parameters in the Garstang and Wallis study.[56] In fact, for most sleep parameters, the effect size of the Garstang and Wallis study[56] was three to four times greater than in most other studies. The exception to this was the impressive effect size of the study by Wirojanan et al.[57] for sleep onset latency. The remainder of the studies demonstrated smaller effect sizes, especially for the treatment versus placebo comparison. Some heterogeneity was evident in the funnel plots for sleep duration and sleep onset latency (see Fig. S3), and the study with the most precision (i.e. Garstang and Wallis[56]) had the largest effect size, resulting in a visually asymmetric funnel. However, linear regression demonstrated non-significant slope coefficients ($p>0.10$), suggesting that this asymmetry was not statistically significant. In addition, publication bias normally results in an asymmetric funnel with smaller studies demonstrating larger effect sizes, rather than smaller effect sizes.[96] Thus, these analyses did not suggest publication bias.

Combining the double-blind, placebo-controlled studies in a meta-analysis was limited by the method used to collect the sleep parameter data. The two studies with the largest effect sizes for the treatment versus placebo condition were very similar as they both used sleep diaries kept by the parents.[56,58] The three remaining studies inferred sleep parameters using wrist actigraphy by quantitatively measuring each child's movements,[57,78,86] with two of these studies combining actigraphy data with a parent sleep diary.[57,78] Overall, the effect of melatonin on sleep duration and sleep onset latency was greater for the studies that used only sleep diaries.

The reason for a greater effect size for studies using parental report (i.e. sleep diaries) compared with actigraphy is not known, but it appears that these two techniques are different in several ways. For example, McArthur and Budden[78] analysed the reliability of the sleep diary data provided by parents. They reported that the data obtained from actigraphy were much more reliable at monitoring long-term sleep–wake cycles than the diaries. Whereas the average data loss from not wearing the actigraphy monitor was 8.4%, the average diary data loss was 57.9%. In addition, parents reported filling in the diaries with sleep parameter data estimated from memory several days after the sleep events in instances when they forgot to consistently maintain the sleep logs. While the mean sleep duration and onset latency were somewhat similar across studies regardless of the two methods used, the mean number of night-time awakenings was clearly much larger for the studies that used actigraphy and much lower for studies that used the diary method. This discrepancy was echoed in the analysis of McArthur and Budden,[78] in which the two methods for measuring the number of night-time awakenings differed by an order of magnitude (diary 0.6 vs actigraphy 13.3). This suggests that each of these two methods measured something different for the number of night-time awakenings. Since some studies[56] did not require parents to go into the children's room at night to check if they were awake, it is also possible that the parents were not aware of minimal night-time awakenings that actigraphy was sensitive enough to record.[78] Alternatively, it is difficult to know if participants were truly awake during the night if they were not observed. For example, actigraphy could mistake parasomnias (e.g. sleep walking) for night-time awakenings. Even though there was significant variation in the number of night-time awakenings, the effect size was very small for night-time awakenings for almost every study except Garstang and Wallis,[56] suggesting that regardless of the method of measuring this sleep parameter, melatonin probably had little effect on night-time awakening.

Studies of the effects of melatonin treatment on daytime behavior in ASD

Six studies reported that the night-time administration of melatonin led to improvements in daytime behavior in some children with ASD.[5,56,58,64,74,83] Improvements included less behavioral rigidity, ease of management for parents and teachers,[56] better social interaction, fewer temper tantrums, less irritability, more playfulness,[74] better academic performance, and increased alertness.[64] One study noted a significant improvement in overall daytime behavior as measured by the Developmental Behavior Checklist when comparing melatonin with placebo.[58] However, a meta-analysis of the effects of melatonin on daytime behavior could not be performed as only two of the six studies[56,58] were placebo controlled and only one[58] reported results for melatonin compared with placebo (the other study[56] reported only that several parents and teachers noted better daytime behavior with melatonin use).

Studies examining side effects of melatonin in ASD

Twelve studies[5,58,64,66,68,71,73,74,78,79,83,86] examined the potential side-effects of melatonin in individuals with ASD. In addition, one parental survey reported the prevalence of 'worse

behavior' with melatonin.[62] Seven of these studies reported that melatonin use was not associated with any side-effects,[5,64,68,71,74,78,79] even with over 4 years of use.[74] Two studies reported mild side-effects in a small number of children,[86] including three of 107 children with ASD who had morning drowsiness or increased enuresis.[66] One study reported that side-effects were not significantly different from those reported with a placebo.[58] Another study reported mild morning tiredness in two children, headache in one child, and tiredness, dizziness, and diarrhea in one child resulting in study discontinuation.[83] The use of melatonin in 21 children with ASD who also had epilepsy was not associated with any increase in seizure activity.[66] In two studies, the use of melatonin was frequently combined with multiple other psychotropic medications without any adverse events.[66,68] Four studies reported evidence of a loss of effect of melatonin in a small number of individuals,[66,73,74,78] including two studies that used relatively high melatonin doses[66,74] and one study that reported loss of positive effect at 4 weeks of treatment.[78] A large parental survey reported that, out of 1105 children with ASD, 8% had 'worse behavior' after melatonin use.[62] Interestingly, in one study of 50 individuals with a sleep and developmental disorder (including 27 with ASD), mild adverse effects were reported in 34% of the participants. These adverse effects included morning drowsiness, night-time awakening, and excitement before going to sleep;[73] however, this adverse event rate is dramatically higher than any of the other studies in this review. Overall, no serious adverse events were reported in any of the studies.

DISCUSSION

Biochemistry and physiology of melatonin in ASD

One of the objectives of this systematic review and meta-analysis was to review the biochemical characteristics of melatonin metabolism in ASD. Abnormalities in the levels of melatonin or melatonin derivatives in ASD were found in nine studies, and four studies reported abnormalities in the circadian melatonin rhythm in ASD. Five studies reported abnormalities in genes in a small percentage of individuals with ASD, which could contribute to the decreased production of melatonin or adversely affect melatonin receptor function. Four studies reported a correlation between concentrations of melatonin or melatonin metabolites and ASD behaviors or clinical findings.

Melatonin is synthesized from serotonin through two reactions. Abnormalities in one of the genes involved in this pathway (*ASMT*) were identified in four studies in a small number of individuals with ASD. Previous studies have reported that some individuals with ASD have elevated blood serotonin (hyperserotoninemia),[97–103] which theoretically would lead to an elevated melatonin level. However, the reviewed studies suggest that a majority of individuals with ASD have low levels of melatonin or melatonin derivatives.[51,85] In fact, in one reviewed study, children with ASD who had an elevated platelet serotonin also had a lower urinary 6-SM level.[80] The reason for this paradoxical finding may be secondary to abnormalities in ASMT function. For example, one reviewed study reported significantly elevated mean serotonin along with lower mean melatonin and ASMT activity in individuals with ASD compared with healthy individuals.[51] Additional studies examining melatonin, serotonin, and ASMT activity levels would be helpful to expand on these findings.

Furthermore, four studies reported evidence of a loss of melatonin effect in a small number of individuals.[66,73,74,78] Some of these studies described this loss of effect as tolerance, and because of this the dose of melatonin was increased over time. However, other investigators have described a loss of effect with melatonin in some individuals without ASD owing to slow melatonin metabolism, which leads to elevated melatonin levels in the daytime, even when melatonin was not recently given, as well as a loss of the normal melatonin circadian rhythm; this problem responds to a reduction in melatonin dose rather than an increase in dose.[53] Interestingly, significantly higher daytime levels of melatonin have been reported in some individuals with ASD compared with healthy individuals [81,84] as has an abnormal melatonin circadian rhythm.[51,77,79,81] Thus, additional studies are needed to determine whether the loss of melatonin effect observed in some individuals with ASD is related to melatonin metabolism and/or abnormalities in circadian rhythms, and to examine the relationship between genetic polymorphisms and these factors.

Since the concentration of melatonin is low in some individuals with ASD, and because some individuals with ASD have abnormalities in genes involved in melatonin synthesis, the use of melatonin may function to replace a deficiency. In the future, routine testing for melatonin levels may be helpful, but currently this testing is not readily available. Overall, the findings of this review and meta-analysis suggest that abnormalities in the physiology and concentrations of melatonin, as well as certain genetic abnormalities, may play a role in both sleep and behavioral problems in individuals with ASD.

Treatment effects of melatonin in ASD

Another objective of this review was to examine the clinical characteristics of melatonin treatment in children with ASD. Three studies reported that the prevalence of melatonin use in the general population of individuals with ASD was about 7%. Three surveys indicated that a recommendation for melatonin use is relatively common in ASD, with approximately 32% of physicians recommending its use, while one survey reported that 14% of physicians discouraged its use. Six studies reported improved daytime behavior with the use of melatonin at night-time. A total of 18 studies reported the effects of melatonin treatment in individuals with ASD who had a sleep abnormality. All of these studies reported improvements in sleep parameters, including improvements in overall sleep, sleep duration, sleep onset latency, and night-time awakenings. A meta-analysis of the five randomized, double-blind, placebo-controlled, crossover studies demonstrated an overall significant improvement in sleep duration (73min compared with baseline; 44min compared with placebo) and sleep onset latency (66min compared with baseline; 39min compared with placebo) but no overall significant improvement in the number

of night-time awakenings. Reported side-effects in these studies were minimal to none.

In the meta-analysis, the effect size was larger for sleep onset latency than for sleep duration, which might be related to the half-life of fast-release melatonin. Fast-release melatonin helps children to fall asleep faster, but controlled-release melatonin helps to maintain sleep better.[104] A few studies used controlled-release melatonin, and some used a combination of controlled-release and fast-release, while others used only fast-release melatonin (Table SI). Some studies did not note which form of melatonin was used, but given the special formulation required for controlled-release melatonin it is likely that these studies used fast-release melatonin. Because of an extended release system, it is possible that controlled-release melatonin might have a better effect on sleep duration than fast-release melatonin. Conversely, fast-release melatonin might help sleep onset latency better than controlled-release melatonin. Unfortunately, there were not enough studies that used controlled-release melatonin to compare changes in sleep parameters with fast-release melatonin. Additional studies are needed to examine the differences in effect between controlled-release and fast-release melatonin in ASD.

Seven studies noted the use of pharmaceutical-grade melatonin or that the melatonin was provided by the hospital pharmacy.[5,56,58,68,78,83,86] Since melatonin is available without a prescription, it is possible that the quality of the melatonin supplement used by children with ASD in the general community (over the counter) may vary from that used in the studies. However, one double-blind placebo-controlled study noted significant improvements with a form of melatonin readily available over the counter.[57]

Potential mechanisms of melatonin treatment in ASD

The potential mechanism of melatonin improvement in individuals with ASD is not clear. It may act to align the circadian rhythm in children with ASD or work as a hypnotic or sedative agent.[52] Alternatively, some children with ASD appear to be deficient in melatonin and, therefore, exogenous melatonin may replace a deficiency. Additionally, melatonin may also ameliorate certain physiological abnormalities that are reported in some individuals with ASD. For example, melatonin is a potent antioxidant[40] and may be beneficial since individuals with ASD, as a group, have been shown to be under higher oxidative stress and have reduced levels of antioxidants compared with healthy individuals.[105–114] Furthermore, melatonin has anti-inflammatory properties,[36] and evidence of inflammation has been described in some individuals with ASD.[115–120] Melatonin may also have a positive effect on the immune system in ASD. For example, a dysfunctional natural killer cell system has been noted in some individuals with ASD,[121–124] and melatonin is known to have a positive effect on natural killer cell production.[43] Melatonin also stimulates the production of $CD4^+$ cells,[43] and several studies have reported lower levels of certain populations of $CD4^+$ cells in individuals with ASD.[125–128]

Furthermore, since melatonin helps regulate synaptic plasticity,[45,46] abnormalities in melatonin pathways could play a role in the development of ASD. For example, an imbalance in the excitatory (glutamatergic) and inhibitory (GABAergic) neurotransmitter systems has been implicated in the pathogenesis of ASD, with a relative increase in the glutamatergic neurotransmitter system.[129] Since GABAergic neurons are inhibitory, they may be especially important between the ages of 12 and 30 months as this window of development corresponds to an overproduction in excitatory neurotransmitters and receptors.[130,131] Thus, without proper GABAergic neuronal function, the brain may be highly susceptible to excitotoxicity during this developmental period. Interestingly, this is the age range when developmental regression most commonly occurs in ASD.[132] In animal models, melatonin is known to enhance brain GABAergic transmission[133,134] and, therefore, a lack of melatonin production could contribute to an imbalance in the glutamatergic to GABAergic systems. Further studies are needed to investigate these possibilities.

Safety of melatonin

The reviewed studies indicate that melatonin is safe and inexpensive, and has been used extensively in children with ASD, sometimes for years at a time.[74] Seven of the reviewed studies reported that melatonin did not have any side-effects, while the remaining studies reported transient or mild side-effects in a small number of individuals. Even when combined with other psychotropic medications, melatonin was well tolerated and had no apparent interactions with these medications. Furthermore, in one study, melatonin did not increase seizure activity in children with ASD who were already taking seizure medication,[66] and no studies were identified in individuals with ASD who reported an increase in seizure activity with melatonin. In fact, one study reported that a lower mean serum melatonin level was associated with an abnormal EEG in individuals with ASD.[81]

Limitations of this review

Some reviewed studies suffered from limitations, including small sample sizes (some were case reports) and variations in protocols for measuring changes in sleep parameters. Five of the reviewed studies contained a mixture of individuals with ASD and individuals with other developmental disabilities. However, we found a similar prevalence of sleep improvements with melatonin when we included case reports and studies containing individuals with other developmental disabilities as when we excluded these studies. In addition, we were able to obtain data on the children with ASD for two of these studies.

The meta-analysis was limited by inconsistencies across studies in the method used to collect the sleep parameter data. For example, some studies relied on a subjective parental report of changes in sleep parameters (parent-report questionnaires and sleep diaries) while others used more objective measures (actigraphy) and some used a combination of both. Additional larger controlled studies of melatonin treatment using a combination of subjective and objective measures of sleep parameters would be helpful to confirm and expand on the findings of this review.

Interestingly, several studies reported improvements with melatonin when other sleep medications had previously failed.[64,68,79,87] However, none of the reviewed studies compared the effects of melatonin with other sleep medications, so conclusions about the effectiveness of melatonin compared with other sleep medications cannot be drawn.

CONCLUSIONS

This systematic review found that, when measured, levels of melatonin or melatonin derivatives are often below average in individuals with ASD compared with healthy individuals. Furthermore, the physiology of melatonin is abnormal in many individuals with ASD, which in some cases correlates to ASD symptoms. Some individuals have abnormalities in genes involved in melatonin production or receptor function. The meta-analysis found that the use of melatonin in ASD is associated with significantly improved sleep parameters (sleep duration and sleep onset latency). Furthermore, melatonin appears to improve daytime behavior in some individuals with ASD and has minimal to no side effects. However, additional studies are needed to examine melatonin metabolism in ASD, including the relationship between melatonin and serotonin in ASD. Some studies reported a higher daytime melatonin level in children with ASD, which could be related to slower melatonin metabolism; this finding needs further investigation. Studies examining optimal effective dosing,[53] optimal timing of melatonin dosing,[53] the potential differential effects of fast-release and controlled-release melatonin, and its long-term adverse effects and safety in ASD[135] are also needed. Additional studies looking at the effects of melatonin on other sleep parameters in ASD, such as early morning awakening, are needed.[135] Finally, studies examining the mechanism of melatonin in possibly ameliorating certain findings associated with ASD, including oxidative stress, inflammation, immune dysregulation, and abnormal neurotransmitter function are needed.

ONLINE MATERIAL/SUPPORTING INFORMATION

Additional material and supporting information may be found in the online version of this article.

REFERENCES

References are available online as Supporting Information.

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INFLAMMATORY AND AUTOIMMUNE DISORDERS OF THE NERVOUS SYSTEM IN CHILDREN

Clinics in Developmental Medicine 184-185

Russell C. Dale and **Angela Vincent**

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Attachment 12

Pharmacoelectroencephalography

Neuropsychobiology

Original Paper

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Klaus Mann
Joachim Röschke

Department of Psychiatry, University of Mainz, Germany

Effects of Pulsed High-Frequency Electromagnetic Fields on Human Sleep

Key Words
Electromagnetic fields
Digital mobile radio telephone
Sleep EEG
REM sleep
Spectral power density

Abstract
In the present study we investigated the influence of pulsed high-frequency electromagnetic fields of digital mobile radio telephones on sleep in healthy humans. Besides a hypnotic effect with shortening of sleep onset latency, a REM suppressive effect with reduction of duration and percentage of REM sleep was found. Moreover, spectral analysis revealed qualitative alterations of the EEG signal during REM sleep with an increased spectral power density. Knowing the relevance of REM sleep for adequate information processing in the brain, especially concerning mnestic functions and learning processes, the results emphasize the necessity to carry out further investigations on the interaction of this type of electromagnetic fields and the human organism.

Introduction

For many years there has been a discussion among both experts and the general public regarding the effects of electromagnetic fields on the human organism, which has recently been stimulated by the introduction of digital mobile radio telephones. Here the points of view are very controversial, reaching from playing down the problem on the one hand to claiming severe health hazards on the other, without having sufficient substantial facts or experimental evidence. This led to a state of uncertainty in the general public and resulted in a lack of clarity of the political and legal situation regarding these new communication systems.

Increasingly in recent years experimental investigations were carried out in order to elucidate the interaction between nonionizing electromagnetic fields and biological systems. For a comprehensive review of the variety of findings see Polk and Postow [1] and Michaelson and Lin [2]. However, the reported phenomena are very heterogenous. Results from various studies can only be compared to a limited extent because of very different physical and biological experimental conditions. Most investigations were performed in cellular model systems or small laboratory animals. Fundamental problems in this area of research are the extrapolation of those results to man and the assessment of observed effects as a potential health hazard.

In principle, one has to distinguish between thermal effects which are caused by heating the tissue through absorption of radiation energy and nonthermal direct radiation effects. Regarding the thermal effects of electromagnetic fields, knowledge has increased considerably. These effects can be estimated quantitatively and can be well controlled by keeping a safe distance. Increasing interest is focused now on the nonthermal effects of weak nonionizing radiation. But there is still a great lack of available data, especially in man, and the fundamental mechanisms of the interaction with biological systems are not yet understood in detail.

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E-Mail karger@karger.ch
Fax +41 61 306 12 34

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Dr. Klaus Mann
Department of Psychiatry
University of Mainz
Untere Zahlbacher Strasse 8
D–55101 Mainz (Germany)

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In the present paper we studied the effects of the electromagnetic field, irradiated by the aerial of a digitale mobile radio telephone, on the sleep of normal subjects. These devices operate on the base of pulsed high-frequency radiation in the lower microwave frequency range. In our design the intensity is weak, not leading to thermal effects. Sleep appears to be an appropriate physiological process to be studied in this area of research. On the one hand, sleep is a very complex biological process, controlled by the central nervous system and reacting very sensitively to external influences. And although the exact biological mechanisms are not known in detail yet, the regular sequences of waking and sleeping states as well as the physiological microstructure of sleep are necessary requirements for correct information processing of the brain, metabolic homeostasis and intact immune function [3]. On the other hand, sleep is a well-defined biological condition, excluding to a large extent other external influences and stressors. Moreover, exposure can be realized over a long period of several hours which might be of relevance due to possible time-integrating interaction mechanisms.

Materials and Methods

Subjects

14 healthy male volunteers, 21–34 years old (mean age 27.3 ± 4.2), participated in the study. To exclude physical or mental illness, a detailed history, psychiatric exploration, physical examination, ECG, EEG, and routine laboratory parameters were taken before beginning the study. Subjects suffering from sleep disturbances were excluded. All subjects were nonsmokers and were not taking any drugs. Consumption of alcohol was forbidden during the period of the study. The subjects were informed in detail about the intention of the study and written consent was obtained. The study design was approved of by the local ethical committee. Two subjects had to be excluded because of technical problems (in one case power failure of the telephone device, in the other case failure of the recording system) and the results are therefore based on the data of 12 subjects.

Experimental Procedure

Each subject spent 3 successive nights in the sleep laboratory. Following an adaptation night, two polysomnographies were performed. EEG signals were measured with Ag/AgCl surface electrodes fixed at the positions F_z, C_z, C_3, C_4, P_z and the mastoid, according to the international 10-20 system. All electrode impedances were below 5 kΩ. Unipolar EEG derivations (vs. mastoid electrodes interconnected by two 5 kΩ resistors) as well as EOG, EMG of the m. mentalis and ECG were recorded.

A digital mobile radio telephone (Motorola, GSM system) was positioned at the head of the bed at a distance of 40 cm to the vertex of the subject. The device was operated from the neighboring room using an extension lead. The telephone aerial emitted a 900 MHz electromagnetic field pulsed with a frequency of 217 Hz and a pulse width of 580 µs. The radiated peak power of the aerial was 8 W, resulting in an average power density of 0.05 mW/cm^2 at a distance of 40 cm. This value is below the permissible limit by a factor of about 20.

The experimental condition was controlled continuously throughout the investigation night. For this purpose, the electromagnetic field emitted by the digital mobile radio telephone was measured by a receiving aerial at a distance of 2 m from the telephone. This aerial was connected to an oscilloscope which was positioned in the neighboring central unit. From here the EEG assistant monitored the function of the telephone device.

Protocol

The subjects had not been allowed to fall asleep until the lights were switched off at 11 p.m. Polysomnographies were performed over 8 h, the registration started at 11 p.m. and finished at 7 a.m. when the subject was woken up. In one night an exposure to the electromagnetic field for each subject occurred from 11 p.m. until 7 a.m., i.e. over a duration of 8 h, in the other night the transmitting aerial was turned off. The order of application was randomized and the subjects were not informed about the experimental condition. In 6 subjects the first polysomnography was performed under exposure to the field and the second without application of the field, while for the other 6 subjects the sequence was vice versa.

Rating scales for measuring subjective perception of sleep quality and well-being during the following day (VIS-M, VIS-A) were filled out by each subject in the morning and in the evening of the day following the sleep laboratory investigations [4]. In addition, side effects were assessed by brief non-standardized interviews.

Data Analysis

All signals were recorded on paper using a Nihon Kohden EEG machine (0.3 s time constant and 50 Hz, 24 dB/octave low-pass filter for EEG signals). The sleep EEGs were scored visually by one experienced rater who was blind with respect to the experimental condition. Sleep stages were defined according to the criteria of Rechtschaffen and Kales [5]: wakefulness (predominating alpha activity), stage I (low voltage, mixed frequency EEG without rapid eye movements), stage II (background EEG similar to stage I, but additionally sleep spindles and K complexes), slow wave sleep (SWS, stage III and IV, characterized by high amplitude, slow wave activity), stage REM (characterized by rapid eye movements and low amplitude EMG).

For further computer analysis, additional 45-Hz low-pass filtering (48 dB/octave) was carried out to avoid aliasing effects. The EEG signal derived from C_z was sampled at a rate of 100 Hz and digitized by a 12-bit analog-digital converter. The data were stored continuously on the disc of a Hewlett-Packard A-900 digital computer in 1440 successive time intervals, each consisting of 2048 data points. Off-line power density spectra of the EEG signal were calculated applying FFT to each of the 1440 time intervals. Afterwards all spectra of a single night, unambiguously corresponding to one of the classical sleep stages, were averaged.

Statistical Analysis

Statistical comparison of the classical sleep EEG parameters (table 1) and of the subjective parameters (table 4) was performed applying Wilcoxon test for paired samples (two-sided). To recognize alterations of sleep stages in the course of the night, the duration of the various sleep stages in the 1st, 2nd and 3rd third of the night was

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determined. For statistical analysis, a two-way ANOVA model with time (1st, 2nd, 3rd third) and field exposure (without field, with field) as within-subject factors was applied for the different sleep stages, preceded by the Kolmogoroff-Smirnoff test in order to prove normal distribution.

For assessment of EEG power spectra, distinct frequency ranges were defined and the mean value of the spectral power density in these frequency bands was calculated. Statistical analysis was performed applying a two-way ANOVA model with sleep stage (awake, stage I, stage II, slow wave sleep, REM) and field exposure (without field, with field) as within-subject factors for distinct frequency ranges. Before, normal distribution was proven by the Kolmogoroff-Smirnoff test. Differences were considered statistically significant with a p value < 0.05.

Preliminary Investigation

The study was preceded by a preliminary investigation to exclude direct effects of the electromagnetic field on the polysomnographic recording device. We studied a spherical model of the head which was positioned at a distance of 40 cm to the digital mobile radio telephone, according to the subject's head position. The electrode impedances were simulated by a passive electrical network, linking the electrodes by 5 kΩ resistors. Corresponding to the study design, the voltage between the electrode positions F_z, C_z, C_3, C_4, P_z versus mastoid was recorded continuously during two sessions of 8 hours' duration each. In one session the electromagnetic field was turned on, in the other it was turned off. Recording on the EEG machine and digitalization were performed in an identical manner as described above.

No direct influence of the electromagnetic field on the polysomnographic recording device was found. In both sessions, without and under exposure to the field respectively, the same noise level was measured in the relevant frequency range. Also, there was no time dependent alteration of the signal under the influence of the field during the registration period of 8 hours' duration.

Table 1. Classical sleep EEG parameters (mean ± standard deviation of 12 subjects)

	Without field	With field
Sleep onset latency, min	12.25 ± 5.96	9.50** ± 4.44
Total sleep time, min	470.04 ± 14.11	473.38 ± 12.80
Sleep efficiency index, %	0.95 ± 0.03	0.95 ± 0.03
Number of awakenings	5.17 ± 5.51	4.58 ± 2.61
Awake, %SPT	2.99 ± 3.45	2.95 ± 2.71
Stage I, %SPT	9.22 ± 2.20	10.22 ± 3.06
Stage II, %SPT	52.98 ± 5.33	54.14 ± 7.03
Slow wave sleep, %SPT	15.70 ± 3.30	16.69 ± 5.86
REM, %SPT	17.07 ± 3.23	13.91* ± 2.88
REM latency, min	84.96 ± 22.69	93.17 ± 27.87

SPT = Sleep period time. * $p < 0.05$; ** $p < 0.005$.

Table 2. Mean power density (dB) of the averaged power spectra of different sleep stages in the frequency range from 1 to 20 Hz (mean ± standard deviation of 12 subjects)

	Without field	With field
Awake	−5.846 ± 1.809	−6.563 ± 1.524
Stage I	−8.109 ± 1.323	−8.717 ± 1.121
Stage II	−6.909 ± 1.574	−7.134 ± 1.438
Slow-wave sleep	−6.703 ± 1.828	−7.131 ± 1.827
REM	−10.133 ± 1.431	−9.595* ± 1.606

0 dB corresponds to 1 $(\mu V)^2$/Hz. * $p < 0.05$.

Results

The results of the classical sleep analysis according to the criteria of Rechtschaffen and Kales [5] are summarized in table 1. The sleep efficiency index was identical under both experimental conditions. Sleep onset latency was significantly reduced from 12.25 to 9.50 min under exposure to the electromagnetic field ($p < 0.005$). REM latency showed a tendency to increase. However, the difference in REM latency did not reach statistical significance. Regarding sleep architecture, in the exposure night a significant decrease of the percentage of REM sleep from 17.07 to 13.91% could be observed ($p < 0.05$), while the other sleep stages were not significantly affected.

The duration of the sleep stages was determined separately for the 1st, 2nd and 3rd third part of the night, in order to recognize possible alterations during the night. For statistical analysis, a two-way ANOVA model with time (1st, 2nd, 3rd third) and field exposure (without field, with field) as within-subject factors was applied. With regard to REM sleep, the statistical analysis revealed a significant main effect for the factor field exposure ($F_{1,22} = 5.64$, $p < 0.05$), which is in accordance with the decrease of the percentage of REM sleep mentioned above. For the non-REM sleep stages, no significant main effect for the factor field exposure was found. In all sleep stages, including REM sleep, no significant interaction between field exposure and time of the night was found. Thus, shortening of the duration of REM sleep under exposure to the field (82.50 min without field, 67.88 min with field) was not restricted to a specific part of the night.

For assessment of the EEG power spectra, first the entire frequency range from 1 to 20 Hz was taken as a basis. Table 2 shows the mean power density of the averaged spectra for each sleep stage. Analysis by a two-way ANOVA (table 3) with sleep stage and field exposure as

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Table 3. F-values of the ANOVA procedures with sleep stage and field exposure as within-subject factors applied to the mean power densities in the given frequency ranges

	Sleep stage	Field	Sleep stage × field
Total	37.99***	1.76	3.58*
Delta	162.43***	0.41	1.35
Theta	71.83***	0.43	1.83
Alpha1	37.35***	2.40	3.80**
Alpha2	36.69***	4.29	4.34**
Beta	83.91***	1.71	1.50

For definition of frequency bands, see text (total = 1–20 Hz). d.f. 1 = 4, d.f. 2 = 44.
* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Table 4. Subjective parameters (selected items of VIS-M concerning subjective sleep perception and VIS-A concerning mental condition during the following day; mean ± standard deviation of 12 subjects)

	Without field	With field
VIS-M		
Alertness in the morning (analog 0–100)	56.75 ± 12.70	60.25 ± 16.76
Sleep quality (analog 0–100)	58.08 ± 19.29	55.66 ± 14.14
Sleep latency, min	31.25 ± 14.00	27.08 ± 12.52
Number of awakenings	2.41 ± 1.44	2.16 ± 1.19
Sleep period, min	432.08 ± 58.56	439.16 ± 51.47
Frequency of 'bad' dreams (0 = not at all; 1 = once or twice, 2 = more than twice)	0.42 ± 0.67	0.25 ± 0.45
VIS-A		
Calmness (analog 0–100)	62.73 ± 15.99	71.09* ± 18.87
Energy level (analog 0–100)	54.27 ± 20.91	65.09 ± 22.33
Concentration (analog 0–100)	65.33 ± 20.01	61.33 ± 23.64
Anxiety (analog 0–100)	67.82 ± 19.55	71.46 ± 18.74

For the analog scales the range is from 0 to 100, 0 = worst and 100 = best (if necessary the variables were transformed). * $p < 0.05$.

within-subject factors revealed, besides a significant main effect for the factor sleep stage which is evident ($F_{4,44}$ = 37.99, $p < 0.001$), a significant interaction between sleep stages and field exposure ($F_{4,44} = 3.58$, $p < 0.05$). Pairwise comparison of the experimental conditions without and with field in the individual sleep stages revealed an increase of the mean power density during REM sleep ($p < 0.05$), while a decreasing tendency of the mean power density was observed in the sleep stages I, II, slow wave sleep and in the waking state. For a more detailed analysis, smaller frequency bands were defined: delta = 1–3.5 Hz, theta = 3.5–7.5 Hz, $alpha_1$ = 7.5–12.5 Hz, $alpha_2$ = 12.5–15 Hz, beta = 15–20 Hz. The results are illustrated in figure 1. During REM sleep, an increase of the mean power density under field exposure was found in all frequency bands, whereas the other sleep stages showed again a decreasing tendency for all frequency bands. Two-way ANOVAs for the different frequency bands (table 3) revealed significant interactions between sleep stages and field exposure for the $alpha_1$ band ($F_{4,44} = 3.80$, $p < 0.01$) and the $alpha_2$ band ($F_{4,44} = 4.34$, $p < 0.01$).

None of the subjects reported any side effects during the investigation nights or the following days. The results obtained from the evaluation of the rating scales are summarized in table 4. Regarding subjective sleep quality and alertness in the morning, no significant effects could be observed. Also, the subjective sleep efficiency parameters sleep latency, number of awakenings and sleep period showed no significant alteration, but the changes were concordant with the corresponding objective sleep EEG parameters. During the day following nocturnal exposure to the electromagnetic field, the subjects felt significantly more calm ($p < 0.05$) and reported a higher energy level ($p = 0.055$) compared to the night without application of the field.

Discussion

Besides a hypnotic effect with significantly reduced sleep onset latency, exposure to the electromagnetic field induced an alteration of REM sleep quantitatively as well as qualitatively. The significant decrease in the duration and the percentage of REM sleep, together with the trend towards an increase of REM latency, points to a REM-suppressive effect of the field. Moreover, spectral analysis revealed a specific alteration of the EEG signal during REM sleep with an increased spectral power density, which was not observed in the other sleep stages and in the waking state. Here the alpha frequency range was mainly affected. Regarding the reports of the subjects, no significant impairment of sleep perception was observed, well-being during the following day was affected in the sense of increased calmness.

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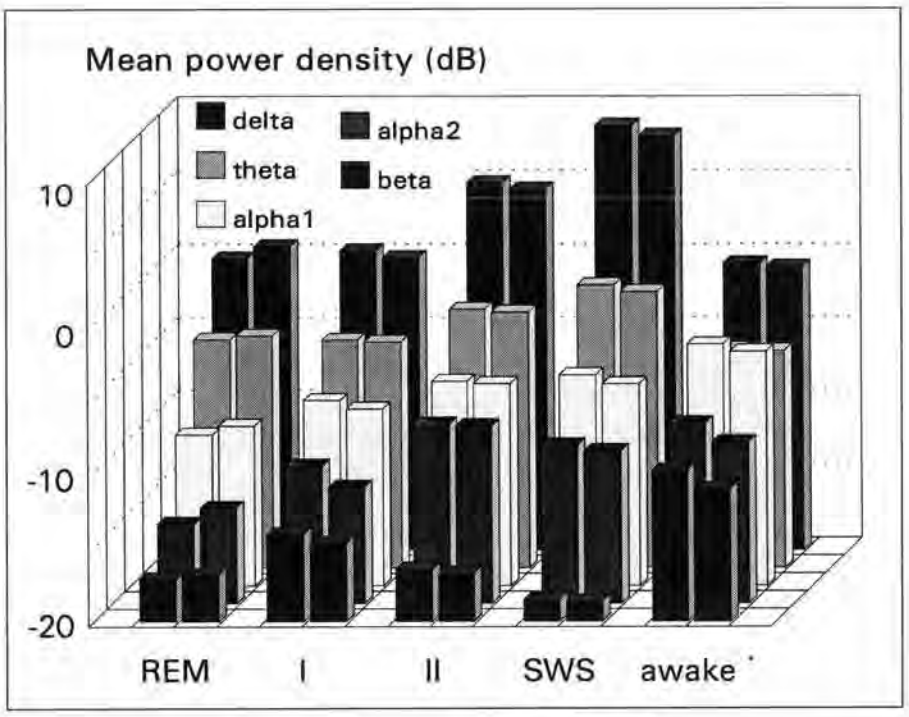

Fig. 1. Mean power density (dB) of the averaged power spectra of different sleep stages in the given frequency ranges. For definition of frequency bands, see text. Mean of 12 subjects (0 dB corresponds to 1 $(\mu V)^2$/Hz). Left columns: without field; right columns: with field.

The results indicate a specific alteration of sleep regulation. To our knowledge, comparable investigations do not exist to date. In accordance with our findings, Reite et al. [6], however, also reported a hypnotic effect during waking EEG registrations in healthy subjects. Exposure to a 27.12-MHz radiofrequency electromagnetic field amplitude-modulated (sine wave) at 42.7 Hz and applied over a 15-min time period resulted in a shortening of sleep latency, an increase in the duration of sleep stages, and the subjects reached a deeper stage of sleep compared to the placebo treatment. As in our study, no appreciable sensations were experienced by the subjects. Direct quantitative comparison of the results (sleep latency, duration of sleep stages) is not possible due to methodical differences. Besides different carrier frequencies and modulation conditions, an older classification of sleep stages was used by Reite et al. [6], based on the Loomis criteria. With regard to sleep architecture, no conclusion can be drawn because the EEG registrations performed by Reite et al. [6] were restricted to 15-min only. Moreover, the underlying interaction mechanisms might be different in the two studies. Although several reports indicate that pulsed microwaves, as used in our study, are more effective than continuous-wave radiation, at present it is an open question whether the two waveforms might elicit different biological effects at the same average power density. However, the occurrence of thermoelastic phenomena in biological tissue is generally accepted in the case of pulsed electromagnetic fields [1], possibly contributing to the observed effects in our study.

Regarding the observed alterations of sleep architecture, some support of our results comes from a clinical study performed by Sandyk et al. [7], although only a very limited comparison can be made due to the greatly varied experimental conditions. In neurological patients exposed to a magnetic field, behavioral effects could be produced which paralleled those observed in REM sleep-deprived subjects. Therefore, the authors hypothesize that the behavioral effects of treatment with magnetic fields may be mediated via REM sleep deprivation. However, no sleep EEG registrations were performed in this study.

The interaction mechanism of the electromagnetic field and the sleep regulation system is unknown. Until now, no comprehensive theory exists which could provide a satisfactory explanation of the variety of reported effects in biological systems under exposure to electromagnetic fields. However, in recent years several hypotheses have been developed based on experimental findings and theoretical considerations [for an overview see 1, 2, 8]. It now appears quite certain that weak nonionizing radiation, not leading to a heating of tissue, is able to cause biological effects. According to theoretical considerations by

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Weaver and Astumian [9], macromolecules are sensitive to very weak external electrical fields due to conformational transitions that involve intramolecular movements of charge or changes in dipole moments. Such field-induced conformational changes could for instance modulate enzyme activities. Considering the time-integrating mechanisms of enzyme-catalyzed membrane-associated reactions, field intensities become relevant which are below the thermal noise due to random fluctuations in the transmembrane potential.

Several studies have identified the cell membrane as the primary site of interaction with electromagnetic fields [10–13]. There is strong evidence for internal nonlinear, non-equilibrium processes, induced by the fields, modifying the coupling of humoral stimuli from surface receptor sites to the cell interior, such as neurotransmitters and hormones. Intramembranous protein particles which form pathways for signalling and energy transfer play a key role, as well as alterations of calcium-ion binding along the membrane surface. Thus, by modulating the inward and outward signal streams through the cell membrane, electromagnetic fields can lead to alterations of intracellular processes as well as intercellular communication.

On the cellular level, a number of phenomena induced by electromagnetic fields in biological systems have been described, which in principle can modify the function of the central nervous system and thus could be accountable for our findings. Especially a disturbance of the calcium homeostasis could be proven, which is important for a multitude of both basic cellular and complex neuronal processes, such as neural excitation, regulating neurotransmitter secretion and hormone molecule binding at membrane receptor sites [14–16]. There are also indications for modifications of information processing in the nervous system. Arber and Lin [17] reported inhibitory as well as excitatory influences of high-frequency electromagnetic fields depending on the kind of signal modulation. Kullnick [18] observed alterations of the membrane potential of central neurons in form of hyperpolarization under exposure to weak non-ionizing radiation. Additionally the threshold of excitation was changed. Moreover, effects on upper physiological levels must be discussed, such as alterations of endocrine systems. Here, especially melatonin has received attention [19, 20]. Also, in several investigations, influences on neurotransmitters were found [reviewed in 2], such as serotonin, noradrenaline and dopamine, which are well known to play an important role in the sleep-wake regulation; but in these studies probably hyperthermia was predominantly responsible rather than direct radiation effects.

The relevance of our results concerning possible health hazards cannot be assessed yet. Although a complete understanding of the physiological function of the different sleep stages is still missing, some principal points concerning their functional meaning have become obvious. While slow wave sleep seems to be associated with recovery processes [21], REM sleep plays a special physiological role for information processing in the brain. Here selecting, sorting and consolidating of new experiences received during the waking state were performed as well as linking them together with old experiences [22]. For this reason, modification of REM sleep parameters induced by the electromagnetic field may be associated with an alteration of mnestic functions and learning processes. But because cognitive functions were not assessed in the present study, no conclusions can be drawn about this from our results.

The study was designed on an exploratory base. From the results the hypotheses of a hypnotic effect and a REM-suppressive effect could be formulated, which have to be tested in further studies. Moreover, the results are based on an exposure time of 8 h and must not be directly applied to everyday exposure to digital mobile radio telephone devices. Regarding telephone calls, exposure times usually are clearly shorter, but here higher power densities may occur due to shorter distances.

Further investigations are necessary for a better assessment of the relevance of our results. Besides studying the long-term effects, neuropsychological tests for measuring cognitive functions in detail should be performed. Moreover, the investigations have to be extended to other subpopulations, possibly revealing stronger susceptibility to the exposure to electromagnetic fields, e.g. older subjects or persons claiming disturbances in their mental or physical condition.

Acknowledgement

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Attachment 13

FINAL REPORT

Risk Evaluation of Potential Environmental Hazards From Low Frequency Electromagnetic Field Exposure Using Sensitive *in vitro* Methods

A project funded by the European Union
under the programme
Quality of Life and Management of Living Resources
Key Action 4 "Environment and Health"
Contract: **QLK4-CT-1999-01574**
Start date: **01 February 2000**
End date: **31 May 2004**
Acronym: **REFLEX**

Table of contents

FOREWORD

Prof. William Ross Adey, who made fundamental contributions to the emerging science of the biological effects of electromagnetic fields (EMFs), died on May 20, 2004, in Redlands, California, USA. He was scheduled to deliver his personal views of EMF research at a REFLEX workshop held in Bologna in October 2002. But by then he was already too frail to travel. Dr. Adey who was an informal advisor of the REFLEX consortium sent us his talk in written form. In memory of his achievements as a scientist and in recognition of his support of the REFLEX work, the consortium decided that his message would be an inspiration to all those scientists who are willing to accept the challenges posed by EMF research, and in addition, make a fitting introduction to the final report.

THE FUTURE OF FUNDAMENTAL RESEARCH IN A SOCIETY SEEKING CATEGORIC ANSWERS TO HEALTH RISKS OF NEW TECHNOLOGIES

The Challenge to Conventional Wisdom

The history of bioelectromagnetics epitomizes a range of problems that arise whenever a community of sciences is confronted with a frontier that delves deeply into the established orthodoxies of biology, the physical sciences and engineering. These conflicts have become even more sharply defined when emerging new knowledge in bioelectromagnetics research has challenged the conventional wisdom in each part of this trinity.

Thirty-five years ago, we, who first voiced our observations of physiological responses to a spectrum of environmental EMFs at levels below thresholds for significant tissue heating, were promptly challenged by acolytes of orthodoxies in the biological and physical sciences. At best, we were euphemistically described as “controversial,” a designation that persists to this day. A Yale physicist recently added the charming term “crackpot” to describe a highly qualified biophysicist investigator.

What is the basis of this deep thorn of discontent? Historically, excitation in biological systems has been modeled and tested in terms of equilibrium thermodynamics. In this classic tradition, it was assumed that the potential effectiveness of an exciting agent could be assessed by its ability to transfer energy to the receptor in excess of its random thermal atomic and molecular collisions. Thus, the physical expression kT, the union of the Boltzmann constant and temperature, has been regarded as an expression of an immutable threshold below which an exciting agent would not be physiologically effective. In like fashion from the quantum realm of the physicist, photon energies of low-frequency magnetic fields, now known to act as effective physiological stimuli, would also fall below this thermal barrier.

Here is one example: The human auditory threshold involves a hair cell vibration of 10-11 meters, or about the diameter of a single hydrogen atom. But, by an as-yet-unknown mechanism, the ear suppresses the vastly larger noise of its thermal atomic and molecular collisions, functioning as an almost “perfect” amplifier close to 0°K.

Clearly, we face a profound paradox, with answers to be sought in cooperative states and nonequilibrium thermodynamics, as first suggested in a biological context almost 60 years ago by Herbert Fröhlich.

The lesson is clear. The awesome complexity of biological organization demands our most careful consideration.

The Recent History of Technology Applications

We also find the heat of controversy in the recent history of technological applications in western societies. At no point in the last 20 years has public school education ensured that a majority of citizens has even a basic understanding of sophisticated communication devices and systems, such as telephones, radio and television. Similarly, automotive engineering remains a sea of vast ignorance for most users. Nor is such knowledge considered appropriate or necessary.

In summary, we have become superstitious users of an ever-growing range of technologies, but we are now unable to escape the web that they have woven around us.

Media reporters in general are no better informed. Lacking either responsibility or accountability, they have created feeding frenzies from the tiniest snippets of information gleaned from scientific meetings or from their own inaccurate interpretation of published research. In consequence, the public has turned with pleading voices to government legislatures and bureaucracies for guidance.

Public Concerns and the Evolving Pattern of Research Funding

We face the problem brought on by the blind leading the blind. Because of public pressure for rapid answers to very complex biological and physical issues, short-term research programs have been funded to answer specific questions about certain health risks.

Participating scientists have all too often accepted unrealistic expectations that, in a matter of a few years, they will provide answers to pivotal questions in cell and molecular biology that can only be achieved slowly, painstakingly and collaboratively over a decade or more.

Using EMFs as tools, we have launched our ship on a vast, uncharted ocean, seeking a new understanding of the very essence of living matter in physical processes at the atomic level. This is an awesome and humbling prospect, surely not to be ignored or forgotten in the pragmatic philosophies of most risk research.

In many countries, and particularly in the USA, the effects of such harassing and troublesome tactics on independent, careful fundamental research have been near tragic. Beguiled by health hazard research as the only source of funding, accomplished basic scientists have diverted from a completely new frontier in physical regulation of biological mechanisms at the atomic level. Not only have governments permitted corporate interests in the communications industry to fund this research, they have even permitted them to determine the research questions to be addressed and to select the institutions performing the research.

These policies overlook the immutable needs of the march of science. In their hasty rush to judgment, they have sought a scientific consensus where none can yet exist. Such a consensus will occur only after experimental convergence emerges from a spectrum of related but certainly not identical experiments.

Defining the Role of Epidemiology in Current Controversies

Much in the fashion of ancient Romans, standing four-square and reading the auguries of future events by noting flight patterns of passing birds, the modern-day epidemiologist has become the high priest in the search for correlates of disease processes with a constellation of environmental observables. It is rare for them to be competent in delving into questions of causality, particularly where no exposure metric has been established for a suspected environmental factor. Nevertheless, in courts of law, in legislatures, and among a concerned public, epidemiological opinions have become a gold standard, typically outranking evidence based on a balanced and often cautionary review of current medical science.

We should remind ourselves that their professional tool is biostatistics —they build endless Byzantine edifices of levels of statistical risk, with little or no commitment to the underlying science or medicine. Their mutual discussions have produced the technique of meta-analysis, the pooling of statistical analyses from a series of epidemiological studies. The method ignores the nuances of both experimental design and epidemiological findings in the separate studies, and blinds us to options for further research based on the possible uniqueness of these separate observations.

It appears reasonable that there should be no more large epidemiological studies on human EMF exposures until essential exposure metrics are established, based on mechanisms of field interactions in tissues.

Repairing the Body Politic of Science: Some Personal Reflections

The passage of time across the years has not diminished in any way the importance, even the urgency, that one feels towards the growing edifice of science. We must not fail to engender in younger minds a passionate curiosity and an imagination sufficient to kindle their commitment to all that is great and good in the scientific method.

As I reflect on major changes wrought in the U.S. national research scene over the past 40 years, I sense a deep and growing concern that research training and the culture of research accomplishment have stifled

the burning thorn of personal discontent that should be the creative option of all young minds entering on a research career.

Graduate students are assigned a project that is typically a segment of their advisor's grand vista. They may not deviate to ask creative “what if?” questions. They emerge from the chrysalis of their training, bearing a parchment for the professional market place, affirming proficiency in certain techniques, but in no way proclaiming the arrival of that precious citadel of a creative mind.

Please allow me to conclude with an urgent proposal that comes from my own research experience. Formal instruction in physics, theoretical and applied, has become the weakest link for those entering on a career in medical research. Bioelectromagnetics research has opened the door to a new understanding of the very essence of living matter in physical regulation at the atomic level, beyond the realm of chemical reactions in the exquisite fabric of biomolecules. Without versatility in biophysics that matches their typical knowledge in molecular biology and biochemistry, none of these students may cross this threshold to the cutting edge of in future medical research. Let us not see this opportunity lost prematurely through prostitution of mechanistic research in the market place of possible health risks.

Thank you for the great privilege of offering these personal reflections.

LIST OF PARTICIPANTS

1. VERUM - Stiftung für Verhalten und Umwelt, München, Germany
   (Scientific person in charge of the project: Prof. Franz Adlkofer)
2. Institut für Klinische Chemie, Universitätsklinikum Benjamin Franklin, Berlin, Germany
   (Scientific person in charge of the project: Prof. Rudolf Tauber)
3. Abteilung für Arbeitsmedizin, Universitätsklinik für Innere Medizin, Wien, Austria
   (Scientific person in charge of the project: Prof. Hugo W. Rüdiger)
4. Institut für Pflanzengenetik und Kulturpflanzenforschung, Gatersleben, Germany
   (Scientific person in charge of the project: Prof. Anna M. Wobus)
5. Insalud, Ramon y Cajal Hospital, Madrid, Spain
   (Scientific person in charge of the project: Dr. Angeles Trillo)
6. STUK - Radiation and Nuclear Safety Authority, Helsinki, Finland
   (Scientific person in charge of the project: Prof. Dariusz Leszczynski)
7. Institut für Biophysik, Universität Hannover, Germany
   (Scientific person in charge of the project: Prof. H. Albert Kolb)
8. Laboratoire PIOM, ENSCPB, Bordeaux, France
   (Scientific person in charge of the project: Dr. Isabelle Lagroye)
9. Universita degli Studi di Bologna, Italy
   (Scientific person in charge of the project: Prof. Fernando Bersani)
10. Institut für Integrierte Systeme, ETH Zentrum, Zürich, Switzerland
    (Scientific person in charge of the project: Prof. Niels Kuster)
11. Cattedra di Farmacologia, Universita degli Studi di Milano, Italy
    (Scientific person in charge of the project: Prof. Francesco Clementi)
12. Ressourcenzentrum für Genomforschung GmbH (RZPD), Heidelberg, Germany
    (Scientific person in charge of the project: Dr. Christian Maercker)

1.0 INTRODUCTION

Based on the state of knowledge acquired during the last 50 years of research on possible biological effects of electromagnetic fields (EMF), the majority of the scientific community is convinced that exposure to EMF below the existing security limits does not cause a risk to the health of the general public. However, this position is questioned by others, who are of the opinion, that the available research data are contradictory or inconsistent and therefore, unreliable. As a consequence, it is necessary that the methodology applied in EMF research to be considerably improved and complemented by the most recent molecular biological techniques. In the REFLEX project, biological effects of extremely low frequency electromagnetic fields (ELF-EMF) and radio frequency electromagnetic fields (RF-EMF) are studied using sophisticated and diverse research methodologies separately since it is assumed that the generation of effects, if verifiable at all, may be based on different mechanisms.

Many laboratory investigations have been performed to test the hypothesis that ELF-EMF exposure may constitute a risk to the health of people. This hypothesis is almost entirely based on epidemiological studies, some of which indicate that ELF-EMF may contribute to the development of leukaemia in children, and other cancers in adults chronically exposed in residential environments or occupational settings (NRPB 2001; California EMF Program 2002; IARC Monographs 2002). The existing uncertainty is a source of increasing concern for the public, the health authorities and also the industry. *In vitro* studies have shown that ELF-EMF induces significant biological alterations in a variety of cells and tissues. These changes concern the up-regulation of several early response genes, including c-myc (Jin et al. 2000), c-fos (Rao and Henderson 1996) and hsp70 mRNA (Goodman and Blank 1998), thus increasing the production of stress inducible heat shock proteins (Goodman and Henderson 1988; Tokalov and Gutzeit 2003). In spite of this, it is still an unsolved issue whether or not exposure to ELF-EMF may promote pathological processes such as carcinogenesis and if so, whether or not the field effects are exerted through mechanisms influencing the genome of cells, cell proliferation, differentiation or programmed cell death (apoptosis). Results from several studies have indicated that ELF-EMF does not exert any direct genotoxic effect, but may promote carcinogenesis indirectly by interfering with the signal transduction pathways of cells (Blackman et al. 1985; Liburdy et al.1993). Of course, the present uncertainty could considerably be diminished by increasing our knowledge on the parameters of the electromagnetic field which are critical for the generation of biological effects and of the biological systems which are crucial for the occurrence of pathological cellular events.

As with ELF-EMF, several epidemiological and animal studies also cast suspicion on RF signals to promote cancer and other diseases in chronically exposed individuals (Stewart Report 2000; Hardell et al. 2003). Because of its overwhelming presence in our society, the potential influence of RF-EMF exposure on the development of adverse health effects has become a major topic of interest for all concerned, including the government, the general public, and the industry. Putatively non-thermal, immediate and reversible responses have been described in the literature for several years (Roschke and Mann 1997; Wagner et al.1998; Borbely et al.1999; Preece et al. 1999; Koivisto et al. 2000; Huber et al. 2000; Krause et al. 2000). However, these effects, because of their unspecific nature have been regarded as indications of potential biological responses to electrical excitation, rather than harmful effects able to produce permanent damage to health. To date, several *in vitro* studies have been carried out to investigate the disease causing potential of RF radiation. While most of these studies using different cell systems, exposure set-ups and molecular-biological and toxicological methodologies did not show any biological effect, increasing numbers of studies have come up with contradicting results (Moulder et al. 1999; Vescovic et al. 2002).

As stated above, although investigations of possible biological effects of EMF have been conducted for decades, reliable answers are still missing. Extensive epidemiological and animal studies commonly expected to provide the answer as to whether or not EMF might be hazardous are in progress. However, this approach alone might not be able to provide certain evidence whether EMF can or cannot contribute to the pathogenesis of diseases such as cancer or neurodegenerative disorders. The low sensitivity of the epidemiological methodology in detecting low risk associations is probably insufficient to reliably identify any risk to health caused by EMF. Therefore, although epidemiological studies will be needed to ultimately validate the extent of any potential health hazard of EMF, such research must be supplemented and supported by data from animal and *in vitro* studies. Therefore, *in vitro* studies using the most modern molecular biological techniques such as. genomics and proteomics are urgently needed in order to create at least a hypothetical basis for the understanding of disease development through EMF-exposure. If it can be determined that such a basis exists, it becomes even more important, to search for marker

substances which are specific for EMF exposure. Such marker substances could considerably increase the accuracy of epidemiological studies, so that even a low health risk due to EMF exposure would not escape epidemiological detection.

The main goal of the REFLEX project is to investigate the effects of EMF on single cells *in vitro* at the molecular level below the energy density reflected by the present safety levels. Most, if not all chronic diseases, including cancer and neurodegenerative disorders, are of diverse and heterogeneous origins. This variability is to a great extent generated by a relatively small number of critical events, such as gene mutations, deregulated cell proliferation and suppressed or exaggerated programmed cell death (apoptosis). Gene mutations, cell proliferation and apoptosis are caused by or result in an altered gene and protein expression profiles. The convergence of these critical events is required for the development of all chronic diseases. The REFLEX project is, therefore, designed to answer the question whether or not any of these disease-causing critical events could occur in living cells after EMF exposure. Failure to observe the key critical events in living cells *in vitro* after EMF exposure would suggest that further research efforts in this field could be suspended and financial resources should be reallocated for the investigation of more important issues.

2.0 MATERIAL AND METHODS

2.1 Exposure setups (Participant 10)

In order to compare the results of investigations carried out in the different laboratories and to ensure the conclusiveness of the data obtained in the studies, it is of the utmost importance that the conditions of exposure to EMF be strictly controlled. It was the task of Participant 10 to evaluate and modify already existing setups, to develop new optimised exposure systems and to provide technical quality control during the entire period of exposure. The latter was realised by (1) the conduct of a thorough dosimetry including an analysis of possible artefacts, (2) the continuous monitoring of exposure and environmental parameters and (3) blinded exposure protocols. Details about this work can be found in the appendix.

2.1.1 ELF-EMF exposure setup

A novel ELF setup was developed, and four copies were installed in the laboratories of Participants 3, 4, 7 and 11. The setup consists of two four-coil systems, each of which is placed inside a µ-metal shielding box. The coils produce a linearly polarised B-field over the area of the Petri dishes with a B-field vector perpendicular to the dish plane. The shielded design of the chamber guarantees non-interference between the two units, such that they can be kept close to each other inside the same incubator in order to guarantee identical ambient conditions for the cell dishes. Two fans per coil system ensure fast atmospheric exchange between the chambers and incubator. The airflow temperature is monitored with accurate Pt100 probes fixed inside the exposure chamber.

The signal is generated by a computer-controlled arbitrary function generator. A custom-designed current source allows arbitrary field variations in the range from MHz to 1.5 kHz. The maximum achievable magnetic flux density for a sinusoidal with a frequency of less than 80 Hz is 3.6 mT RMS. Sinusoidal signals with a frequency range from 3 Hz up to 1000 Hz can be applied, controlled and monitored. A powerline signal was defined which represents a worst-case scenario with respect to spectral content and corresponds to the maximum accepted distortions for power systems by the International Electrotechnical Commission (IEC 1995) (Figure 1). In addition to these waveforms, arbitrary field on/off intermittently in the range from seconds to hours can be applied.

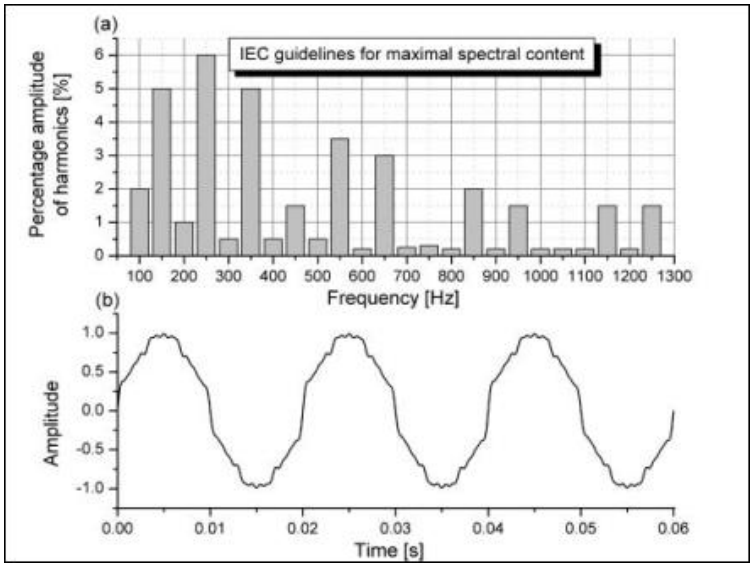


Figure 1. (a) Frequency composition of the powerline signal, corresponding to the maximum allowed spectral content according the IEC guidelines for low and medium voltage power networks (IEC 1995). Shown are the spectral amplitudes of the harmonics in relation to the main 50 Hz component. (b) The resulting powerline signal in the time domain.

The coil current and consequently the magnetic field is quasi-continuously (10s intervals) recorded and regulated using resistors providing low-temperature sensitivity. The currents in the bifilar coils can be randomly switched parallel for field exposure or nonparallel for sham control by the computer. This procedure is used to apply blind protocols and additionally to avoid temperature artefacts between exposed and control coils, since they are heated by the same current.

The evaluation and optimisation of the coil configuration was performed using numerical techniques (Mathematica V4.1) and experimental methods (3-axis Hall meter, FH49 Magnet Physik, Germany). A non-uniformity of less than 1% for the magnetic field over the exposure area of 16 cm x 16 cm x 23 cm is achieved. An uncertainty of 4.3% for the B-field assessment and a B-field variability of 1.6% were found. The average deviation of 2.9% between simulation and measurement is integrated in the uncertainty assessment. Parasitic electric fields generated by the coil system are reduced to less than 1 V/m by a grounded, metallic shielding box between the coil and Petri dishes. The temperatures inside the cell media have been analysed and no temperature differences due to field or sham exposures could be detected (i.e., temperature differences were below 0.1°C). The induced electric fields resulting from a sinusoidal exposure can be expressed as E = 3.2*f*B*r [V/m] and for the powerline exposure as E = 664*B*r [V/m], whereby f is the frequency of the sinusoidal [Hz], B is the average B-field [T] and r is the radial distance from the dish centre [m]. The estimated vibration of the exposed cells is less than 1 m/s^2 (= 0.1 g), which is a factor of 20 above the minimal background level for sham. If an elastically damped dish holder is used (as provided for Participant 4) the vibration load can be further reduced by a factor 12 to 0.1 m/s^2.

In addition to the newly developed exposure systems, it was decided to use two existing setups (Participants 5, 8).

The ELF setup of Participant 5 is based on a pair of Helmholtz coils placed inside a μ-metal shield; exposure and sham are kept in different incubators (no blinded protocols); and sinusoidal B-fields (50 Hz) up to 0.1 mT can be applied.

The ELF setup of Participant 8 is based on two unshielded 4-coil systems arranged in the same incubator; B-fields up to 1 mT (50 Hz) can be applied under non-blinded exposure conditions.

2.1.2 RF-EMF exposure setup

A novel RF setup (GSM) was developed, and four copies were installed in the laboratories of Participants 2, 5, 6, and 8. The system enables EMF exposure of cells under defined conditions with respect to field strengths, polarisation, modulation and temperature and is operated within the GSM DCS mobile frequency band. The setup consists of two single-mode resonator cavities for 1.8 GHz that are placed within an CO_2 incubator. Up to six 35 mm diameter Petri dishes can be exposed in one waveguide resonator. A dish holder guarantees that the dishes are placed exactly in the H-field maximum of the standing wave inside the waveguide.

Each waveguide is equipped with a fan for rapid environmental atmospheric exchange. In order to ensure stable exposure independent of the loading and drifts, monopole antennas are integrated to monitor and control the incident field. The system enables the exposure of monolayers of cells with a non-uniformity of SAR of less than 30% and an efficiency of better than 20 W/kg per W input power.

Much care has been taken to avoid artefacts due to temperature differences between exposed and sham exposed cells. The temperature response of the medium has been assessed by measurements in terms of the incident field strength, cell medium volume and air flow. A temperature load of less than 0.03°C per W/kg SAR was found. The air flow temperature is monitored with accurate Pt100 probes, resulting in differences of less than 0.1°C between the air flow temperature of the exposed and sham waveguides. A numerical heat flow analysis has shown that the possibility of temperature hot spots inside the medium can be excluded.

Field strengths, temperatures and fan currents as well as all commands are continuously logged to encrypted files which are evaluated after the experiments in order to ensure studies under 'blind' conditions (exposure and sham conditions are blindly assigned to the two waveguides by the computer-controlled signal unit).

Field, SAR and temperature characterisations were performed with numerical methods (FDTD simulation platform SEMCAD, SPEAG, Switzerland) and were experimentally verified using the near field scanner DASY3 (SPEAG, Switzerland) equipped with dosimetric field and temperature probes. An uncertainty and variability analysis resulted in an absolute uncertainty for the SAR assessment of 20% and a variability of 5%. The average deviation of 15% between SAR measurement and simulation is within the range of the uncertainty and therefore verifies the reliability of the numerical dosimetry.

The signal unit allows the application of the following five different exposure signals (Figure 2):

- Continuous Wave (CW): An unmodulated CW signal can be applied as a reference (same thermal load, but no ELF modulation components).
- GSM-217Hz: GSM signals are amplitude modulated by rectangular pulses with a repetition frequency of 217 Hz and a duty cycle of 1:8 (pulse width 0.576 ms), corresponding to the dominant modulation component of GSM. The ratio between slot average SAR and time average SAR is 8.
- GSM-Basic: In addition to this basic GSM-217Hz TDMA frame, every 26th frame is idle, which adds an 8 Hz modulation component to the signal. The ratio between slot average SAR and time average SAR is 8.3.
- GSM-DTX: The discontinuous transmission mode (DTX) is active during periods without speaking into the phone. To save battery power, the transmission is reduced to 12 frames per intermediate multiframe of 104 frames (compared to 100 frames for GSM Basic). The frame structure of the DTX signal results in 2, 8 and 217 Hz components. The ratio between slot average SAR and time average SAR is 69.3.
- GSM-Talk: GSM-Talk generates temporal changes between GSM-Basic and GSM-DTX and simulates a conversation with an average duration of 97s and 50s for Basic and DTX, respectively. The ratio between slot average SAR and time average SAR is 11.9. Furthermore, arbitrary field on/off intermittence in the range from seconds to hours can be applied.

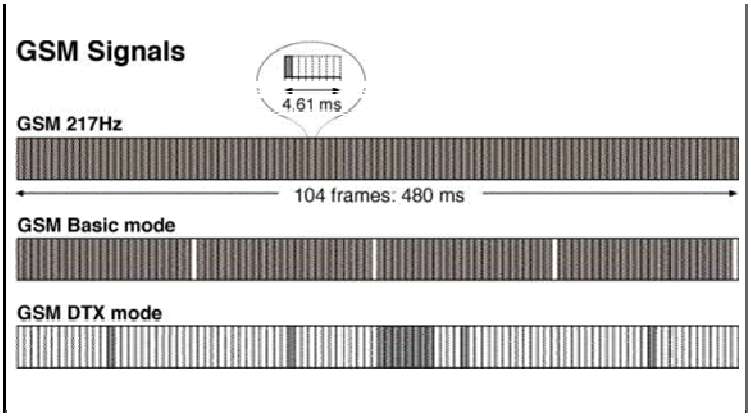


Figure 2. Pulse structure of the applied GSM signals. The basic frame has a period of 4.61 ms and contains a 576 µs burst including 15 µs rising and falling edges. 26 frames make up a GSM multiframe (MF) and 104 frames a GSM intermediate multiframe (IMF). GSM-217Hz is composed of a repetition of basic frames (104 bursts per IMF), whereby every 26th frame is blanked for the GSM-Basic signal (100 burst per IMF). The GSM DTX mode is active during periods of silence and transmission is reduced to 12 bursts per IMF.

In addition to the newly developed exposure systems, it was decided to use three existing setups (Participants 4, 6, 9) and to modify and improve the two setups of Participants 4 and 9.

The RF setup of Participant 4 is based on two R14 waveguides operated at 1710 MHz (Schönborn et al. 2000). Eight 60 mm diameter Petri dishes are exposed with the same concept as for the RF setup (GSM). An identical signal unit is applied, and similar performance is achieved. However, different settings for GSM-Talk were used: Average duration of Basic and DTX was 50s and 97s (instead of 97s and 50s). Therefore the ratio between slot average SAR and time average SAR is 19.8 (instead of 11.9).

The existing RF setup of Participant 6 is based on a R9 resonator cavity operated at 900 MHz. Active water cooling of the Petri dishes is integrated; for details see Toivo et al. (2001). The signal unit can apply GSM-217Hz. Blinded exposure protocols are not possible. In the course of the project, Participant 6 was additionally equipped with the standard RF-setup (GSM).

The RF setup of Participant 9 is based on the Wire-Patch cell and is operated at 900 MHz (Laval et al. 2000). The system was equipped with a new signal unit, allowing the full spectrum of GSM signals. However, also this setup does not allow for computer-controlled blinded exposure conditions. On the other hand, Participant 9 assures that exposure using this system was done blinded (see 2.6.2 below)

2.2 Experiments with human fibroblasts, lymphocytes, monocytes, melanocytes and muscle cells and with granulosa cells of rats (Participant 3)

2.2.1 ELF and RF-EMF exposure setups

See 2.1

2.2.2 Cell culture and exposure conditions

Human diploid fibroblasts (obtained from healthy donors) and SV40 transformed GFSH-R17 rat granulosa cells (Keren-Tal 1993) (provided by Participant 7) were cultivated in Dulbecco´s modified Eagle's medium (DME) supplemented with 10% fetal calf serum (FCS), 20 mM Hepes buffer, 40 µg/ml neomycin, 2 mM L-glutamine, 100 IU/ml penicillin and 100 µg/ml streptomycin. Human melanocytes (male, 3 years old) and skeletal muscle cells (male, 63 years old) were received from Promocell (Heidelberg, Germany) and cultured according to the supplied protocol. Cells were incubated at 37°C in an atmosphere of 5% CO_2 and at 90-100% relative humidity and supplied with fresh culture medium every 48h.

Leukocytes from a healthy donor (female, 31 years old) were isolated from venous blood using Ficoll Paque gradient centrifugation. Cells were washed twice with PBS, resuspended in DME medium with additives and seeded into 35 mm Petri dishes. After 2 hours monocytes had become completely attached on the bottom surface and were separated from lymphocytes by decantation. Monocytes were washed twice with PBS and taken up in 3 ml DME medium.

Lymphocytes from a healthy donor (female, 27 years old) were isolated from venous blood with Ficoll Paque gradient centrifugation. Cells were resuspended in fresh culture medium (DME, 25% FCS, 20 mM Hepes buffer, 40 µg/ml neomycin, 2 mM L-glutamine, 100 IU/ml penicillin and 100 µg/ml streptomycin) with or without stimulation with phytohemagglutinine (1%). The cells were seeded into 35 mm Petri dishes at a density of 2×10^5 cells/3 ml, 24 hours prior to ELF-EMF exposure.

The cells mentioned above were exposed or sham-exposed in suitable waveguides connected with an ELF-EMF generation system provided by Participant 10 within a Heraeus incubator (model Kendro BBD 6220). After exposure cells were detached with trypsin and suspended in fresh culture medium for Comet assay analysis or maintained in culture for cytogenetic testing. Each exposure level was tested in duplicate. In another series of experiments human fibroblasts (cell strain ES-1) and SV40 transformed GFSH-R17 rat granulosa cells (Keren-Tal 1993) (provided by Participant 7) were exposed or sham-exposed in suitable waveguides connected with a RF-EMF generation system (RF 1800 MHz) provided by Participant 10 within a Heraeus incubator (model Hera cell 150l). These experiments were first performed by E. Diem in the laboratories of Participant 2 and later on continued in our own laboratories with a comparable RF-EMF exposure setup, but a RF of 1950 MHz. After exposure cells were detached with trypsin and suspended in fresh culture medium for Comet assay analysis or maintained in culture for cytogenetic testing. Each exposure level was tested in duplicate.

Combined exposures to UVC and ELF-EMF were performed on ice using a germicidal lamp (60 W, Desaga, Heidelberg, Germany), the output of which predominantly contains UVC (253.7 nm). Exposure to UVC prior to ELF-EMF was carried out at an intensity of 2 W/m^2 (measured with a radiometer, Blak-

ray®, Ultra-violet products. Inc., model J225, San Gabriel, USA) for 10 minutes, which equals 1.2 kJ/m^2. Exposure to UVC post to ELF-EMF exposure was performed at an intensity of 2.5 W/m^2 for 30 minutes, which equals 4.5 kJ/m^2.

For thermal exposure cells were incubated at 38.5°C for 4 hours in a commercial incubator (BBD 6220, Kendro, Vienna, Austria). To study repair kinetics, cells were further incubated at 37°C for up to 24 hours.

2.2.3 Comet assay

We followed the technique described by Östling and Johanson (1984) with minor modifications by Singh et al. (1988, 1991). EMF-exposed and sham-exposed cells (10,000 – 30,000) were mixed with 100 µl low melting agarose (0.5%, 37°C) to form a cell suspension, pipetted onto 1.5% normal melting agarose pre-coated slides, spread using a cover slip, and maintained on a cold flat tray for about 10 minutes to solidify. After removal of the cover slip the third layer of 0.5% low melting agarose was added and solidified. The slides were immersed in freshly prepared cold lysis solution (2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris, pH 10, 1% sodium sarcosinate, 1% Triton X-100, 10% DMSO, pH 10) and lysed for 90 minutes at 4°C. Subsequently, the slides were drained and placed in a horizontal gel electrophoresis tank side by side, nearest the anode. The tank was filled with fresh electrophoresis buffer (1mM Na_2EDTA, 300 mM NaOH, pH>13 or pH=12.1 in case of alkaline Comet Assay and 100 mM Tris, 300 mM sodium acetate, 500 mM sodium chloride, pH 8.5 in case of neutral Comet Assay) to a level approximately 0.4 cm above the slides. For both, alkaline and neutral Comet assay, slides were left in the solution for 40 minutes for equilibration and unwinding of the DNA before electrophoresis. Electrophoresis conditions (25 V, 300 mA, 4°C, 20 min, field strength: 0.8 V/cm) were the same for neutral and alkaline Comet assay. All steps were performed under dimmed light to prevent the occurrence of additional DNA damage. After electrophoresis the slides were washed 3 times with Tris buffer (0.4 M Tris, pH 7.5) to neutralise, then air-dried and stored until analysis. Comets were visualised by ethidium bromide staining (20 µg/ml, 30 seconds) and examined at 400 X magnification using a fluorescence microscope (Axiophot, Zeiss, Germany). One thousand DNA spots from each sample were classified into 5 categories corresponding to the amount of DNA in the tail according to Anderson et al. (1994) with modifications. The proposed classification system provides a fast and inexpensive method for genotoxic monitoring. Due to the classification to different groups by eye, no special imaging software is required. The different classification groups are not weighted equally, due to the fact that they do not represent equal grades of damage. Moreover, the technique becomes more sensitive, because many cells can be scored in a short time (1000 cells instead of 50-100 cells with image analysing). The subsequent calculation of a "Comet tailfactor" allows quantifying DNA damage as a single figure, which makes it easier to compare results. Due to the scoring of 1000 cells in one experiment, which are tenfold the cells processed with image analysing, standard deviations are very low. Reproducibility has been thoroughly checked. Results expressed as "Comet tailfactors" were calculated according to Diem et al. (2002). All experiments were performed in duplicate by the same investigator.

2.2.4 Micronucleus assay

Micronucleus (MN) assay was performed according to Fenech and Morley (1985) and Fenech (1993). Fifty thousand cells were seeded into slide flasks (Nunc, Roskilde, Denmark) and exposed to ELF-EMF. In order to block cytokinesis, cytochalasin B (3 µg/ml, Sigma, St. Louis, USA) was added four hours before the first round of replication. After termination of the culture, fibroblasts were treated with hypotonic KCl solution (0.075 M, 30 min.) and fixed with a mixture of methanol : aqua bidest. (7:3) for 10 min. Slides were air-dried and stained with 4,6-diamidino-2-phenylindole (DAPI, Sigma, St. Louis, USA) for 3 minutes. MNs were visualised under a fluorescence microscope and 2000 binucleated cells were scored according to criteria published by Lasne et al. (1984). The results are expressed as MN events/500 binucleated cells.

2.2.5 Chromosomal aberrations

For evaluation of chromosomal aberrations 2 x 10^5 cells were seeded into 35 mm petri dishes (Nunc, Roskilde, Denmark) and exposed to EMF at conditions producing maximum effects in the Comet assay.

After EMF exposure, fibroblasts were trapped at metaphase by incubation with colcemid (0.2 µg/ml, Invitrogen Corporation, Paisely, Scotland) for the last 4h prior to harvesting. Subsequently, the cells were detached with trypsin (Invitrogen Corporation, Paisely, Scotland) and subjected to a hypotonic treatment (0.075 M KCl, 37°C, 30 min.). Thereafter, cold fixative (methanol : acetic acid 3 : 1) was slowly added and cells were collected by centrifugation. Fixation procedure was repeated twice. Finally, the cells were resuspended in 0.5 ml of fixative, dropped on clean slides, air dried, stained for 12 minutes with 4% GIEMSA, prepared in Sorensen's buffer (38 mM KH_2PO_4, 60 mM Na_2HPO_4 x 12 H_2O, pH=7) and rinsed with aqua bidest. Chromosomal aberrations were evaluated in 10,000 well-spread and complete (46 chromosomes) metaphases (5,000 ELF-exposed, 5,000 sham-exposed). The identification of chromosome aberrations was carried out following the criteria recommended by the WHO (1985). Different types of aberrations (chromosome gaps, chromosome breaks, ring chromosomes, dicentric chromosomes and acentric fragments) were scored separately. Five independent experiments were performed. Results are expressed as percent chromosomal aberrations per cell.

2.2.6 Fluorescence *in situ* hybridisation (FISH)

For evaluation of stable translocations, cells were seeded into 35 mm petri dishes at cell density of 2 x 10^5/3ml. After ELF-EMF exposure and an additional repair time of one replication round, fibroblasts were trapped at metaphase by incubation with colcemid (0.2 µg/ml, Invitrogen Corporation, Paisely, Scotland) for the last 4h prior to harvesting. Subsequently, the cells were detached with trypsin (Invitrogen Corporation, Paisely, Scotland) and subjected to a hypotonic treatment (0.075 M KCl, 37°C, 30 min.). Thereafter, cold fixative (methanol : acetic acid 3 : 1) was slowly added and cells were collected by centrifugation. Fixation procedure was repeated twice. Finally, the cells were resuspended in 0.5 ml of fixative, dropped on clean slides and air-dried. Subsequently, slides were denatured (70% formamide/2×SSC pH 7, 72°C, 2 min) and immediately dehydrated through a cold (-20°C) ethanol series (70%, 80%, 90%). The rhodamine-labelled whole chromosome probes (chromosome 1-22, X, Y) were prepared according to the recommendation of the manufacturer (Appligene Oncor Q-biogene, Illkirch, France). Aliquots of 30 µl were applied to the metaphase preparations, the slides were covered with a cover slip and incubated overnight in a moist chamber at 37°C. The following day, slides were immersed in 0.5×SSC at 72°C for 5 min, transferred to 1×PBD at room temperature for 2 min and counterstained with DAPI (4,6-diamidino-2-phenylindole, 0.02 µg/ml, Sigma, St. Louis, USA). Fluorescence signals were evaluated using a fluorescence microscope equipped with filters capable of simultaneously passing DAPI/Rhodamine. 1,000 well spread and complete (46 chromosomes) metaphases were scored for translocations for each labelled chromosome.

2.2.7 Changes in mitochondrial membrane potential (JC-1 staining)

Changes in the mitochondrial membrane potential ($\Delta\Psi_m$) upon ELF-EMF exposure were assessed by staining mitochondria with 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethyl-benzimidazolcarbo-cyanine iodide (JC-1, Molecular Probes, Leiden, The Netherlands), a fluorescent dye with high sensitivity to $\Delta\Psi_m$ in intact cells (Cossarizza et al. 1993b; Salvioli et al. 1997). This lipophilic cation forms J-aggregates in the matrix of intact mitochondria (emitting at 590 nm) or is released in a monomeric form (527 nm) from depolarised mitochondria. A good correlation between the J-aggregate fluorescence of JC-1 and $\Delta\Psi_m$ has been reported previously (Smiley et al. 1991). Immediately after exposure to ELF-EMF (50 Hz, 1 mT, 15h, intermittent 5 min on/10 min off) or RF-EMF (GSM basic 1950 MHz, SAR=1 W/kg, 15h, intermittent 5 min on/10 min off), cells were trypsinated, centrifuged (700 g, 5 min) and resuspended in medium (RPMI 1640 with 10% fetal calf serum). Subsequently, the cells were incubated in triplicates at a density of 0.45 x 10^6 cells/ml in complete medium for 15 min at 37°C in the dark with 10 µg/ml JC-1. After incubation with JC-1 the fibroblasts were washed twice with phosphate buffered saline (Gibco, Vienna, Austria) and adjusted to 2.15 x 10^5 cells/ml. From each of the three stained samples per ELF-EMF exposure and sham exposure, respectively, 8 x 250 µl cell suspension have been transferred to a 96 well sample plate. Measurement of red fluorescence (excitation 540 nm, emission 590 nm) and green fluorescence (excitation 485 nm, emission 535 nm) was done on a Wallac Victor 2 fluorescence plate reader (EG&G Wallac, Turku, Finland). Results were expressed as ratios of red/green fluorescence. For positive controls cell cultures were treated for 18h with 20 µM camptothecin (Sigma-Aldrich, Vienna, Austria), a well known inducer of apoptosis.

2.2.8 Statistical analysis

Statistical analysis was performed with STATISTICA V. 5.0 package (Statsoft, Inc., Tulsa, USA) and SPSS 10.0 package (SPSS Inc., Illinois, USA). All data are presented as mean ± standard deviation (SD). The differences between exposed and sham-exposed, as well as between different exposure conditions were tested for significance using independent Student's t-test or one-factorial ANOVA with post hoc Student´s t-test Bonferroni-correction. A difference at $p<0.05$ was considered statistically significant. Correlation was assessed by multiple regression analysis using linear regression.

2.3 Experiments with human HL-60 cells (Participant 2)

2.3.1 RF-EMF exposure setup

See 2.1.2

2.3.2 Cell culture and exposure conditions

Human HL-60 cells (ATCC, Rockville, MD, USA) were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum (Promocell, Heidelberg, Germany), 1% L-glutamine, 1% HEPES buffer and 2% penicillin/ streptomycin (Gibco BRL Life Technologies, Rockville, MD, USA) under temperature- and pH-control conditions. The cell line was maintained in logarithmic growth phase at 37°C in a 5% CO_2 atmosphere. For radio-frequency (RF) exposure experiments the initial seeding density per 35 mm petri dish was 7.5×10^5 cells. In addition to sham-exposed cells, cells incubated under these normal cell culture conditions without the waveguides being connected to the generator system (see below) were examined as incubator controls. In positive control cells DNA breakage was induced either by incubation of cells for 60 min in cell culture medium containing hydrogen peroxide at a final concentration of 100 µmol/l or by 6 MeV γ-irradiation (0.5 Gy, exposure time: 5.2 s). In case of assessing indirectly the generation of reactive oxygen species and directly the modulation of detoxifying capacities of HL-60 cells, culture medium was supplemented with ascorbic acid (10 µmol/l) prior to RF-exposure.

The following exposure conditions were examined with respect to direct and indirect genotoxic effects in HL-60 cells using the alkaline Comet assay, the cytokinesis-block micronucleus assay, the flow cytometric measurement of micronuclei induction and DNA alterations, cytotoxicity testing, assessment of cell viability and cell growth:

- 1800 MHz, continuous wave (C.W.) exposure, 24 hours, SAR=0.2 W/kg, 1.0 W/kg, 1.3 W/kg, 1.6 W/kg, 2.0 W/kg and 3.0 W/kg, compared with the corresponding sham exposed cells, incubator control cells and positive controls.
- At 1800 MHz, SAR 1.3 W/kg, C.W., different periods of exposure, ranging from 2h up to 72h exposure.
- 1800 MHz, SAR of 1.3 W/kg different RF-signals: C.W., 5 min on/10 min off; GSM-217Hz; GSM-Talk were examined.
- 1800 MHz, GSM-DTX, 24 hours, 5 min on/5 min off, SAR 1.0 W/kg, compared with the corresponding sham exposed cells, for gene expression profiling studies.
- 1800 MHz, C.W., 24 hours, SAR1.3 W/kg, compared with the corresponding sham exposed cells and incubator controls for gene expression profiling studies.

Cells were exposed or were sham-exposed in waveguides connected with a RF-generator system in a Heraeus incubator (Model Hera Cell). After each run the cells were immediately taken out of the incubator for subsequent analyses unless otherwise stated. Experiments were performed under blinded conditions in the following way: after the cells were placed in the two waveguides, and the incubator chamber was closed, the selection which of the two waveguides was connected to the RF generator and which remained disconnected, i.e. served as a sham control, was controlled by the computer system provided by Participant 10 and remained concealed to the experimentalists until analyses by the Comet assay and the micronucleus assay were completed and results documented. For decoding which of the two waveguides was connected to the RF-generator or remained disconnected, respectively, and for control of

the experimental conditions of each run, technical data were mailed independently to Participant 10 and returned electronically with electronic documentation of the time points of dispatching and return. Temperature within the waveguides was monitored during each run for as well sham-exposed and RF-field exposed cells and documented electronically. Additionally, at the end of the experiment pH values were controlled within the cell culture medium of sham-exposed, RF-field exposed and control cells.

An independent experiment consisted of 6 exposed and 6 sham-exposed petri dishes (35 mm diameter) with an initial seeding density of 7.5 x 10^5 cells per petri dish. In order to rule out potential differences of the six positions within the waveguide, the cells from the six exposed petri dishes and of the six sham-exposed dishes, respectively, were pooled prior to further analysis. Differences between exposed cells and corresponding controls were tested for significance, employing the Student´s t-test at a level of $p \leq 0.05$.

2.3.3 *in vitro* genotoxicity tests

The cytokinesis-block in vitro micronucleus (MN) assay

The MN assay was carried out as described by Natarajan and Darroudi (1991) according to the guidelines developed by Fenech (1993, 2000), Fenech and Morley (1985, 1986), Fenech and Rinaldi (1995), Fenech et al. (1994) and Garriott et al. (2002). In order to evaluate the frequency of MN in binucleated (BN) human HL-60 cells, cytochalasin B (final concentration 3.0 µg/ml) was added to the growth medium after exposure and washing. Cytochalasin B prevents the cells from completing cytokinesis resulting in the formation of multinucleated cells (Fenech and Morley 1985). The cells were fixed after 24 hours. For fixation the cells were washed and treated with cold hypertonic KCl solution (5.6 g/l). Then the cells were fixed 3 times with a solution of acetic acid/methanol (1:3) and subsequently air dried preparations were made. For the detection of MN in binucleated cells (BNC) the slides were stained with 2.0% Giemsa solution. To determine the frequency of MN of RF-exposed, sham-exposed or control cultures the number of MN in 1000 BNC cells were scored microscopically at 400 fold magnification by one person in 2 slides for each experimental point. All particles about the size smaller than one-third that of the main nuclei, round-shaped with similar staining characteristics as the main nuclei were counted as micronuclei. In particular after high doses of γ-irradiation (2 and 3 Gy, respectively), it was sometimes difficult to distinguish between "true" micronuclei and apoptotic bodies that occurred also in BN cells. Experiments were repeated at least three times independently.

To study the effects of RF-EMF on cell division, the number of BNC relative to the number of mono-, bi-, tri- and tetranucleated cells (% BNC) was determined according to Fenech (2000). Furthermore, apoptotic cells can be recognised by a characteristic pattern of morphological changes, which may be broadly defined as cell shrinkage, cell shape change, condensation of cytoplasm, nuclear envelope changes, nuclear fragmentation and loss of cell surface structures.

Alkaline single cell gel electrophoresis assay (SCGE, Comet assay)

The alkaline SCGE assay was carried out as described by Singh et al. (1988) according to the guidelines developed by Tice et al. (1990, 2000), Fairbairn et al. (1995) and Klaude et al. (1996). After exposure and washing, a single cell suspension of 1 x 10^4 cells was mixed with 100 µl of 0.7% LMP agarose in PBS and transferred to the fully frosted slides precoated first with 1% and then with 0.5% NMP agarose in PBS. Finally, a covering layer (0.7% LMP agarose) was transferred to the slide. All procedures were performed under dimmed light. Subsequently, the slides were covered with a coverslip and allowed to solidify in the refrigerator. Then the coverslips were removed and the slides were immersed for 1 hour at 4°C in lysing solution (2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris, pH 10) containing 1% Triton-X 100 and 10% DMSO added just before use. Thereafter, the slides were exposed to 0.3 M NaOH for 20 min to allow the DNA to unwind. After this, the slides were placed in a horizontal gel electrophoresis tank containing freshly prepared cold electrophoresis buffer (1 mM Na_2EDTA and 300 mM NaOH, pH 13.5) following electrophoresis at 0.8 V/cm (25 V, 300 mA) for 20 min. After electrophoresis, the slides were rinsed 2x with 400 mM Tris, pH 7.5 and were stained with 80 µl ethidium bromide (0.02% in water), covered with a coverslip. To prevent additional DNA damage all steps described above were conducted under dimmed light or in the dark. 50 randomly chosen cells per slide (two slides per culture) were analysed using a 400-fold magnification with a Zeiss fluorescence microscope (Zeiss Axioplan). A computerized image analysis system (Kinetic Imaging 4.0, Optilas, München, Germany) was employed to measure different Comet parameters. As a measure of DNA damage tail length (in µm), Tail Extent Moment, Olive Tail Moment (OTM) and % of DNA in tail were automatically calculated. To determine

DNA migration of exposed, sham-exposed or control cultures, 100 cells were scored microscopically for Comet formation on 2 slides for each experimental point. As a positive control hydrogen peroxide at a final concentration of 100 µmol/l for 1h was used. Experiments were repeated at least three times independently. The results reported are the mean values ± standard deviation (SD).

Positive control through Gamma-Irradiation

Irradiation was administered with 6 MeV X-rays on an linear accelerator to the HL-60 cells (0.75×10^6) in dishes at doses of 0.5, 1.0, 2.0 and 3.0 Gy (dose rate: 5.8 Gy/min). A control dish received no irradiation. Then, both irradiated and non-irradiated samples were returned to the incubator and cultured until analyses at each relevant point were performed.

Viability test

Viability of the cell samples was assessed using the trypan blue exclusion test. The percentages of viable cells were then determined by placing aliquots of the treated cells in a Neubauer chamber and scoring cells for either the absence (viable cells) or the presence (dead cells) of blue staining. Only cultures with a viability more than 90% were analysed.

2.3.4 *in vitro* cytotoxicity testing

In order to exclude in vitro cytotoxic effects different approaches were used to verify cell viability, including trypan blue staining, flow cytometry tests, by which cells with reduced viability are marked by nuclear propidium iodide and the MTT assay, a colorimetric assay, that is based on the ability of viable, i.e. metabolically active cells to cleave tetrazolium salts to form formazan dye.

Cell viability and cell cytotoxicity were assessed by using the MTT assay. MTT is a sensitive first indicator of mitochondrial damage induced by oxidative stressors (Wasserman and Twentyman 1988). To analyse HL-60 cell proliferation, the MTT assay (Cell Proliferation Kit I, Roche, Mannheim, Germany) was used according to the manufacturer's protocol, and the data reported as OD units. This assay is very sensitive for the measurement of cell proliferation based upon the reduction of the tetrazolium salt 3,[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT).

Briefly, around 2×10^3 cells per well were plated in 96-well microtiter plates with 100 µl of medium. 10 µl of a MTT (5 mg/ml) solution (Roche, Mannheim) was added. Incubation occurred for 4h at 37°C. 100 µl of solubilisation solution was added to each well. The plate was allowed to stand overnight in the incubator (37°C, humidified atmosphere). Absorbance was measured at 570 nm. Performing analysis on micronuclei induction, the ratio of BNC against mono-, bi-, tri- and tetranucleated cells is determined, giving a measure of cell division and cell cycle progression. Performing the flow cytometric analysis of micronuclei induction, an assessment of cell viability and also of DNA distribution and therefore of cell cycle alterations become feasible.

2.3.5 Preparation of nuclei suspensions from cells for flow cytometry analysis

The method was performed according to Nüsse and Kramer (1984), Nüsse and Marx (1997) and Wessel and Nüsse (1995). After exposure of cells and subsequent incubation for 24h (recovery time) the medium was removed and cells were washed twice with PBS and counted. Cells were then spun at 100 x g for 5 min at room temperature. Supernatants were removed carefully, the remaining cell pellet was re-suspended by gently shaking. FACS solution I (10 mM NaCl, 3 mM sodium citrate, 10 mg/l RNase A, 3 ml/l of 10% Igepal solution in water, 25 µg/l ethidium bromide freshly prepared before use) was added to the cell pellet and cells were suspended to a density of approximately 1×10^6 cells per ml. The suspension was stirred for 2 sec and was kept for 30-60 min at room temperature in the dark. After adding FACS solution II (70 mM citric acid, 250 mM sucrose, 40 mg/l of ethidium bromide) the suspension was stirred for 2 sec and subjected to FACS analysis.

2.3.6 Flow cytometric exclusion of apoptosis via Annexin V assay and TUNEL assay

Annexin V assay

After exposure to RF-EMF 10^7 cells were centrifuged, washed several times with PBS and the pellet was resuspended with Annexin V binding buffer (Becton Dickinson Biosciences, Heidelberg, Germany), 1 ml of this suspension was incubated for 20 min in the dark in the presence of 100 µl Annexin V binding buffer and 3 µl Annexin V FITC (Becton Dickinson Biosciences, Heidelberg, Germany). After washing with Annexin V binding buffer, cells were resuspended in 300 µl Annexin V binding buffer + 5 µl Propidium Iodide (PI) solution (50 µg/ml) and were analysed by flow cytometry using a FACSCalibur Analytic Flow Cytometer (Becton Dickinson Biosciences, Heidelberg, Germany).

TUNEL assay

After exposure to RF-EMF 10^7 cells were centrifuged, washed several times with PBS and then fixed for 1h by PBS/formaldehyde (4%). Then the cells were washed again, resuspended with PBS and permeabilised with 100 µl Triton X solution (0.01% Triton in 0.1% sodium citrate solution) for 2 minutes, followed by labelling with 50 µl TUNEL reaction mixture (Roche, Mannheim) for 1 h at 37°C. After this incubation the cells were washed and resuspended with 500 µl PBS. The cells then underwent flow cytometric analysis in order to determine the number of green stains (representing apoptotic DNA fragmentation). DNA content analysis was performed on a Becton Dickinson FACScan according to the manufacturer's protocol.

2.3.7 Reactive oxygen species (ROS) and antioxidant enzyme activity

Reactive oxygen species (ROS), including superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl free radical (OH^-) and singlet oxygen ($^1O_2$) have powerful oxidative potential. ROS are capable of attacking lipids, nuclear acids and proteins, resulting in certain degree of oxidative damage. The cell possesses an efficient antioxidant defence system, mainly composed of antioxidative enzymes such as superoxide dismutase, and glutathione peroxidase. These enzymes are able to scavenge excessive ROS to cellular metabolism, and thereby lead to a relative stabilisation of the ROS level under physiological conditions. To evaluate the ROS level differences in RF-exposed and sham-exposed HL-60 cells the Nitric Oxide Assay, the oxyDNA assay, the direct detection by flow cytometry using the fluorescent dye Dihydrorhodamine 123 and the Lipid Peroxidation Assay were used. For measuring the antioxidative enzyme capacity the activities of superoxide dismutase and glutathione peroxidase were evaluated. The tests mentioned above were chosen within a first screening approach in order to assess qualitatively gross changes in ROS levels and antioxidative enzyme activities. All analyses were performed at room temperature unless otherwise stated.

Nitric oxide (NOx)

Nitric oxide (NOx) was measured using the colorimetric Nitric Oxide Assay Kit from Calbiochem (Cat. No. 482650, Calbiochem-Novabiochem GmbH, Bad Soden, Germany). Briefly, nitrate in aqueous solutions (supernatant after centrifugation of 7 x 10^5 cells/ml) was reduced to nitrite by enzymatic conversion by nitrate reductase and was estimated spectrophotometrically at 540 nm using the Griess reaction. The absorbance obtained is compared against a standard curve of known concentrations of NOx (1-25 µmol/l) and the results were expressed as µmol/l NOx. Detection limit is <1 µmol/l for NOx (Miles et al. 1996).

oxy DNA

Oxidative DNA damage, with 8-oxoguanine as the major oxidative DNA product, was measured using the fluorogenic OxyDNA Assay Kit from Calbiochem (Cat. No. 500095, Calbiochem-Novabiochem GmbH, Bad Soden, Germany). The assay utilizes a direct fluorescent probe directly binding to the DNA adduct of 8-oxoguanine (de Zwart et al. 1999, Kasai 1997, Cooke 1996). Briefly, cells (1 x 10^6) were washed first in 1× PBS, then in wash solution, and then by the addition of 100 µl blocking solution with a 1-hour incubation at 37°C. After 2 washes in working solution, cells were incubated with 100 µl FITC conjugate for 1 hour in the dark at room temperature before they were washed twice in washing solution and once in PBS. The FITC labelled protein conjugate binds to the 8-oxoguanine moiety present in the 8-oxoguanosine of oxidized DNA. Finally, cells were resuspended in FACS buffer and were analysed by flow cytometry (FACScan, Becton Dickinson). The presence of oxidized DNA is indicated by a

green/yellow fluorescence. A partial augmentation (shoulder at the right side of the signal) of FL-1 fluorescence intensity indicates an increase in level of oxidative DNA damage, i.e. 8-oxoguanine. In the present study, assays for the screening of oxidative DNA damage were performed after exposure to RF-field (1800 MHz, continuous wave, SAR 1.3 W/kg, 24h) or sham-exposed cells. Oxidatively damaged DNA was quantified by determination of the area under the curve (AUC) of the shoulder at the right side of the signal fluorescence intensity in RF-field exposed cells.

Detection of ROS level with Dihydrorhodamine 123

7.5 x 10^5 cells were incubated with 5 µmol/l dihydrorhodamine123 (DHR123, Sigma, Germany), as a ROS capture (Lopez-Ongil et al. 1998), during sham- or RF-exposure for 24h. Additionally, positive controls were run, in which 100 µmol/l of hydrogen peroxide (H_2O_2) was added for 1h prior to the end of the experiment. Intracellularly, DHR123 is oxidized by ROS to form the fluorescent compound rhodamine123 (Rh123), which is pumped into mitochondria and remains there. After the experiment, cells were harvested, washed with PBS and immediately analysed for Rh123 fluorescence intensity by flow cytometry (FACScan, Becton Dickinson). The percentage of oxidative damage was defined as the percentage of gated HL-60 cells with Rh123 fluorescence. The results presented represent the means of three independent experiments.

Lipid Peroxidation Assay

Lipid Peroxidation was measured using the colorimetric Lipid Peroxidation Assay Kit from Calbiochem (Cat. No. 437634, Calbiochem-Novabiochem GmbH, Bad Soden, Germany). Malondialdehyde (MDA) and 4-hydroxy-2(E)-nonenal (4-HNE), products of lipid peroxidation, can be estimated spectro-photometrically at 586 nm after reaction with a chromogenic reagent at 45°C to obtain an index for lipid peroxidation (Melchiorri et al. 1995, Öllinger and Brunmark 1994, Sewerynek et al. 1995).

Briefly, 3 x 10^6 cells were used per assay. The cells were lysed by repetitive freezing/thawing in 1000 µl distilled water. The cellular membranes were not removed until after the incubation with reagent R1 and R2. The samples were centrifuged at 15.000 x g for 10 minutes to clarify the homogenate supernatant. Immediately prior to reading the absorbance at 586 nm, 200 µl of sample solution, 650 µl of diluted reagent R1 and 150 µl of diluted reagent R2 were mixed to a volume of 1000 µl. A least-square linear regression demonstrates that the standard curve (concentration range 0-20 µmol/l) is a linear function of the concentration of either MDA or 4-HNE. The absorbance values obtained were compared against a standard curve of known concentrations of MDA/4-HNE (1-20 µM). The results were reported as µmol (MDA + 4-HNE)/l. Detection limit is 0.1 µmol/l for (MDA + 4-HNE).

To screen the possible effect of RF-EMF on endogenous antioxidant enzyme activity, the activities of superoxide dismutase (SOD) and glutathione peroxidase (GPX) were determined in HL-60 cells that exposed to RF-EMF (1800 MHz, continuous wave at SAR 1.3 W/kg for 24h) or sham-exposed

Superoxide dismutase (SOD) activity

Superoxide dismutase (SOD) activity of cell homogenates was determined using the Superoxide Dismutase Assay Kit from Calbiochem (Cat. No. 574600, Calbiochem-Novabiochem GmbH, Bad Soden, Germany). Briefly, 4 x 10^6 cells were washed with PBS buffer, diluted in 250 µl of PBS buffer and extracted with 400 µl of a chloroform/ethanol mixture (62.5/35.5 v/v). 40 µl of the aqueous layer of this sample extract was mixed with 30 µl of diluted chromogenic reagent (R1) and 30 µl reagent R2 in 900 µl of an aqueous alkaline solution buffer. The SOD-mediated increase in the rate of auto-oxidation of this reaction mixture was utilised to yield a chromophore with maximum absorbance at 525 nm (Wang et al. 1991, Vilim and Wilhelm 1989). Results were expressed as SOD units. Detection limit for SOD activity is 0.2 U/ml.

Glutathione peroxidase (GPx) activity

Glutathione peroxidase (GPx) activity of cell homogenates was determined using a cellular glutathione peroxidase assay Kit from Calbiochem (Cat. No. 354104, Calbiochem-Novabiochem GmbH, Bad Soden, Germany). Briefly, to assay cellular glutathione peroxidase, 70 µl of cell homogenisate of 1 x 10^6 cells is added to a 1050 µl of a solution containing glutathione (GSH, 1 mmol/l), as a source of reducing equivalents, GSH reductase (0.4 U/ml) and NADPH. The reaction is initiated by the addition of 350 µl of the diluted organic peroxide t-butyl hydroperoxide and the absorbance at 340 nm was recorded over a period of 5 minutes. The rate of decrease in the absorbance (NADPH is converted to NADP) is directly proportional to the GPx activity in the cell homogenisate. Therefore, the difference in absorbance per min

was used to calculate the enzyme activity and results were expressed as GPx units/ mg protein. As a positive control cellular glutathione peroxidase at an activity of 288 mU/ml was assayed at 23°C.

2.3.8 Analysis of cellular growth behaviour

Cellular doubling time

Cellular growth behaviour of HL-60 cells following RF-exposure for 24h (1800 MHz, continuous wave, SAR 1.3 W/kg) with respect to growth velocity as compared to sham and incubator controls was assessed by determination of the cellular doubling time td:

$$\mathrm{td} = \frac{\log 2 \cdot dt}{\log N - \log No}$$

with *dt* = time of exposure with RF field or sham-exposure, *No* = number of cells at the beginning of the experiment and *N* = number of cells at the end of experiment.

Thymidine kinase (TK) assay

Thymidine kinase activities were determined by radioenzyme assay Prolifigen® TK-REA (AB Sangtec Medical, Bromma, Sweden) with ^{125}I-deoxyuridinemonophosphate as substrate. Briefly, assay buffer containing ^{125}I-labelled substrate was added to the HL-60 cell lysate (lysate diluted 1:100) and incubated for 4h. Lysis was performed with 5 x 10^4 cells and a NP40/Tween 20 containing lysis buffer. The reaction was stopped by addition of a separator tablet which binds the phosphorylated product. After washing, radioactivity was measured. The level of radioactivity is directly proportional to the enzyme activity in the original sample. The TK value was calculated from the standard curve and expressed as U/l.

2.3.9 Statistics

To compare the results of the different groups listed above, the Student´s t-test (two-sided test) was used.

2.3.10 Proteomics

Different optimisation strategies for enhancement of 2-dimensional resolution of highly complex cellular protein mixtures were performed during the project. First of all we started with the IPG-strip approach/Pharmacia. Alternatively we also tested the tube gel approach and found, that for our system this proofed to be the most suitable one with the best resolution. This is essential for the following identification steps. Therefore we here focus on the description of the tube gel technology. In general, the methodology for two-dimensional electrophoresis of protein mixtures described by Klose and Kobalz (1995), was performed. Alternatively, a protocol with slight modifications was applied, described below.

IEF-sample preparation

For separation of cellular proteins by 2-dimensional gel electrophoresis cells were lysed after thorough washing with PBS by repeated freezing and thawing. Proteins were solubilised in: 56 mg urea lyophilised with 16.8 µl 0.6 M DTE, 41 µl protein (corresponds to 250 µg of protein)/water, 5 µl Ampholyte 9-11, 8 µl 25% CHAPS, mixed for 30 minutes at room temperature, centrifuged 6 minutes at 14000 g, then the supernatant was taken off as sample.

IEF/2-D PAGE

High resolution 2-dimensional SDS-polyacrylamide gel electrophoresis (2-D PAGE, 23 x 30 cm gels, pI 2-9.5; 14 x 16 cm gels alternatively, pI 2-11) was performed as follows. For the isoelectric focussing of the small gels a pH-gradient extending from pH 2 to pH 11 was generated by means of carrier ampholytes. For the 14x16 cm 2D-gels six 13 cm long and 2.2 mm thick isoelectric focusing gels were prepared with 3.3 g urea, 780 µl acrylamide / N,N'-methylene-bis-acrylamide (30% T, 5.4% C), 480 µl 25% CHAPS, 1.92 ml water and 300 µl ampholyte mix. The ampholyte mix, which can be stored at -20°C, consisted of 340 µl servalytes 5-7, 340 µl ampholines, 113 µl servalytes 3-10, 113 µl servalytes 2-11 and 113 µl servalytes 3.5-10. The IEF-samples were loaded on the tube gel, overlaid with 10 µl sample overlay (12 M urea, 8% w/v CHAPS, 4% ampholyte 9-11, 0.16 M DTE) and with the upper chamber

buffer consisting of a sodium hydroxide solution (20 ml 1N NaOH in 980 ml water). The lower chamber buffer contained 0.6 ml 85% phosphoric acid in 900 ml water. Gels were run in a tube gel apparatus at room temperature. The samples are focused for 9000 Vh. After extrusion gels were equilibrated for 5 minutes in SDS equilibration buffer (10% v/v 0.5 M Tris HCl pH 6.8, 5% v/v 20 mM EDTA 2Na, 20% of a 10% SDS solution, before usage 1.54% w/v DTE was added) and then loaded onto the 14 x 16 cm second dimension vertical slab gel. This gel consisted of a 12.5% SDS separation gel (7.5 ml 2M Tris HCl pH 8.8, 0.4 ml 10% SDS, 1.9 ml 20 mM EDTA 2Na, 12.7 ml water, 15.1 ml of an acrylamide/bisacrylamide solution (30% T, 2.67% C), 12.7 ml water; polymerisation with 20 µl TEMED and 200 µl of a 10% ammonium persulfate solution) and a stacking gel (2 ml Tris HCl pH 6.8, 80 µl 10% SDS, 400 µl 20 mM EDTA 2 Na, 1.1 ml of an acrylamide/bisacrylamide solution (30% T, 2.67% C), polymerised with 8 µl TEMED and 80 µl of a 10% ammonium persulfate solution). The lower chamber buffer for the second dimension consisted of 400 ml Tris/glycine 10x stock solution, 40 ml 10% SDS, diluted to 4000 ml with water, the upper chamber buffer consists of 50 ml Tris/glycine 10x stock solution, 5 ml 10% SDS, diluted to 500 ml with water, 230 mg DTE and 50 µl 0.5% BPB. The isoelectric focussing gel was layered on top of the SDS stacking gel and overlaid with top chamber buffer. Each gel was run for approximately 4 h beginning with 20 mA, 30 mA within the stacking gel and finally 50 mA in the separation gel. After finishing electrophoresis the gels were fixed in 40% ethanol/10% glacial acetic acid overnight, then silver stained according to the method applied by Klose and Kobalz (1995) and then dried.

Two-dimensional polyacrylamide gels were first qualitatively analysed on a light box visually. Clear changes in the protein secretion pattern like newly expressed or disappearing proteins can be detected by this means. The human eye is capable of registering even very small variations like an increasing or decreasing secretion of a characteristic protein, whereas a quantitative analysis of changes protein secretion is not possible. Protein spots of the 2-D gels were displayed by standard staining procedures with silver for image analysis. The corresponding gels were digitised. The spots were detected and the master gel image was calibrated. Image analysis was performed with Proteom Weaver (Definiens, München, Germany). Known proteins serve as "landmark proteins". With the help of their isoelectric points and molecular weights an internal two-dimensional co-ordinate system can be generated. In this system an evaluation of the isoelectric point and molecular weight of any protein of interest together with the determination of its amount expressed in the sample is possible. Qualitative and quantitative analysis of single gels and a direct gel-to-gel comparison was performed by this method.

2.3.11 Gene expression profiling

In an approach to examine effects of RF-EMF on gene expression on the transcriptional level (transcriptome), changes of cellular RNA profiles were analysed by use of the array technology in collaboration with the Resource Center of the German Human Genome Project (Participant 12). Methodological details and detailed presentations of the results obtained together with Participant 12 are provided under 2.10.3 and 2.10.4.

2.4. Experiments with the human neuroblastoma cell line NB69 (Participant 5)

2.4.1 ELF-EMF exposure setup

50 Hz, sine wave magnetic fields (MF) at 10 µT, 100 µT or 2000 μT_{rms} were generated by a pair of coils in a Helmholtz configuration energised by a wave generator Newtronic Model 200MSTPC, (Madrid, Spain). The exposure setup used in these experiments was reproduced from that described by Blackman et al., (1993). Each exposure system consisted of two 1000-turn, 20-cm-diameter coils of enamelled wire, aligned coaxially 10 cm apart and oriented to produce vertically polarised magnetic fields. Cell culture dishes were placed in the uniform MF space within the coils for exposure or sham-exposure. Currents in the coils were adjusted and monitored using a multimeter (Hewlett Packard, model 974A, Loveland, CO) after the flux density was established with fluxgate magnetometers (Bartington, model MAG-3, GMW Assoc and Wandel and Goltermann S.A, EFA-3, Model BN 2245/90.20. Two pairs of coils were mounted

in the centre of magnetically shielded (co-netic alloy) boxes (Amuneal Corp., Philadelphia, PA) housed in incubators (Forma, Models 3121 and 3194) with a 5% CO, 37ºC. The magnetic shielding allowed for reduced environmental fields at the samples' locations. With DC MF = 0.02-0.08 μT and AC MF = 0.07 - 0.1 μT. Each incubator contained a coil system and shielding box, but only one set was energised for each experiment.

Two sets of coils, shielding rooms and incubators were used. In each experimental run, one set of coils was energised at random. The samples in the unenergised set were considered sham-exposed control. See also 2.1.1

2.4.2 RF-EMF exposure setup

See 2.1.2

2.4.3 Cell culture and EMF-Exposure

The human neuroblastoma cell line NB69 was obtained from Dr. M.A. Mena, (Hospital Ramón y Cajal, Madrid) and cultured in Dulbecco's Minimum Essential Medium (D-MEM) supplemented with 15% (ELF-EMF) or 10% (RF-EMF) heat inactivated foetal calf serum (FCS, Gibco), 2 mM L-Glutamine and 100 U/ml penicillin / 100 U/ml streptomycin. The cells were grown at 37ºC in a CO_2 incubator. In each experiment, cells were seeded at a density of 4.5 x 10^4 cells/ml in ∅ 60 mm plastic dishes. NB69 cells cultured in D-MEM were exposed and/or incubated in the presence or absence of retinoic acid (RA). In the experiments with RA, 40 dishes were supplemented with 0.0 μM (20 dishes) or 2.0 μM all trans RA (20 dishes) in absolute ethanol dissolved (1:1000) in culture medium. This vehicle was proven not to affect significantly cell growth when compared to cultures treated with the same volume of medium alone. In the RF-EMF experiment, cells were seeded at a density of 4.5 x 10^4 cells / ml in ∅ 35 mm dish (NUNC) in 12 dishes, supplemented with 0.0 ng/ml (6 dishes) or 20 ng/ml of bFGF (human recombinant, Boehringer Mannheim GmbH, 6 dishes). Immunocytochemical and *in situ* hybridisation studies were carried out 4 days post-plating.

Isolated embryonic neural stem cells

Striata from E15 Sprague-Dawley rat embryos were dissected and mechanically dissociated. Cell suspensions were grown in a defined medium (DF12), composed of Dulbecco's modified Eagle's medium and Ham's F-12 (1:1), 2 mM L-glutamine, 1 mM sodium piruvate (all from Gibco BRL, Life Technologies Inc, Grand Island, New York), 0.6% glucose,25 mg/ml insulin, 20 nM progesterone, 60 μM putrescine, and 30 nM sodium selenite (all from Sigma Chemical Co, St Louis, MO), 100 mg/ml human transferrin and 50 ng/ml human recombinant EGF (both from Boehringer Mannheim GmbH, Germany). After a minimum of five passages, cells were plated at a density of 500.000 cells/dish (Ø 35 mm) on 12 mm glass coverslips coated with 15 μg/ml poly-l-ornithine (immunocytochemistry) or 50 μg/ml poly-l-lysine (in situ hybridisation). The cultures were maintained in DF12 and EGF for 3 days and then switched to DF12 without EGF for longer culture periods. Immunocytochemical and in situ hybridisation studies were carried out at day 3 post-plating. For additional information see Reimers et al., (2001). Neural stem cells (NSCs) are self-renewable, multipotential cells capable of differentiating into the three major neural cell types. The mechanisms, involved in the regulation of NSC's differentiation are not fully understood.

Test for cellular response to retinol (ROL) or retinoic acid (RA)

Different concentrations of ROL and RA were selected within the physiological range in mammals: 0.1 μM to 5 μM. Cells were seeded and supplemented with ROL concentrations of 0.0, 0.1, 0.5, 1.0, 2.0, or 5.0 μM or with RA concentrations of 0.0, 0.5, 1.0, 2.0, or 5.0 μM. Seventy-two hours after plating, the medium was removed and replaced with fresh medium supplemented with the corresponding ROL or RA concentrations. Each of the retinoid concentrations was triple tested (a total of 15 petri dishes per experimental replicate), and a total of 3 experimental replicates were carried out. At the end of five days of incubation in the absence or presence of ROL or RA the cells were scrape-collected in 1 ml of culture medium. Aliquots of the cell suspensions received 1 ml of 0.4% Trypan Blue, and the number of total cells and of viable cells were calculated using a Neubauer chamber. Each sample was double counted.

ELF-EMF exposure conditions

In each experimental replicate 20 dishes with cells (10 with 0.0 µg/ml RA and 10 with 2.0 µg/ml RA) were incubated for three days inside pairs of unenergised Helmholtz coils placed in a shielded chamber, inside a 5% CO_2 incubator, at a 37ºC and 100% RH atmosphere. At the end of day 3 the media were renewed and 10 dishes (5 with 0.0 µg/ml RA and 5 with 2 µg/ml RA) were placed in one incubator; the remaining ten dishes (5 with 0.0 µg/ml RA and 5 with 2 µg/ml RA) were placed in an identical incubator. Both incubators were used, in a random sequence, alternatively for MF exposure and sham-exposure. The exposed group was treated intermittently, 3h on/3h off, to 50 Hz ELF-EMF at 10 or 100 µT magnetic flux densities (MFD) for 42h or 63 hours. At the end of this period the cells were checked for appropriate viability and proper inmunocytochemical characteristics before being processed for analysis of their responses to the physical and chemical treatments. Spectrophotometric analysis of total protein and DNA contents were done following the methods described in Mena et al., (1995). As we described below, cell counting by Trypan Blue exclusion, BrdU incorporation in DNA, PCNA labelling and flow cytometry were assayed in cells exposed to 10 or 100 µT as an estimation of the proliferative activity of NB69 cell line. The percent of apoptotic cells was estimated with TUNEL-labelling (TdT-mediated dUTP nick labelling) and the expression of the phosphorilated cyclic adenosine monophosphate response-element binding protein (p-CREB) was analysed using phosphorylation site-specific antibodies. Assays and analysis were performed blinded to treatment condition. The statistical test used was ANOVA followed by Student's T-test for unpaired data.

RF-EMF exposure condition

Neural stem cells (NSC) and NB69 cells grown on coverslips were exposed for 21 or 24 hours, respectively to 1800 GSM signals (Talk, Basic, CW and DTX signals), at 1-2 W/kg SAR), in 5 min On/10 min Off cycles. In the experiments with neural stem cells a total of 10 replicates were carried out. In each replicate a total of 12 dishes (with three coverslips per dish) were RF-EMF-exposed or sham-exposed, in groups of 6 samples. In the experiments with human neuroblastoma cells a total of 27 replicates were carried out. In each replicate a total of 12 dishes were exposed, in groups of 3 samples, to one of the following treatment combinations: untreated controls, bFGF alone, EMF alone, bFGF + EMF. The sham-exposure and the RF-EMF exposure were carried out inside shielded chambers (IT'IS Setup, Schuderer et al., 2001), in a 5% CO_2, 37ºC and 100% humidity atmosphere. At the end of the 21-h (NSC) or 24-h (NB69) period of RE-EMF exposure or sham-exposure the cell responses were checked for appropriate viability and proper inmunocytochemical characteristics before being processed for analysis of their responses to the physical and chemical treatments through Trypan Blue exclusion, immunocytochemical and in situ hybridisation studies.

Cell counting and cell viability

After treatment the cells were detached from culture dishes and resuspended in 1 ml of media. The cell number was determined in aliquots of 50 µl using a haemocytometer and each sample was double-counted by Trypan Blue exclusion. Doubling time (DT) of proliferating cells was calculated according to Falasca et al., (1999) using the formula DT = T2 - T1 / 3.32 log (X2 / X1), where T1 and T2 are the culture times in hours, and X1 and X2 are number of cells at the corresponding time. The ANOVA test followed by Student's T-test for unpaired data was used for statistical significance ($p < 0.05$).

2.4.4 Immunocytochemical characterisation of NB69 Cells

Cells were grown in Ø 60 mm dishes onto 12 mm-diameter round coverslips for immunocytochemical detection. After fixation with 4% (wt/vol) paraformaldehyde in 0.1 M phosphate buffer, the cells were incubated for 30 min in a blocking solution containing 2.5% (wt/vol) BSA in phosphate-buffered saline (PBS) to prevent non-specific antibody binding. The same solution was used to dilute the different antibodies. Cells were successively incubated with a mouse monoclonal antibody raised against the neuron-specific intermediate filament BIII-tubulin, and for the astrocyte-specific glial fibrillary acidic protein, GFAP), (1:1000, Promega; 1:300, Sigma, respectively). Nestin immunostaining was carried out by 1:5000 dilution of a rabbit antiserum, followed by an anti-rabbit IGL labelled with FITC (1:200; Jackson ImmunoResearch, West Grove, PA, USA).

2.4.5 Immunocytochemical characterisation of neural stem cells (NSC)

EGF-expanded neurospheres were seeded onto adherent substrate and treated with EGF during their first 3 days in culture, in order to enhance expansion of precursor cells. After this period the mitogen was withdrawn, and cells grew in a defined medium, which promoted cell differentiation to neurons, astrocytes and oligodendrocytes. Between 2h and 3 days, the cultures, mainly contained nestin-positive, undifferentiated precursors. At later stages, the total number of cells dropped, as a gradual loss of nestin content occurred, together with and an enhancement in the differentiation processes of neurons, oligodendrocytes and astrocytes.

2.4.6 Immunocytochemical staining for the Cell Nuclear Antigen (PCNA).

PCNA, the auxiliary component of DNA polimerase delta, is a proliferation-induced, 36 kD nuclear protein. The expression of PCNA in tissues has been found to be correlated with proliferative activity. In fact, it has been suggested (Kawasaki et al. 1995) that PCNA levels may reflect differences in the proliferative activity of neuroblastomas, as they evolve through different stages of the disease. However, PCNA is also necessary for nucleotide-excision repair of DNA. In the present work, we estimate PCNA positive cells in eight experiments with cells exposed to the MF at 10 μT on day 3 after plating. At the end of the 42-h exposure period, PCNA positive cells were determined by immunostaining, using PCNA-labelling and Hoechst for quantification of total number of cells. Cells were stained with 2 μg/ml Hoechst dye 33342 for 10 min at room temperature, studied and photographed with fluorescence microscope. In a total of 13 experiments the cells were exposed to the MF at 100 μT on day 3 after plating, and processed for PCNA labelling at the end of the 42 and 63-h exposure (day 5 and 6 postplating). The proportion of PCNA+ cells was quantified by counting 15 microscope fields per coverslip, in a total of 4 coverslips (two control and two exposed) per experimental replicate. All determinations were carried out following blind protocols.

2.4.7 5-bromo-2'-deoxyuridine (BrdU) labelling for identification of cells synthesising DNA.

Samples exposed to 100μT were labelled with BrdU at different times during the exposure period: Time 0 of exposure, (day 3 after plating); time 21h (day 4) or time 42h (day 5). The cells were always analysed 21 hours after the BrdU application, i.e. at 21, 42 or 63 hours of exposure. The results were compared to those in the respective controls (BrdU-treated, MF-unexposed). The analysis was performed through total cell number counting (Trypan Blue exclusion protocol) and by quantification of anti-BrdU antibody positive cells. The proportion of BrdU+ cells was quantified by counting 15 microscope fields per coverslip, in a total of 4 coverslips per experimental replicate. All determinations were carried out following blind protocols.

2.4.8 Flow cytometry assay

DNA content and cell cycle phase distribution were analysed by flow cytometry with propidium iodide DNA staining, in cells exposed to 100 μT for 42 or 63 hours (day 5 or 6 post-plating, N= 3 or 6 experiments, respectively, with two replicates per experimental condition). Cells were harvested, fixed with 70% ethanol and incubated with RNAse A (100 μg/ml) and the DNA intercalating dye propidium iodide (20 μg/l) in citrate buffer (3.4 mM). The cell cycle phase analysis was performed by flow cytometry using a Becton Dickinson FAC flow cytometer and Becton Dickinson CellQuest software. All determinations were carried out following blind protocols.

2.4.9 Apoptosis assay

In order to investigate the potential influence of 50 Hz MF on apoptosis the percent of apoptotic cells was estimated with TUNEL-labelling (TdT-mediated dUTP nick labelling) after 63 hours of exposure. Additional assays for apoptosis were carried out through flow cytometry in propidium iodide-stained neuroblastoma cells, exposed to the MF for 42 or 63 h (N= 7 experimental replicates).The distribution pattern of apoptotic nuclear DNA was determined using a Becton Dickinson FAC flow cytometer and Becton Dickinson CellQuest software. Three additional experiments were conducted and the cellular

response was analysed through TUNEL procedure. After fixation in 4% paraformaldehide, cells were washed with PBS, permeabilised with 0.1% Triton X-100 in 0.1% sodium citrate, washed again with PBS, and incubated for 60 min at 37ºC with biotin-conjugated dUTP in a TdT (terminal deonucleotidyl transferase, 25 U/ml) catalysed reaction (Roche Molecular Biochemicals) in a humidified atmosphere in the dark. The labelled nuclei (dUTP) were revealed with 3,3'-diaminobenzidine. The cells were counterstained with methyl green. *In situ* labelled nuclei were quantified by image analysis and photographed under light microscope. All determinations were carried out following blind protocols.

2.4.10 Immunocytochemical staining for the expression p-CREB

CREB is a nuclear transcription factor that regulates expression of genes controlling cell proliferation, differentiation, and survival. In fact, this protein is known to play an important role in neuronal survival and plasticity. Besides, different alterations of the CREB family of transcription factors have been observed in tumours. The cells were grown on coverslips. The MF-exposed samples (50 Hz; 100 μT, 30 or 60 minutes exposure) and their respective controls were labelled at the end of the exposure period for 30 min, 60 min, or 2 hours using phosphorylation site-specific antibodies. As positive controls, samples were treated with bFGF at a concentration known to activate p-CREB immunoreactivity in neuronal cells. The proportion of p-CREB cells was quantified by counting 15 microscope fields per coverslip, in a total of 4 coverslips per experimental replicate. All determinations were carried out following blind protocols. For analysis of Ser^{133}- phosphorilated CREB and total CREB, Western blotting was performed on 2 $x10^6$ cells per experimental point. Cell pellets were added at 4ºC with a lysis buffer containing 1% deoxycholate, 1 μg/ml aprotinin, 2 μg/ml leupectin, 1 mM phenylmethylsulfonyl fluoride, and 1mM sodium orthovanadate for 10 min. Cell lysates were sonicated and either immediately processed by Western or kept frozen until assayed. Protein concentration in the samples was estimated by the method of Lowry et al., (1951). Equivalent (50 μg) amounts of proteins per sample were subjected to electrophoresis on a 10% sodium dodecyl sulphate-acrilamide gel. The gel was then blotted onto a nitrocellulose membrane. Blotted membranes were blocked for 1h in a 4% suspension of dried skimmed milk in PBS and incubated overnight at 4ºC with a rabbit polyclonal anti-P-CREB serum; against the phosphorilated Ser133 form of CREB (1:1000 dilution). The membranes were washed and incubated for 1h at room temperature with peroxidase-conjugated anti-rabbit immunoglobulin G (dilution 1:1000). Specific reactions were revealed by the ECL Western blotting detection reagent (Amersan Biosciences).

2.4.11 Indirect immunocytochemistry

Rabbit polyclonal antibodies and mouse monoclonal antibodies against neural antigens and receptors for FGF were used as primary antibodies for indirect immunocytochemistry. Polyclonal anti-FGFR1, anti-FGFR2 and anti-FGFR3 were purchased to Santa Cruz Biotechnology Inc. Burlingame, CA. Anti-GFAP was obtained from Dakopatts a/s, Glostrup, Denmark. Monoclonal antiβ-tubulin isotype III and anti-GFAP were obtained from Developmental Studies Hybridoma Bank (University of Iowa), Sigma Chemical Co, and Boehringer Mannheim GmbH, respectively. Secondary antibodies raised in goat against rabbit, and in sheep against mouse immmunoglobulins, conjugated to alexafluor were purchased to Jackson Boehringer Mannheim GmbH. After appropriate culture time, cells grown on poly-l-ornithine coated coverslips were fixed with 4% paraformaldehyde for 10 min, rinsed 3 times in phosphate-buffered saline, then blocked with 10% foetal calf serum, and subsequently incubated in primary antibodies for 1 hour at room temperature (for growth factor receptors). Permeabilisation for intracellular antigens was achieved by incubation with ethanol acetic solution at –20ºC for 20 min. To assess non-specific binding for each antibody, adjacent cultures were incubated in buffer, without primary antibody. For dual labelling, primary antibodies generated by different species were added together. Secondary antibodies were administered for 45 min in the dark at room temperature. The coverslips with cells were mounted in a medium containing p-phenylenediamine and bisBenzimide (Hoechst 33342, Sigma Chemical Co). In selected experiments, FGFR1 was immunoperoxidase detected, using a biotin-linked (Vector Laboratories Inc, Burlingame, CA), instead of a fluorescence-linked secondary antibody, followed by incubation with an avidin-biotinylated horseradish peroxidase complex (Vectastain Elite ABC Kit, Vector Laboratories Inc). Finally, peroxidase was developed with 0.05% DAB, 0.005% hydrogen peroxide. The samples were counterstained with haematoxylin.

2.4.12 Hybridisation histochemistry

The hybridisation protocol has been adapted for all cell cultures from that reported by Simmons et al. (1989). The cells were fixed with 4% paraformaldehyde for 30 min at room temperature, dehydrated, air dried for 2 hours, and stored at –80ºC. Before hybridisation, the cultures were treated with proteinase K at a doses ranging from 0.1 to 1 μg/ml for 10 min at 37ºC, acetylated with acetic anhydride, dehydrated and air dried. Each coverslip was overlaid with 50 μl of hybridisation solution (50% formamide, 0.3M ClNa, 10mM Tris pH 8.0, 1mM EDTA, 0.5 mg/ml transfer RNA, 0.5 mg/ml total yeast RNA, 1x Denhardt's solution, 10% dextran sulphate, 10 mM DTT) containing $1x10^7$cpm/ml [^{35}S]-radio-labelled probe (FGFR1, FGFR2, FGFR3, FGFR4 cRNAs). In each experiment parallel cultures were hybridised with the complete probes and with the hydrolysis product of each type of probe. Alkaline hydrolysis was performed by incubating probes at 60ºC with bicarbonate buffer, pH 10.2, during appropriate periods of time, in order to obtain fragments of 250 bp. Hybridisation was carried out at 55ºC overnight, and thereafter coverslips were treated with 20 μg/ml ribonuclease A for 30 min at 37ºC. Coverslips were washed at increasing stringency with the final wash in 0.1x SSPE (10mM NaH_2PO_4, pH 7.4, 0.15M NaCl, 1mM EDTA), containing 1mM DTT, for 1 hour at 45ºC. After dehydration, cultures were air dried for 2 hours. In order to facilitate subsequent manipulation of coverslips, they were fixed to slides with DPX, so that 2 similar cultures, hybridised with the antisense and sense probes respectively, were attached to the same slide. Cultures were exposed to X-ray film for 5 days, and then dipped in Kodak NTB-2 liquid autoradiographic emulsion, and exposed for 4 weeks at 4ºC prior to development in Kodak D-19. Finally, the cultures were counterstained with haematoxylin and eosin and analysed.

2.4.13 Nucleic acid probes

[^{35}S]-UTP (ICN Pharmaceuticals Inc, Irvine, CA) labelled probes were synthesised in a run-off transcription reaction, using T3 or T7 and SP6 or T7 polymerases to generate antisense or sense RNAs, respectively.

2.4.14 Analysis of inmunocytochemical data

The results were expressed as mean ± SEM from 4 to 6 independent experiments (per treatment) done in duplicates (two coverslips). Where indicated, data are normalised in relation to their own controls, and represent the mean ± SEM of two coverslips from 4 independent experiments. In each coverslip, 30 predetermined visual fields (400X amplification) were counted under fluorescence microscopy through a program of Image Analysis. Statistical analyses were performed using Student's T test, and differences were considered significant when $p \leq 0.05$.

2.5 Experiments with human lymphocytes and thymocytes and embryonic stem cells of mice during cardiac differentiation (Participant 8)

2.5.1 ELF-EMF exposure setup

The ELF-EMF exposure setup used by Participant 8 is based on two unshielded 4-coil systems arranged in the same incubator; B-fields up to 1 mT (50 Hz) can be applied (see 2.1.1). It was composed by two systems, one used for the active exposure and one used as sham. Each system was composed by four circular coils; each coil being double-wrapped, in order to obtain a wound (active) or counter-wound (sham) configuration. The characteristics of the coils were the following: internal radius of the top and bottom coils 9.2 cm, numbers of turns 40 (20+20); internal radius of the two central coils 6.6 cm, numbers of turns 40 (20+20); distances between the coil centres 7.9 cm. The coil configuration was calculated in order to have a large zone of high uniformity (1%). The two systems were powered in series by an home-made DC amplifier connected to a function generator (Beckman FG3A). The ELF set up was kept inside a commercial CO_2 incubator (HeraCell) and the temperature was monitored by means of an high precise thermoresistor. At variance with the ELF set up used by Participant 4 the automated blind protocol was not implemented in the system by means of a suitable switcher and the experiments were

done in blind by the experimental protocol.

2.5.2 RF-EMF exposure setup

See 2.1.2

2.5.3 Cell proliferation by ^{3}H-TdR incorporation test

Peripheral blood mononuclear cells (PBMCs) from 20 young donors, were separated by centrifugation on Histopaque-1077 (Ficoll Histopaque, Sigma Chemical, St. Louis, MO, USA) discontinuous density gradient (Böyum 1968). One hundred µl of cell suspension containing 10^5 PBMC in complete medium (RPMI 1640 with 2 mM glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin and 10% heat-inactivated AB serum from a pool of 10 human donors) was distributed in microplate wells (Costar, Cambridge, Ma, USA) and added with 0.1 ml of medium with or without mitogen. The following mitogens were used to promote lymphocyte proliferation: phytohemoagglutinin (PHA-P, Difco, Detroit, MI, USA) at the final concentrations of 0.1, 1, 5 and 10 µl/ml; anti-CD3 monoclonal antibody (mAb) (OKT3, an IgG2a mAb, from ATCC, Rockville, MD, USA) at the final concentration of 10 ng/ml. Each point was performed in quadruplicate. Cultures were incubated and sham-exposed or exposed to 50 Hz magnetic field (50 µT) for 2 up to 6 days (5% CO_2 in a humidified atmosphere). ^{3}H-methyl-thymidine (^{3}H-TdR, Amersham Int., UK, specific activity 5 Ci/mM) was added for the last 6 hours of culture (0.5 µCi/well). At the end of the incubation period, PBMC were harvested and washed on fibre filters by a multiple cell culture harvester (Skatron, Norway); ^{3}H-TdR incorporation was measured by liquid scintillation counting (*b*-counter, Beckman).

2.5.4 Cell proliferation by flow cytometry

PBMCs from young donors were marked by using the fluorescent cell tracer Carboxyfluorescein diacetate, succinimidyl ester (CFDAse, Serotec, UK). It couples irreversibly intracellular proteins by reaction with lysine side-chains and other available amine groups. Cells become fluorescent and after mitogenic stimulation (with 1 or 0.1 µl/mL PHA, phytohaemoagglutinin; in some experiments anti-CD3 10 ng/mL was also used), the dye is divided between the daughter cells. Each division results in generation of a population of cells that is marked by half of the cellular fluorescence intensity. PBMCs were cultured in petri dishes (35mm) at the concentration of $1x10^6$/mL of medium and exposed to RF. Two intermittent types of exposure were applied using Talk modulated RF signal (SAR 2 W/kg): (1) 10 min on/20 min off for 44 h; (2) 2h on/22h off for 72 hours. We performed experiments with cells from 6 donors using the former, from 11 donors using the latter. All cells were acquired and analysed after 72 h and 120 h of culture. At the end of time culture, cells were harvested and labelled by anti-CD4, anti-CD8 and anti-CD28 monoclonal antibodies (Serotec, UK), in order to discriminate helper and cytotoxic T cells with or without the co-stimulating molecule CD28, fundamental for the activation of lymphocytes.

2.5.5 Cell cycle analysis by flow cytometry

Cell cycle was analysed in PBMCs exposed either to ELF and RF-EMF. In ELF experiments, PBMCs from 9 young donors were stimulated by PHA (optimal dose, i.e. 1 µl/ml) or anti-CD3 (10 ng/mL) and exposed or sham-exposed for 24, 48, 72 and 96 hours. In RF experiments, PBMCs from 8 young donors were exposed to GSM basic signal (SAR=2W/Kg), PBMCs from 10 young and 8 elderly donors to TALK signal (SAR=2W/Kg) and PBMCs from 8 young subjects were exposed to DTX signal (SAR= 1.3 W/Kg).The exposure time was 10 minute on and 20 minute off for 44 hours.

Cell cycle analysis was performed by the method of 5-bromo-2'-deoxyuridine (BrdU) incorporation and propidium iodide (PI) staining. Briefly, at the end of the incubation period, cells were labelled with 20 µM BrdU for 30 min., centrifuged for 1 min., washed twice with 1 ml of PBS solution containing 0.5% Tween 20, and resuspended in 1 ml HCl 1N. After a 30 min. incubation at room temperature, cells were centrifuged at 300g for 1 min. washed once in 0.1 M $Na_2B_4O_7$, and added with 5 µl of anti-BrdU mAb (Becton Dickinson, San Josè, CA, USA). Cells were incubated for 60 min. at 4°C, washed twice and

resuspended in 200 µl of diluted secondary antibody (goat-anti-mouse IgG conjugated with fluorescein isothiocyanate, FITC). After a 30 min. incubation at 4°C, cells were washed twice and resuspended in 200 µl of PBS solution with 0.5% Tween 20 and 200 µl of PI working solution (50 mg/ml in 3.4 mM trisodium citrate, 9.65 mM NaCl plus 0.03% Nonidet P40). After 15 min. at 4°C in the dark, cells were acquired and analysed by flow cytometer.

2.5.6 Expression of membrane receptors on T lymphocytes by flow cytometry

Phenotypical analysis of T lymphocytes was performed in PBMCs exposed either to ELF and RF. In ELF experiments, HLA-DR and CD25 membrane expression were analysed on CD3+ T and CD4+ T helper lymphocytes respectively. The analysis was performed on cells from 9 young donors, before and after stimulation with PHA (1 µl/ml) or anti-CD3 mAb (10 ng/ml). Briefly, cells were stimulated and exposed from 24h to 72 h, collected, washed twice with cold PBS and stained with different mAbs directly conjugated with FITC or phycoerythrin (PE). The following mAbs from Becton Dickinson and from Serotec (Oxford, UK), were used: anti-CD3, recognising all T cells; anti-HLA-DR, recognising B cells and activated T cells; anti-CD4, reactive with helper/inducer T cell subset; anti-CD25, reactive with the p55 chain of IL-2 receptor. The expression of HLA-DR and CD25 molecules was studied on CD3+ and CD4+ lymphocytes at 24, 48, 72h after mitogen stimulation. In RF experiments, PBMCs from 8 young donors were exposed to GSM basic signal (SAR=2W/Kg), PBMCs from 10 young and 8 elderly donors were exposed to TALK signal (SAR=2 W/Kg) and PBMCs from 8 young subjects were exposed to DTX signal (SAR=1.3 W/Kg). CD25, CD95 and CD28 in CD4+ helper and CD8+ cytotoxic T lymphocytes, respectively, were analysed by flow cytometry technique. (In specific: CD95 is the receptor activating the pathway of programmed cell death). Lymphocytes before and after exposure to RF were phenotypically analysed. Cells, unstimulated or stimulated with anti-CD3 mAb (10 ng/ml), immediately after exposure or sham-exposure, were collected, washed twice with cold PBS and stained with three different mAbs (CD25, CD95, CD28, CD4 and CD8, Serotec, Oxford, UK) directly conjugated with FITC or phycoerythrin (PE), or tricolor fluorocromes. The analysis was performed on 10,000 lymphocytes for each sample and the three fluorescences were analysed using "paint-a-gate" software (Becton Dickinson).

We performed also from 5 up to 8 replications, using PBMCs from the same young donor. Cells were un-stimulated or anti-CD3-stimulated (10 ng/mL) and exposed or sham-exposed to TALK modulated RF (SAR 2 W/kg) for 44h (10 min on/20 min off). After exposure, cells were stained using the same protocol describe before. The following membrane molecules were analysed: CD25, CD95, CD28, CD45RO, HLA-DR on CD4+ and CD8+ T lymphocytes, respectively. Moreover, a more sophisticated analysis was performed on fluorescence distribution of CD4+ helper T lymphocytes from 10 young and 8 elderly donors

2.5.7 Spontaneous and induced apoptosis by flow cytometry

PBMCs from 8 young donors were exposed to GSM basic signal (SAR=2W/Kg), PBMCs from 10 young and 8 elderly donors to TALK signal (SAR=2W/Kg) and PBMCs from 8 young subjects were exposed to DTX signal (SAR=1.3 W/Kg). PBMCs were induced to undergo apoptosis by 2-deoxy-D-ribose (dRib) (Barbieri et al, 1994), which acts through an oxidative pathway (Monti et al. 2000) and the early stage of apoptosis was assessed by Annexin-V and propidium iodide kit (ANX-V , Bender, Vienna, Austria) using flow cytometry technique. Briefly, cells were collected, washed in PBS and resuspended in 200 µL of ANX-V binding buffer (10 mM Hepes/NaOH pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$), stained with 5 µL of ANX-V and incubated for 10 minutes at room temperature. Then, cells were washed with binding buffer to remove the excess of ANX-V, resuspended in PBS, counterstained with 5 µg/ml PI and analysed by flow cytometry. ANX-V, the probe used for the detection of early stage apoptotic cells, is able to recognise phosphatidylserine (PS) when present on the outer leaflet of the plasma membrane. It is well known that PS is normally found only on the inner leaflet of the cell membrane double layer, but it is actively transported to the outer layer as an early event in apoptosis and becomes available for annexin binding (Green and Steinmetz 2002).

2.5.8 MMP modifications in induced and spontaneous apoptosis

PBMCs from 8 young donors were exposed to GSM basic signal (SAR=2W/Kg), PBMCs from 10 young

and 8 elderly donors to TALK signal (SAR=2W/Kg) and PBMCs from 8 young subjects were exposed to DTX signal (SAR=1.3 W/Kg). Changes in MMP have been evaluated by using the lipophilic cationic probe JC-1, which changes reversibly its colour from green to orange as MMP increases (over values of about 80-100 mV). This property is due to the reversible formation of JC-1 aggregates upon membrane polarisation that causes a shift in the emitted light from 530 nm (*i.e.*, emission of JC-1 monomeric form) to 590 nm (*i.e.*, emission of J-aggregates) when excited at 490 nm; the colour of the dye changes reversibly from green to orange/red as the mitochondrial membrane becomes more polarised. Both colours can be detected using the filters commonly mounted on flow cytometers, so that green emission can be analysed in one fluorescence channel, and orange/red emission in the other. Briefly, cells were stained with 2.5 μg/mL JC-1 and kept at room temperature for 10 minutes, washed twice with PBS, resuspended in a total volume of 400 μL PBS and analysed (Cossarizza et al. 1993b; Salvioli et al. 1997).

2.5.9 Cytokine production by ELISA

The production of interleukin-1 β (IL-1 β) and interleukin-6 (IL-6) in unstimulated and stimulated PBMCs from young donors was determined in the surnatant of cultures. Two types of stimuli were used: a) 10 ng/mL anti-CD3, b) 10 ng/mL TPA (12-O-tetradecanoylphorbol-13-acetate) plus 1 μ/mL PHA (phytohaemoagglutinin). PBMCs from 26 donors were stimulated with the former and 24 were stimulated with the latter. The second stimulus is stronger than the first, since it is directed on monocytes. PBMCs were exposed both to GSM talk (2 W/kg SAR) and DTX only (SAR 1.4 W/kg) RF for 44 hours (10 min on/20 min off). At the end of time culture, surnatants were harvested and frozen. After the collection of all the samples we analysed the presence of cytokines by ELISA (Enzyme linked Immuno-adsorbant assay), a well known immuno-enzimatic method, by which cytokine is revealed by a coloured end-product. By using an ELISA plate reader final data are produced as pg/mL units.

2.5.10 Hsp70 levels in induced and spontaneous apoptosis by flow cytometry

PBMCs, obtained from 7 healthy young donors, were treated by 2-deoxi-d-Ribosio (dRib, 10 mM) for 44 h and in the meantime cells were exposed to RF (GSM Talk signal, SAR 2W/kg; 10 min ON and 20 min OFF). Detection of intracellular Hsp70 was performed by flow cytometry techniques (Bachelet et al., 1998). At the end of culture, cells were collected, fixed and permeabilised by ethanol 95% and acetic acid 5% for 15-30 minutes, at -20°C. Then, cells were washed with PBS with 1% of BSA and labelled with Hsp70 primary antibody (Mouse IgG, 70 KDa for human target, Pharmingen, BD, San Josè, CA, USA) for 1h at 4°C in the dark. Then, cells were incubated with a secondary fluorescent antibody (GOT anti-mouse, Becton Dickinson), FITC conjugated (isothiocyanate of fluorescein) for 30 min at 4°C in the dark. At the end of the incubation cells were washed and analysed by flow cytometer (FCScalibur, BD). Fluorescence intensity was evaluated by CellQuest® programme.

2.5.11 Thymocyte development and apoptosis by HTOC and flow cytometry

Pieces of human thymus were obtained by cardio-surgery from S.Orsola-Malpighi Hospital (Bologna, Italy) from 6 human newborn. (5 days-8 months). Small fragments of tissue (2-3 mm^3) were cut and cultured above a sterilised filter on a small piece of gelfoam which was embedded of medium (20% of FCS, 1% of penicillin-streptomycin, 79% of DMEM). Each gelfoam was placed inside the Petri dishes containing the DMEM medium. This is a standard technique used to analyse in vitro thymocyte differentiation (Anderson and Jenkinson 2000). Human thymus organ cultures (HTOC) were incubated for 48 h, but the exposure (or sham-exposure) was performed only during the first 24h (DTX only at SAR 1.4 W/kg; 10 min on / 20 min off). At the end of culture (48 h), thymocytes were separated by gentle pressing through a fine stainless steel screen submerged in PBS. Single cell suspensions were obtained by passing the cells through a steel filter, and washing twice in PBS. Thymocytes were directly labelled with FITC or PE conjugated mAbs; such as, CD4, CD8, αβTCR (T cell receptor), γδTCR, CD71 (transferrin receptor on proliferating cells) and CD16 (receptor for IgG) in order to discriminate different phenotypical phases of differentiation. Moreover, apoptosis and viability were assessed by annex-V (see the methods described above) and PI staining, respectively. 10,000 cells from each sample were acquired by flow cytometer and analysed by paint-a gate-software.

2.5.12 T lymphocyte gene expression by microarray technology

Quiescent T lymphocytes were separated by MACS® (Magnetic Cell Sorting, Miltenyi Biotec, Germany). Three samples were obtained from control cultures, sham-exposure and exposure to DTX only RF for 44h (10 min on/20 min off). These samples were sent to Participant 12 for gene expression analysis by microarray technology. Data analysis were performed in Bologna by Participant 8.

2.5.13 Cell culture of embryonic stem cells and EMF-Exposure

GTR1 ES cells, a derivative of R1 ES mouse cells (Nagy 1993) bearing the puromycin-resistance gene driven by the cardiomyocyte-specific MHC promoter (GTR1 cells were kindly provided by Dr. William L Stanford (University of Toronto and Centre for Modelling Human Disease, Canada). ES cells were maintained in the undifferentiated state by culturing in DMEM containing 15% FBS, supplemented with a final concentration of 1000 U/ml ESGRO-LIF (LIF). To induce cardiac differentiation, cells were plated onto bacterial Petri dishes, containing DMEM lacking supplemental LIF. After 2 days of culture, the resulting embryoid bodies (EBs) were plated onto tissue culture dishes. When spontaneous contractile activity was noticed, puromycin (2 µg/ml) was added to eliminate non-cardiomyocytes. After 2 days, puromycin-selected myocytes were transferred to new tissue culture dishes. As indicated in the legend of each figure, EBs, collected at several stages after plating, as well as puromycin-selected cells, were processed for gene expression analyses. Following LIF removal and throughout puromycin selection, GTR1 cells were exposed to ELF-EMF (50 Hz, 0.8 mT_{rms}).

2.5.14 Analysis of mRNA expression

Expression of GATA-4, Nkx-2.5, prodynorphin, alpha-myosin heavy chain and myosin light chain-2V mRNA was assessed by RT-PCR as previously described (Ventura 2000), using GAPDH mRNA as a measure of equal loading and mRNA stability. GATA-4 mRNA levels were also quantitated by RNase protection assay, as described elsewhere (Ventura 1997, Ventura 2003a, Ventura 2003b). Briefly, fragments of the main exon of the mouse GATA-4 (292 bp) gene was inserted into pCRII-TOPO (Invitrogen, CA). Transcription of the plasmid linearised with *BamHI* generated a sense strand of GATA-4 mRNA, which was used to construct a standard mRNA curve. Transcription in the presence of [^{32}P]CTP (800 Ci/mmol) (Amersham International) of plasmids linearised with *Xba*I produced an antisense strand of GATA-4 mRNA (radio-labelled cRNA probe). Samples were then incubated with a combination of RNase A and T1 and exposed to proteinase K. The protected fragments were recovered after phenol chloroform extraction and electrophoretically separated in a polyacrylamide non-denaturing gel. Autoradiographic exposure was performed for 48h. The individual bands were counted for radioactivity by liquid scintillation spectrometry, and cpm values were translated to pg values on a correlated standard curve. Data were expressed as pg of mRNA/µg of total RNA.

2.5.15 Transcriptional analysis in isolated nuclei

Nuclear run-off was performed. 90 µl of nuclear suspension were added with 100 µl of 2 x reaction buffer (10 mmol/L Tris/HCl, pH 7.5, 5 mmol/L $MgCl_2$, 0.3 mol/L KCl, 5 mmol/L dithiothreitol, 1 mmol/L each of ATP, GTP, and CTP), and 5 µl of [α-^{32}P]UTP (3000 Ci/mmol), followed by incubation at room temperature for 15 min. DNA was digested by incubating the transcription mixture for 5 min at room temperature in the presence of 1 µl of 20,000 units/ml RNase-free DNase. Equal counts of ^{32}P-labeled nuclear RNA (about 5 x 10^6 cpm) were then subjected to a solution hybridisation RNase protection assay and were hybridised for 12h at 55°C in the presence of unlabelled antisense GATA-4 mRNA Samples were then incubated with a combination of RNase A and T1 and exposed to proteinase K. The protected fragments were recovered after phenol chloroform extraction and electrophoretically separated in a polyacrylamide non-denaturing gel. Autoradiographic exposure was for 48h. ^{32}P-labeled nuclear RNA was also hybridised with unlabeled antisense cyclophilin mRNA synthesised from a *Nco*I-linearised pBS vector containing a 270-base pair fragment of plB15, a cDNA clone encoding for rat cyclophilin (6). Cyclophilin mRNA was utilised as a constant mRNA for control.

2.6 Experiments with brain cells of different origin and human monocytes and endothelial cells (Participant 9)

2.6.1 Exposure setup and exposure conditions

The wire-patch cell (WPC) is the setup that was used for exposure to frame-scheme (FS) GSM-900 signals. This exposure system accommodates 8 Petri dishes to be built as a double Petri dish, i.e. a 3.5-cm diameter Petri dish (where cells are cultured) is positioned inside a 5-cm diameter dish (outer dish with distilled water inside). Two double Petri dishes are pilled-up and placed in the wire-patch antenna (Figure 3) so that a total of 8 dishes can be exposed at a time. Two successive WPC models were used. The second one shown in Figure 1, built by Participant 10, was fitted with electric field probes that allowed monitoring of the SAR in real time.

Cells were cultured in 3.2 ml of culture medium and the outer Petri dishes are filled with 5 ml of distilled water. Cultured cells in Petri dishes were placed in a standard CO_2 air-flow incubator inside a WPC for a minimum of 3 hours to allow for temperature stabilisation. Sham exposed samples were treated in the same way in a non-activated WPC placed in a second, identical incubator. Each WPC was fitted with a square annular ring made of absorbing foam (Figure 3) to block emission sideways towards the metallic walls of the incubators and allow for a good adaptation at 900 MHz. The foam is sealed with waterproof lining.

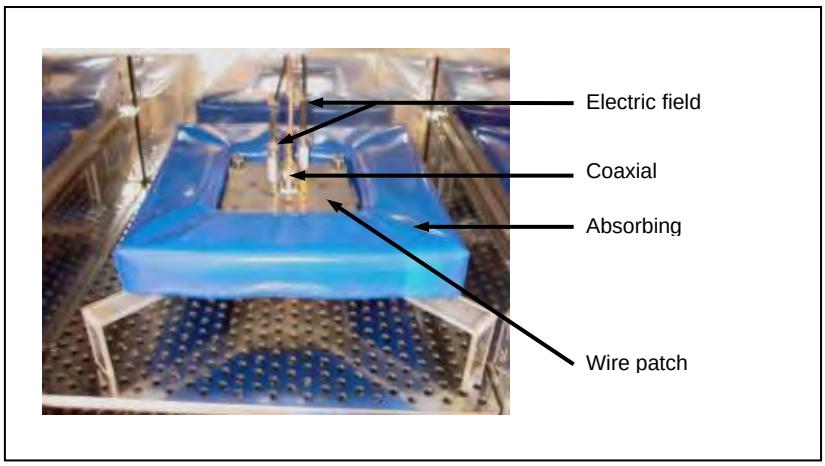


Figure 3. Image of a wire-patch antenna surrounded by the absorbing foam in a dedicated incubator

Dosimetry

The WPC's dosimetry (temperature measurement and modelling) was performed in collaboration with French experts (P. Lévêque, IRCOM, France and J. Wiart, France Télécom R&D). A good efficiency was found (around 0.6 W/kg per incident watt) and the uniformity for cell exposure was found to be very good (ca. 15%) (Figure 4). Experimental Specific Absorption Rate (SAR) evaluation was undertaken in order to validate the data obtained by numerical dosimetry (see Participant 10). Based on the measurement of temperature increase after the RFR generator was turned on, this technique gives also information on the thermal consequences at the level of the whole setup. Temperature was recorded in the inner part of a double Petri dish using optical fibres (Luxtron probes, that are immune to the microwaves), as well as in the incubator throughout the first hours of exposure (until temperature stabilisation was reached). Temperature measurements also showed that it took at least 2 hours for temperature to equilibrate, after the cell cultures were introduced, in the absence of microwaves. This needed to be taken in account in the exposure protocol. Experimental dosimetry showed that the mean SAR was 0.77 W/kg at the level of the cell monolayer, which is in good agreement (within 15%) with numerical data. The corresponding temperature rise was 0.2°C (i.e. temperature difference between the Petri dish inner part and the incubator).

Since heating is produced by absorption of the microwaves by the samples, care was taken to keep the temperature of the exposed and sham-exposed samples identical during the experimental trial. For that purpose, the "exposed " incubator temperature was first set at a lower value than that of the "sham" side, depending on the SAR level chosen for a given experiment. For a SAR of 2 W/kg for instance, the temperature difference was 0.5°C for an input power of 3.4 W, in very good agreement with numerical FDTD modelling (Figure 4) Those data have been confirmed for quality control purposes by Participant 10.

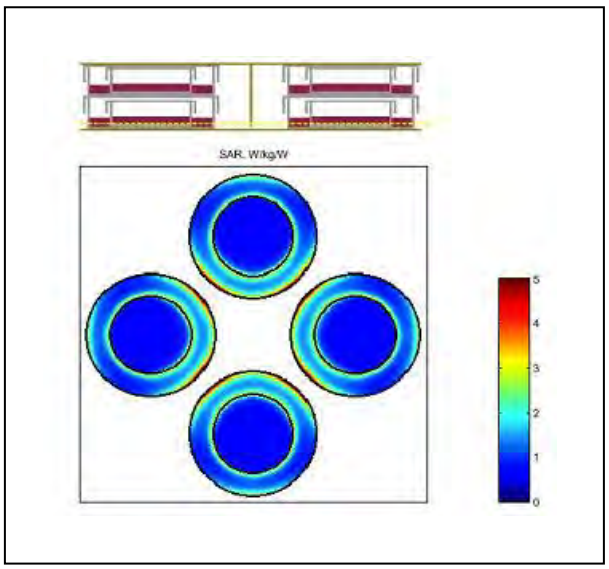


Figure 4. FDTD simulation of SAR in the Petri dishes (outer and inner parts) using an input power of 1 W. Upper panel shows the Petri dishes disposition in the wire-patch antenna.

2.6.2 Cell culture and RF-EMF exposure

For each exposure condition, at least three independent experiments were performed in a blind manner. For that purpose, all samples are coded prior to exposure, one researcher is in charge of exposure and codes are broken after completion of the analysis of all parameters investigated in the experiment by researchers unaware of the exposure conditions.

Culture of nerve cells

Rat primary neurons (granule cells) - a very critical cell type in the central nervous system-, rat primary astrocytes and rat C6 astrocytic cell line were used. Both primary cell types were prepared from newborn rat cerebella. Human nerve cell lines were also used to look at possible species specificity as well as at differences between transformed and normal cells. Human U87 astrocytic- and SH-SY5Y neuronal- cell lines were chosen as models.

i) Rat brain primary cultures: Primary cultures were prepared from postnatal day 4-9 (P4-P9) rat cerebella. Two types of cultures were prepared: neuronal-astroglial and glial (astrocytes) cultures.

Neuronal-astroglial cultures, with approximately 80% neurons and 20% glia in the cell population, were obtained from P4 rat cerebella. The cells were plated on polylysine-coated dishes at 10^6 cells/dish in Hanks Minimum Essential Medium supplemented with 10% horse serum. They were maintained in 3% CO_2 in air at 37°C for 4-6 days. Astrocyte cultures, where very few neurons survived, were obtained from P8 rat cerebella and plated at 10^6 cells/dish in Dulbelco Miminum Essential Medium (DMEM) supplemented with 10% fetal calf serum (FCS) and antibiotics (AB, 100 U/ml penicillin and 100 µg/ml streptomycin) and maintained in 9% CO_2 in air at 37°C for 13-15 days. For the experiments, all primary cells were plated in 35 mm diameter Petri dishes and used as it, without any further handling. The endpoint investigated in these cells was apoptosis. Rat primary neurons were sham-exposed or exposed to GSM-900 for one or 24 hours and apoptosis was evaluated following a time-kinetics (4, 8 and 24 hours

after exposure began) or immediately after exposure, respectively. Rat primary astrocytes were sham-exposed or exposed to GSM-900 for 1 hour and apoptosis was evaluated as described previously. Three to six independent experiments were performed for each exposure condition. Results are expressed as the percentage of apoptotic cells in GSM-900- exposed versus sham-exposed samples. The Student t test was used for statistical analyses.

ii) Human neuroblastoma cells: Human SH-SY5Y neuroblastoma cells (ECACC N° 94030304) were cultured in Ham's F12 medium supplemented with 15% FCS, 1% non-essential amino-acids and AB The endpoint investigated in these cells was apoptosis. Human SH-SY5Y neuronal cells – as rat primary neurons – were sham-exposed or exposed to GSM-900 for one or 24 hours and apoptosis was evaluated following a time-kinetics (4, 8 and 24h after exposure began) or immediately after exposure, respectively. Three to six independent experiments were performed for each exposure condition. Results are expressed as the percentage of apoptotic cells in GSM-900- exposed versus sham-exposed samples. The Student t test was used for statistical analyses.

iii) Human glioblastoma cells: Human U87 glioblastoma cells (ECACC N° 89081402) were grown in Eagle Minimum Essential Medium supplemented with 10% FCS, 1% non-essential amino-acids and AB. The endpoint investigated in these cells was apoptosis. Human U87 astrocytic cells -as rat primary astrocytes - were sham-exposed or exposed to GSM-900 for 1 hour and apoptosis was evaluated following a time-kinetics (4, 8 and 24h after exposure began). Three to six independent experiments were performed for each exposure condition. Results are expressed as the percentage of apoptotic cells in GSM-900- exposed versus sham-exposed samples. The Student t test was used for statistical analyses.

iv) Rat glioma cells : Rat C6 glioma cells were obtained from the European Collection of Cell Cultures (ECACC N° 85040101, UK) and maintained in DMEM-F12 medium (Biomedia, France) containing 10% fetal bovine serum (Gibco), 2 mM sodium pyruvate (Biomedia, France) and 1% antibiotics (100 U/ml penicillin and 100 µg/ml streptomycin, Biomedia, France) at 37°C in a standard culture incubator. The endpoint investigated in these cells was expression and activity of the inducible isoform of the Nitric Oxide Synthase (iNOS or NOS_2). C6 cells were sham-exposed or exposed to GSM-900 alone (0.2 W/kg or 2.0 W/kg, 48h) or in the presence of a cocktail of lipopolysacharide and cytokines CK (see below). Following RFR or sham-exposure the cells were harvested for western blot analysis and culture medium collected for the determination of nitrite accumulation, to test iNOS expression and activity, respectively. Randomised sham/sham exposures were included in the schedule of exposure, so that the engineer responsible for the analysis never was aware of any exposure condition. A total of four sham/sham exposures, six sham/exposed experiments at 0.2 W/kg and three sham/exposed experiments at 2 W/kg were conducted. The Student t test was used for statistical analyses.

Culture of immune cells

i) Human monocytes: Human U937 monocytic cells (ECACC N° 85011440) are grown as a cell suspension in RPMI 1640 medium complemented with 10% FCS plus AB. Two endpoints were investigated in these cells:

- Apoptosis in cells submitted either to a 48 hour-exposure to GSM-900 at a SAR of 0.7 W/kg or to a 1 hour-exposure at 0.7 W/kg and 2.0 W/kg followed by a treatment with camptothecin (CPT). Three to six independent experiments were performed for each exposure condition. Results were expressed as the ratio of apoptotic cells in GSM-900-exposed versus sham-exposed samples with or without CPT treatment. The Student t test was used for statistical analyses.
- Gene expression in cells submitted to a 1 hour-exposure to GSM-900 at a SAR of 2.0 W/kg.

ii) Human microglial cells: The human cloned microglial cells (CHME 5) were plated at a density of 10^6 cells/35mm diameter dishes in 2 ml of complete Dulbecco's MEM medium. Cultures were carried out for 3 days in water-saturated 5% CO_2 in air at 37°C before GSM exposure. The endpoint investigated in these cells was gene expression in cells after a 1 hour-exposure to GSM-900 at a SAR of 2.0 W/kg.

Culture of endothelial cells

Two EA.hy926 cell lines were tested: one was a generous gift from Participant 6, the other one from Dr. Cora-Jean S. Edgell who first developed the cell line and gave permission to use these cells in Bordeaux. The purpose for using both cell lines was to look at potential different behaviour of cells cultured in slightly different conditions that may have led to possible genotypic drift. Human EA.hy926 endothelial cells were cultured according to the provider's instructions.

i) EA.hy926 (a gift from Participant 6): Cells were grown in DMEM supplemented with 1% penicillin-streptomycin, 2% L-glutamine (200 mM), HAT-supplement and 10% FCS.

ii) EA.hy926 (a gift from Dr. Cora-Jean S. Edgell, North Carolina University at Chapel Hill, NC, USA): Cells were grown in DMEM supplemented with 1% penicillin- streptomycin, 2% L-glutamine (200 mM) and 10% FCS. For the RF-EMF experiments, cells were removed from culture flasks with trypsin, washed and seeded at a density of 0.26 x 10^6 cells/12 mm-diameter glass coverslips corresponding to 1.2 x 10^6 cells/55 mm-diameter dishes (as mentioned in Leszczynski et al. 2002). After an overnight culture, coverslips were transferred to 35-mm diameter Petri dishes and EA.hy926 cells were sham-exposed or exposed to RF-EMF for one hour at 2.0 W/kg. The endpoint to be studied in these cells is the expression of the heat-shock protein 27 (hsp27). EA.hy926 cells were sham-exposed or exposed to RFR for one hour at 2.0 W/kg.

2.6.3 Chemicals and other treatments

Positive controls used chemicals or other treatments. In order to look at possible interactions between RFR and chemicals, different protocols used RFR exposure combined to or prior chemical treatment.

Lipopolysaccharide plus cytokine treatment

Lipopolysaccharide plus cytokine treatment was used as a positive control for iNOS expression in C6 glial cells (Hewett et al., 1993; Nomura 1998). Two days before RFR exposure, C6 cells are plated in custom-made Petri dishes at a density of $5x10^4$ cells/dish. At the day of experiment, Petri dishes are filled with culture medium containing 4% FCS for cell deprivation. Half of the samples are treated with a cocktail of ***e.coli*** lipopolysacharide (LPS 10 µg/ml) and cytokines IFNγ (50 U/ml) plus TNFα (50 ng/ml) before Petri dishes are placed in the wire-patch antenna. Cells were then put in the exposure-dedicated incubators during 3 hours for temperature stabilisation before exposure to RFR started.

Camptothecin treatment

The apoptosis-inducer camptothecin (4 µg/ml, 4 hours) as a positive control in U937 monocytic cells. Camptothecin is a topoisomerase I inhibitor. As such, it inhibits the topoisomerase molecule from religating DNA strands after cleavage. This leaves a cell with DNA breaks, which if not repaired, become lethal (Holden 2001). When cells of the immune system are exposed the topoisomerase I inhibitor campthothecin, they rapidly undergo cell death via apoptosis, irrespective of what phase of the cell cycle a cell is in (Cotter 1992).

Heat shock

Positive controls for heat shock proteins (Hsp27 and Hsp70) induction were performed by exposing the different cell lines (U87, C6 and SH-SY5Y cell lines) to a heat shock at 43°C for 20 min.

2.6.4 Detection of apoptosis

The occurrence of apoptosis was assessed using two markers and flow cytometry.

Double staining with Annexin-V/FITC and propidium iodide

During apoptosis, phosphatidyl-serine is exposed on the outer leaflet of the plasma membrane that causes a loss of membrane asymmetry. Annexin V preferentially binds to phosphatidylserine (Van Engeland et al. 1998) and can be detected by flow cytometry using the APOPTEST^{TM}-FITC kit (Dako, France) according to manufacturer' instructions. Immediately after the complete treatment, cells were harvested so that all cells, including floating cells, were taken in account for the apoptotic test. Where needed, cells were scrapped (nerve cells) before being washed with PBS, and centrifuged at 200 g for 5 minutes. Cell pellet was resuspended and 10^6 cells were incubated for 15 minutes in 100 µl of cold labelling solution (1 µl of Annexin-V/FITC and 2.5µl of propidium iodide (PI) 250 µg/ml) in 96 µl of the kit's labelling buffer. Then 250 µl of labelling buffer were added and samples are analysed on a flow cytometer.

Double staining with $DiOC_6(3)$ and propidium iodide

Mitochondrial physiology is disrupted in cells undergoing apoptosis via intrinsic pathways. Mitochondrial membrane potential ($\Delta\Psi_m$) decrease has been largely described which can be measured using the

carbocyanine dye ($DiOC_6(3)$, Zamzami et al., 1995). Briefly, immediately after exposure, cells were washed and centrifuged as indicated above. Then 10^6 cells were incubated for 10-15 minutes in 500 µl of PBS containing 40 nM of $DiOC_6(3)$. Propidium iodide (2.5 µl of PI; 50 µg/ml) was added before analysis on a flow cytometer.

Data acquisition was performed using a FacScan® flow cytometer (Becton Dickinson) with the following parameters: 488 nm excitation, 515 nm bandpass filter for the Annexin V and $DiOC_6(3)$ dyes and filter > 560 nm for PI detection. Analysis was performed on 10000 events using the Cell-Quest® software. Analysis was performed blindly.

2.6.5 Western Blot analysis

Western Blotting was used for the detection of iNOS expression in C6 cells. C6 cells were lysed using RIPA buffer [0.5 mM Tris (pH 8.0), 0.5% Sodium Deoxycholate, 10% SDS,150 mM NaCl, 1% Triton X100 and protease inhibitors (16 mg/ml Benzamidin, 10 mg/ml Aprotinin, 10 mg/ml Pepstatin, 10 mg/ml Leupeptin, 10 mg/ml Phenanthroline and 1 mM Phenylmethylsulfonyl Fluoride)] using methods adapted from Schreiber et al. (1989).

Proteins were extracted from cell lysates and the concentration was determined by Bradford reaction (Biorad Protein Assay®). Protein samples (10-20µg) were electrophoretically separated through a 7.5% polyacrylamide SDS-page gel, electroblotted to polyvinylidene difluoride membranes and probed with mouse anti-iNOS (Transduction Laboratories N-39120, 1/5000°). In addition, we used ß-actin as an internal control for protein loading (all blots were de-hybridised and reprobed for ß-actin detection). Immunoreactive bands were visualised using ECL Western Blotting System® (Amersham-Pharmacia Biotech, RPN 2108) followed by exposure to autoradiography film (Biomax, Kodak). The NIH Image 1.54 software was used for blot quantitative analysis (based on OD measurements).

2.6.6 Griess reaction

iNOS activity in C6 cells was quantified as nitrites accumulation in culture media by the colorimetric assay based on the Griess reaction. 50µl of culture medium collected in triplicate from the samples were added to 60µl of Griess A solution (sulfanilamide 1% in 1.2N HCl) and 60µl of Griess B solution (Naphtylene Diamine Dichlorhydrate 0.3% in distilled water). The mixture was incubated 10 min at room temperature and red at 540 nm with a spectrophotometer. Fresh corresponding culture medium served as blank for NO_2^- content determination. Results were expressed as µg of nitrite per million cells.

2.6.7 Hsp immunolabelling and image analysis

Hsp70 expression was evaluated in human neuronal (SH-SY5Y) and rat (C6) or human (U87) astrocytic cell lines. Three days before the experiment, cells were plated on glass coverslips in 24-well plates at a density of 0.5×10^5 cells/well. The day before the experiment, coverslips were transferred to 35-mm diameter Petri dishes before being placed in the sham- and RFR-dedicated incubators.

U87, C6 and SH-SY5Y cell lines were sham-exposed or exposed to GSM-900 for 24 hours and the expression of Hsp70 was evaluated at the end of exposure. Hsp27 expression was evaluated in human EA.hy926 cells at the end of a-one hour sham- or RFR exposure at 2.0 W/kg. Following RFR or sham exposure, the cells were fixed in PBS-paraformaldehyde (4%) for immunocytochemistry. Anti-hsp70 and anti-hsp27 antibodies were obtained from Stressgen®. The first antibody was revealed using a FITC-labelled antibody. Coverslips were mounted on slides with Mowiol® before microscopy observation. For each exposure condition, three (hsp70) to five (hsp27) independent experiments have been performed in a blind manner. After immunocytochemistry labelling, fluorescence analysis was performed using the Aphelion® image software. Results are expressed as arbitrary units of fluorescence intensity.

2.6.8 RNA extraction and cDNA array hybridisation

Based on the data available within the REFLEX consortium on the effect of RF-EMF exposure on gene expression (see Participant 12), we chose two human cell lines involved in inflammatory processes (brain

human microglial and monocytes cells). Indeed, one of the gene families that were shown to be sensitive to exposure to electromagnetic fields is the gene family involved in inflammation. The human cloned microglial CHME-5 cells and the monocytic U937 cells were sham-exposed or exposed for one hour at 2 W/kg. Immediately after exposure, they were harvested for RNA extraction using Nucleospin® RNA purification kit (BD Biosciences Clontech, Palo Alto, USA). Total RNA purification was performed following user manual instructions. The amount of total RNA was measured by spectrophotometry. RNA samples were then frozen at –80°C before being sent to Participant 12 who performed cDNA array hybridisation.

2.7 Experiments with embryonic stem cells of mice (Participant 4)

2.7.1 Exposure setups

See 2.1.1 and 2.1.2.

2.7.2 Cell culture and EMF exposure

Pluripotent R1 ES cells (Nagy et al. 1993), wild type (wt) D3 (Doetschman et al. 1985) and p53-deficient ES cells (p53-/-; a gift of Dr. T. Jacks, Howard Hughes Medical Institute, Cambridge, MA, see Jacks 1994) derived from D3 cells were cultured as described (Wobus et al. 2002) except that p53-/- ES cells were maintained on neomycin-resistant SNL feeder cells (a gift of Dr. A. Bradley, Baylor College of Medicine, Houston, TX) in presence of 300 µg/ml G418. EC cells of line P19 (Edwards and McBurney 1983) were cultured without feeder cells (Wobus et al. 1994). For differentiation, P19, R1, and wt or p53-/- D3 cells were cultivated as EB in hanging drops in Dulbecco's modified minimal essential medium (DMEM, Gibco) supplemented with 20% FCS, L-glutamine, non-essential amino acids (NEAA) and β-mercapthoethanol (β-ME) as described (Wobus et al. 2002). Briefly, cells (n=400) in 20 µl of differentiation medium were incubated in hanging drops as embryo-like aggregates ("embryoid bodies", EBs) for 2 days and in suspension for 3 days. EBs were plated separately onto gelatin-coated 24-well microwell or tissue culture plates (∅ 6cm) at day 5 for morphological and reverse transcriptase polymerase chain reaction (RT-PCR) analyses, respectively. For the induction of P19 cell differentiation, EBs were cultivated in the presence of 1% DMSO (Sigma) during the first 2 days of EB development (Wobus 1994).

EBs derived from p53-/- and wt D3 ES cells were RF- or ELF-EMF- exposed in hanging drops for 6 or 48 hours (Figure 5). For 6h experiments, samples were collected immediately after exposure and used for RT-PCR analysis (Figure 5). After 48h exposure, EBs were further cultivated and samples were sequentially collected during differentiation for RT-PCR analyses. EBs derived from R1 cells were EMF (GSM-217)- or sham-exposed in hanging drops (SAR: 1.5 W/kg) for 2 days and in suspension (SAR: 2.0 W/kg) for 3 days. P19 cells (n=200,000) were seeded into 0.1% gelatin-coated tissue culture dishes, cultured in DMEM (see above) and after 2h pre-incubation at 37°C placed into the exposure setup for EMF and sham-exposition. As control, non-exposed cells were cultured in a separate humidified 5% CO2 incubator at 37°C. P19 cells were exposed to EMF at SAR value of 2.0 W/kg for 22 or 40h. After exposure, cells were immediately processed for flow cytometric analysis (P19), and in parallel, R1 and P19-derived EBs were prepared for differentiation and RT-PCR analysis

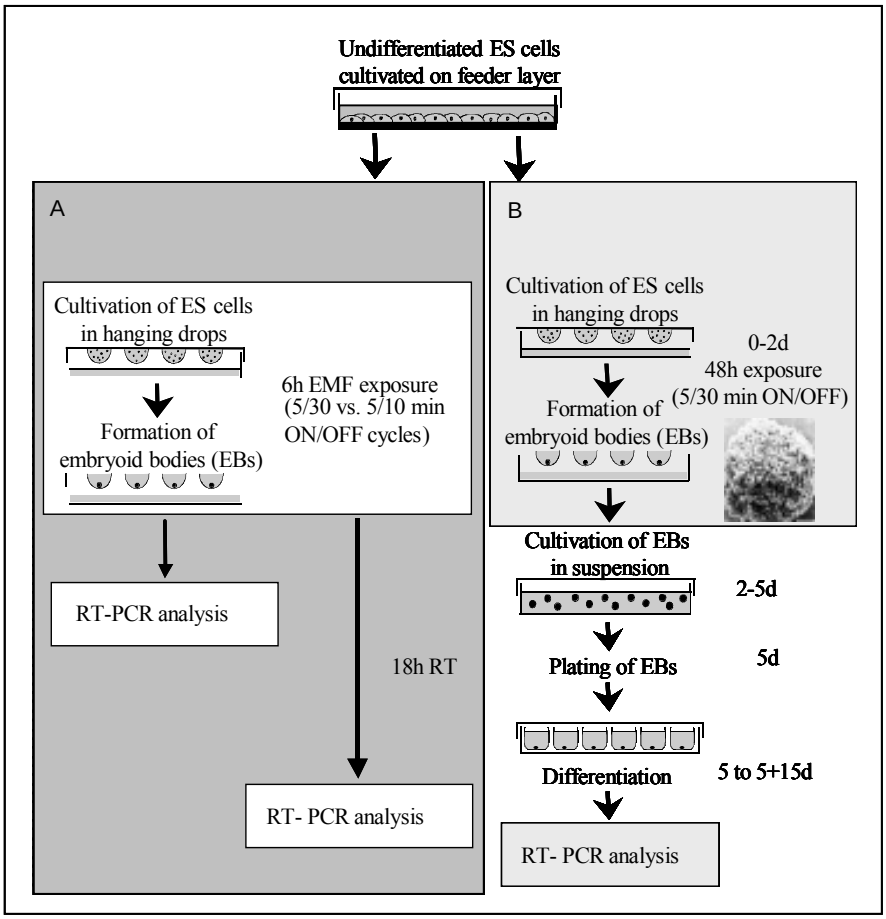


Figure 5. Experimental protocol for the exposure of undifferentiated and differentiating ES cells to EMF. Wild type (wt) and p53-deficient (p53-/-) D3 ES cells were grown on feeder layer. (A) ES cells were RF-EMF or PL-MF exposed for 6 h at the initial stage of hanging drop formation, when the differentiation processes are initiated. mRNA levels of genes encoding egr-1, p21, c-jun, c-myc, hsp70 and bcl-2 were analysed immediately after exposure (or after 18 h recovery time for PL-MF). (B) ES cells were exposed to EMF in hanging drops for 48 h and were monitored at different stages of the differentiation process.

For differentiation of neural phenotypes, R1 ESs were cultivated in 'hanging drops' (n = 200 cells/drop) for 2 days. EBs were transferred to bacteriological petri dishes (Greiner, Germany) and cultivated for two days in Iscove's modification of DMEM (IMDM, GIBCO) containing 20% FCS and supplements as described (Wobus et al., 2002), with the exception that ß-mercaptoethanol was replaced by 450 µM α-monothioglycerol (Sigma, Steinheim, Germany). EBs (n=20-30) were plated onto tissue culture dishes (∅ 6cm) at day 4 and cultivated in IMDM +20% FCS. The selection of neural precursor cells was carried out according to (Rolletschek 2001). After attachment of EBs, one day later, the medium was exchanged by DMEM/F12 medium supplemented with 5 µg/ml insulin, 30 nM sodium selenite (all from Sigma), 50 µg/ml transferrin and 5 µg/ml fibronectin (all from GIBCO) referred as "nestin-selection media". The culture medium was replenished every 2 days. RF-EMF or ELF-EMF exposure was performed for 48h between 4+4d and 4+6d. Nestin-positive neural precursor cells were selected after cultivation for 7 days (= 4+7d). At day 4+8, EBs were dissociated by 0.1% trypsin (GIBCO)/0.08% EDTA (Sigma) in PBS (1:1) for 1 min, collected by centrifugation, and replated onto poly-L-ornithine/laminin-coated tissue culture dishes into DMEM/F12 containing 20 nM progesteron, 100 µM putrescin, 1µg/ml laminin (all from Sigma), 25 µg/ml insulin, 50 µg/ml transferrin and 30 nM sodium selenite, referred to as "nestin-expansion media", for six days until day 4+13. The medium was changed every 2 days. 10 ng/ml basic fibroblast growth factor (bFGF) and 20 ng/ml epidermal growth factor (EGF; Strathmann Biotech, Hannover, Germany) were added daily. At day 4+14, the differentiation of neurons was induced by 'Neurobasal' medium plus 2% B27 (GIBCO), 10% FCS and maintained by the addition of survival

promoting factors such as interleukin-1ß (IL-1ß, 200 pg/ml daily; PeproTech, London, UK) and db-cAMP (700 µmol every four days; Sigma). Glial cell line-derived neurotrophic factor (GDNF, 2 ng/ml; R&D Systems) and transforming growth factor-ß3 (TGF-ß3, 2 ng/ml) were applied at day 4+18 and at day 4+21, respectively. Neurturin (NTN, 10 ng/ml; all from PeproTech) was applied at day 4+21. The application of survival promoting factors during terminal stages was combined with medium changes at three-day intervals. The total time of cultivation was 4+23d (Figure 6).

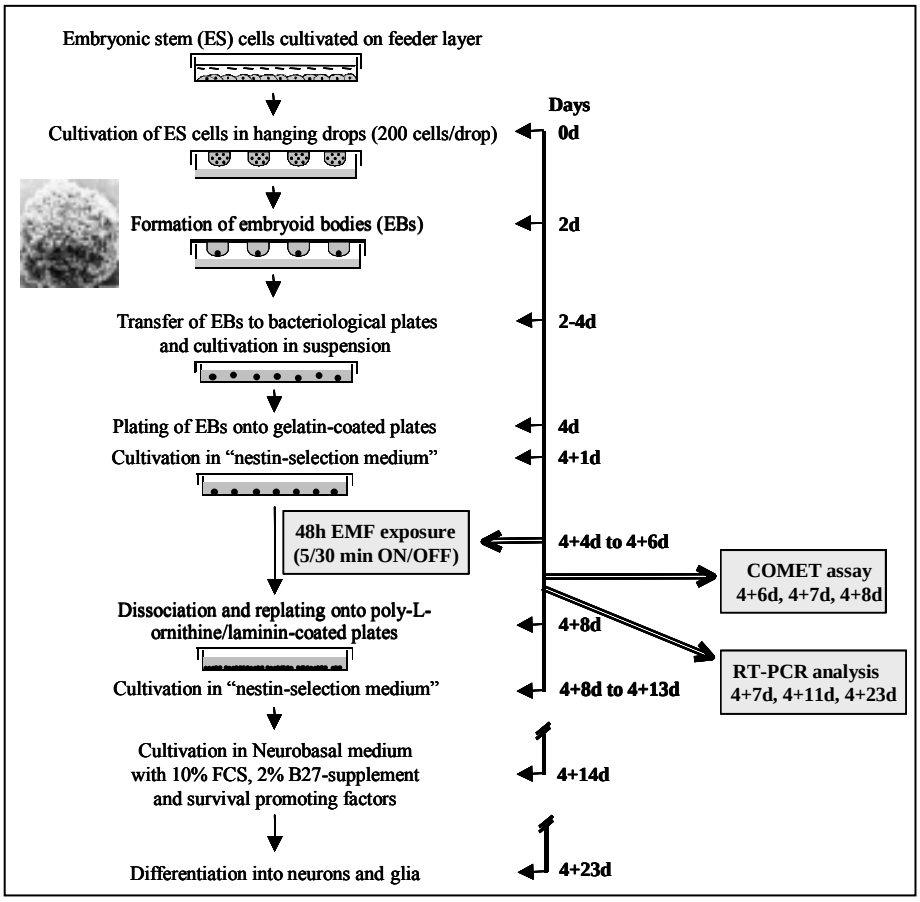


Figure 6. Experimental protocol for EMF exposure of R1 ES cells differentiating into the neural lineage. For differentiation of neural phenotypes, ES were cultivated in 'hanging drops' as EBs (embryoid bodies) for two days, then transferred to bacteriological petri dishes and cultivated two more days. EBs (n = 20-30) were plated onto tissue culture dishes (∅ 6cm) at day 4. After attachment of EBs, one day later, the medium was exchanged with medium supporting the development of neural precursor cells (replenished every 2 days). Nestin-positive neural precursor cells were selected after cultivation for 7 days (= 4+7d). Cell samples were analysed for primary DNA damage (24 and 48h after exposure) measured by the Comet assay, and mRNA levels of various regulatory, tissue-specific and neuronal genes at different stages of differentiation.

2.7.3 Detection of mRNA levels by semi-quantitative RT-PCR analysis

The expression of early response and growth regulatory genes as well as genes involved in neural and cardiac differentiation was analyzed in ES and EC cells after differentiation by semi-quantitative RT-PCR as described (Wobus et al. 2002). EBs or cells differentiating after the dissociation of EBs collected at days 4+4, 4+7, 4+11 and 4+23 were suspended in lysis buffer (4 M guanidinium thiocyanate, 25 mM sodium citrate, pH 7; 0.5% sarcosyl, 1% ß-mercaptoethanol). Total RNA was isolated by the single step extraction method according to (Chomczynski, 1987). RNA was reverse transcribed using Oligo $d(T)_{16}$ primers (Perkin-Elmer, Überlingen, Germany) for the genes encoding c-fos, c-jun, c-myc, early growth response-1 (egr-1), hsp70, p21, p53, bcl-2, bax, growth arrest and DNA-damage inducible –45

(GADD45), engrailed-1 (en-1), nurr-1, nestin, tyrosine hydroxylase (TH), GFAP (glial fibrillary acidic protein), α-myosin heavy chain (α-MHC) (primer sequences and the number of PCR cycles are available on request).

Reverse transcription was performed with MuLV reverse transcriptase (Perkin-Elmer) for 1h at 42°C, followed by denaturation for 5 min at 99°C and cooling to 4°C according to the protocol supplied by the manufacturer. For semi-quantitative determination of mRNA levels, PCR analyses were carried out with Ampli Taq DNA polymerase (Perkin-Elmer). For determination of relative mRNA levels, two separate PCR reactions, either using primers of the analyzed gene or primers specific for ß-tubulin were performed with 3 µl from each RT reaction.

One third of each PCR reaction was electrophoretically separated on 2% agarose gels containing 0.35 µg/ml ethidium bromide. Gels were illuminated with UV light and the ethidium bromide fluorescence signals were stored by the E.A.S.Y. system (Herolab, Wiesloch, Germany) and analysed by the TINA2.08e software (Raytest Isotopenmeßgeräte, Straubenhardt, Germany). Data of the target genes were plotted as percentage changes in relation to the expression of the housekeeping gene ß-tubulin. Gels of four independent experiments were analysed.

2.7.4 Detection of mRNA levels by quantitative RT-PCR (Q-RT-PCR)

Quantitative RT-PCR was performed with specific primers and TaqMan probes designed with Primer Express 2.0 (Applied Biosystems). All oligonucleotides were obtained by Metabion (Germany). The TaqMan probes for Q-RT-PCR were 5´-labelled with FAM (6-carboxyfluorescein) and with 3' prime quencher, TAMRA. The primer and TaqMan probe sequences are available on request. The mouse GAPDH gene was used as endogeneous reference. Reactions were carried out in 96-well plates using iCycler, Version 3.0.6070 (BioRad). The threshold cycle (Ct) , which is the cycle number, at which the amount of the amplified product of the analysed genes reached a fixed threshold, was determined subsequently. The relative quantitaion of bcl-2 and GADD45 mRNA levels was calculated using the comparative Ct method. In order to avoid amplification of contaminating DNA, all primers were designed with an intron sequence inside the amplicon, template-free controls were used as negative controls, the melting temperature of the TaqMan probe was adjusted to be at least 10° higher than the melting temperatures of the sense and anti-sense primers. After Real-time RT-PCR, gel electrophoresis was performed to confirm the correct size of the amplified product.

2.7.5 Single cell gel electrophoresis (Comet assay)

The lkaline version of the Comet assay was applied for detection of single-strand break and alkali-labile site induction, and neutral Comet assay for detection of double-strand breaks.In order to obtain single-cell suspensions, control, sham-exposed or exposed EB outgrowths were trypsinised by addition of 2 ml prewarmed at 37°C mixture of 0.1% Trypsin : 0.01% EDTA (1:1, v/v) per 6 cm tissue culture dish, incubated for 60 sec at room temperature, then the mixture was carefully aspirated as previously described (Wobus et al. 2002). The procedure was repeated. Thereafter, the cells were resuspended into cold Dulbecco's modified Eagle's medium (Gibco BRl, cat. No. 52100-039) with 15% FCS, the cell density was adjusted at ~$5x10^5$ cells/ml and the test-tubes were placed on ice.

For the Comet assay procedure, the protocol of the original technique described by Östling and Johanson (1984) was followed with minor modifications by (Singh et al. 1991, Morris et al. 1999, Speit et al. 2000, Ivancsits et al. 2002b). Briefly, 20 µl of cell suspension (~10 000 cells) was mixed with 200 µl prewarmed (37°C) 0.5% low melting point agarose in PBS. The cell suspension was rapidly pipetted onto slides with frosted ends precoated with 1.5% normal melting point agarose in PBS and evenly spread using a coverslip. The slides were incubated at 4°C for 15 min. to allow the microgel to solidify. Then the coverslips were removed by pulling them carefully aside. The slides were immersed in precooled (4°C) lysis solution (2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris, 1% sodium sarcosinate, 10% DMSO, pH 10 for the alkaline Comet assay and pH 7.5 for the neutral COMET assay) and lysed for 60 min at 4°C in the dark. After lysis, the slides were removed from the lysis buffer, drained, placed into a horizontal electrophoresis tank and covered with the precooled (4°C) electrophoresis buffer (1 mM Na_2EDTA, 300 mN NaOH , pH 13 for the alkaline Comet assay and 100 mM Tris, 300 mM sodium acetate, 500 mM sodium chloride, pH 8.5 for the neutral Comet assay, respectively). After 20 min incubation for DNA

alkaline denaturation and expression of the various types of alkaline labile sites (alkaline Comet assay) and for washing away the lysis buffer and equilibration (neutral Comet assay), the electrophoresis was performed in the dark at 4°C for 10 min. The electrophoretic conditions (25 V, 300 mA, field strength 0.7 V/cm) were identical for both versions (alkaline and neutral) of the single cell gel electrophoresis assay. After removal from the electrophoresis tank, the slide surface was carefully covered 3x with a neutralization buffer (400 mM Tris, pH 7.5, 5 min. incubation), then the slides were rinsed briefly in distilled water and fixed in 100% ethanol for 5 min. The slides were air-dried at a slanted angle for at least 2 hours and stained with 50 µl ethidium bromide solution (20 µg/ml in bidistilled water). The analysis was done using the fluorescence microscope ECLIPSE E600 (Nikon, Germany), appropriate excitation and barrier filters (excitation 540-580 nM, barrier 600-660 nm) at 200 fold magnification and the imaging software Lucia (Version 4.71 for Windows). A total of 1000 nuclei were scored (500 per slide) for exposed, sham exposed and control cells and classified into 5 groups according to tail length and intensity using the classification proposed by Anderson et al. (1994): Group A corresponding to <5% DNA damage; B (5-20%); C (20-40%); D (40-95%) and E (>95%). All analyses were performed by the same investigator blind. Results were expressed as 'tail factors', calculated according to Ivancsits et al. (Ivancsits et al. 2002a; Ivancsits et al. 2002b) by the following formula: Tailfactor (%) $=AF_A+BF_B+CF_C+DF_D+EF_E/n$, where A is the number of nuclei classified to group A, F_A is the average DNA damage of group A, which is 2.5% in Anderson's classification; B is the number of nuclei classified to group B, F_B is the average DNA damage of group B (12.5%); C is the number of nuclei classified to group C, F_C is the average DNA damage of group C (30%); D is the number of nuclei classified to group D, F_D is the average DNA damage of group D (67.5%); E is the number of nuclei classified to group E, F_E is the average DNA damage of group E (97.5%); n – the number of scored nuclei (n= 1000).

The results on DNA damage in the alkaline and neutral Comet assay were obtained from 6 separate experiments. The statistical analysis was performed with the SigmaPlot for Windows Version 3.06 package (Jandel Corp.). All data are presented as mean values ± standard error of the mean (SEM). The differences between exposed, sham exposed and control cells were checked for statistical significance using the independent Student's *t* test.

2.7.6 Analysis of cardiac differentiation

Cardiac differentiation of EC or ES cells was used as a parameter of differentiation according to the embryonic stem cell test, EST (Spielmann et al. 1997), established for in vitro analysis of embryotoxic agents. Spontaneously beating cardiomyocytes were estimated at various stages after EB plating. The percentage of EB containing beating cardiomyocytes and mRNA levels of α-MHC were used for the estimation of the degree of cardiac differentiation.

2.7.7 Flow cytometric analysis of cell cycle phases

RF-EMF- and sham-exposed P19 cells were processed according to the two-step procedure of DNA staining (Sehlmeyer et al. 1996). Cells were analysed with a FACStar^{PLUS} flow cytometer (Becton Dickinson, Heidelberg, Germany). Data from 3 (22h) and 4 (40h) independent experiments with 3 to 6 parallels were subjected to statistical analysis performed with the 'ModFitLT' software (Verity Software House, Inc.).

2.8 Experiments with the human neuroblastoma cell line SY5Y (Participant 11)

2.8.1 ELF-EMF exposure setup

See 2.1.1

2.8.2 Cell culture and exposure conditions

The human neuroblastoma cell line was grown in RPMI, 10% foetal calf serum, 100 units/ml penicillin, 100 µg /ml streptomycin, and 2 mM L-glutamine at 37°C and 5% CO_2. Cells were plated one day prior to exposure at densities varying with the exposure protocol: $2.6x10^6$ cells per 100 mm dish for 16 hours exposure protocol; 2 x 10^6 cells per 100 mm dish for 48 hours exposure protocol followed by recovery of the cells immediately after the end of the exposure; and 10^6 cells per 100 mm dish when cells were exposed to the electromagnetic field for 48 hours and harvested 48 hours after the end of the exposure. This was to ensure the collection of the same amount of cells at the end of the different exposure protocols.

Human neuroblastoma cells (SY5Y) were exposed to ELF-EMF (50 Hz, powerline) in a "blind trial" system that allows direct comparison to control unexposed cells. Different exposure protocols, varying in the density of ELF-EMF and in the time of exposure, were applied: a) 2 mT magnetic flux density, intermittent exposure of 5 min on/5 min off, duration 16h; b) 1 mT magnetic flux density, intermittent exposure 5 min on/5 min off, duration 16h; c) 2 mT magnetic flux density, continuous exposure, duration 16h; d) 1 mT flux density, continuous exposure, duration 16hs; e) 1 mT magnetic flux density, continuous exposure, duration 48h. Harvesting of the cells was performed immediately after the end of exposure, unless indicated otherwise.

2.8.3 RNA preparation and Northern blot analysis

Total RNA from exposed and sham exposed SY5Y cells was extracted using RNA Fast II (Molecular Systems, San Diego, CA, USA) according to manufacturer's instructions and size fractionated on 1% agarose gel containing 2.2 M formaldehyde as described in Sambrook et al. (1989). RNA was subsequently transferred and cross-linked to a nylon membrane (Biodyne A, Pall Europe Ltd., UK). After two hours of pre-hybridisation at 65°C in 0.125 M Na_2HPO_4, 1 mM EDTA, 0.25 M NaCl, 7% SDS, 10% polyethylene glycole and 1% BSA, RNA was hybridised with 10^6 cpm/ml of ^{32}P labelled cDNA probe corresponding to the cytoplasmic domain of the human α3 (nucleotides +975/+1404; Fornasari et al. 1997) and α5 (nucleotides +1005/+1263, Chini et al. 1992) nAChR subunits and to the full length coding region of the human α7 nAChR subunit (Groot Kormelink and Luyten 1997). Following hybridisation, membrane was washed at a final stringency depending on the probe used: α3, 0.1x SSC/0.1% SDS at 50 °C; α5, 0.2x SSC/0.1% SDS at 55 °C; α7, 0.1x SSC/0.1 % SDS at 65°C.

The human Phox2a probe corresponds to the 5'UTR region (nucleotides +26/+219) obtained by digestion of the construct SacI-NcoI (Flora, 2001) with Eag I and NcoI. The human Phox2b probe corresponds to the 5'UTR specifying region (nucleotides -299/-90 with respect to the ATG; GenBank accession number NM_003924) and was obtained by RT-PCR. The primers used were: upper primer 5'-GTG CCA GCC CAA TAG ACG GAT G-3'; lower primer 5'-CTC AAC GCC TGC CTC CAA ACT G-3'. The human DβH probe (nucleotides +728/+1337; GenBank accession number NM_000787) was obtained by RT-PCR using the following primers: upper primer 5'-GCT TCT CTC GGC ACC ACA TTA TC-3'; lower primer 5'-TGA GGG CTG TAG TGA TTG TCC TG-3'. The final stringency washings were Phox2a, 0.2x SSC/0.1% SDS at 55°C; Phox2b, 0.2x SSC/0.1% SDS at 50°C; DβH, 0.1x SSC/0.1% SDS at 55°C, respectively.

After stripping the probe, blots were re-hybridised to a human 18S cDNA probe (nucleotides 715-794; Ambion, Austin, TX, U.S.A.) to check the quality of the RNAs and normalise the amount of RNA loaded.

2.8.4 Radioligand assay with ^{125}I-α Bungarotoxin and ^{3}H-Epibatidine

After the end of the exposure, the cells were detached with buffer A (50 mM Tris-HCl pH 7, 150 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 2.5 mM $CaCl_2$, 2mM PMSF (buffer A) and centrifuged at 10000g for 60 min. The pellets were washed and then homogenised using an Ultra Turrax homogeniser in an excess of buffer A containing 10 µg/ml of a mixture of the protease inhibitors leupeptin, bestatin, pepstatin A and aprotinin in order to block possible proteolysis during the incubation time of the assays.

^{125}I-αBungarotoxin (αBgtx) was from Amershan, England, and had a specific activity of 200 Ci/mmol; ^{3}H-Epibatidine (Epi; NEN, Boston, USA) had a specific activity of 56 Ci/mmol.

In preliminary experiments we determined the affinity of ^{125}I-αBgtx by performing saturation binding experiments on the cell homogenate. The ^{125}I-αBgtx concentrations ranged from 0.1 to 20 nM, and aspecific binding was determined, after overnight incubation at room temperature, using 1 µM unlabeled αBgtx. The affinity of ^{3}H-Epi was determined by performing saturation binding experiments on the cell homogenate using ^{3}H-Epi concentrations between 0.005 and 10 nM, diluted in buffer A, and incubated overnight at 4°C.

After having determined the affinity of nicotinic ligands, the determination of the number of nicotinic receptors was performed using ^{3}H-Epi binding and ^{125}I-αBgtx-binding to membrane homogenates using saturating concentrations of nicotinic ligands (2 nM ^{3}H-Epi or 10 nM ^{125}I-αBgtx) and subtracted for the aspecific binding performed in parallel using 2 nM ^{3}H-Epi or 10 nM ^{125}I-αBgtx and 100 nM cold Epi or 1 µM cold αBgtx. For total and aspecific ^{3}H-Epi binding membranes were always preincubated with 2 mM cold αBgtx.^{125}I-αBgtx binding was performed overnight at room temperature and the ^{3}H-Epi binding overnight at 4°C. At the end of the incubation, the samples were filtered on GF/C filters and radioactivity counted in a α- or β- counter, respectively. The number of receptor present was expressed as fmol of ^{3}H-Epi or ^{125}I-αBgtx bound/mg of protein. Protein measurement was done using the BCA protein assay (Pierce) with bovine serum albumin as the standard.

2.8.5 Protein preparation and Western blot analysis

Total protein extract was prepared from sham and exposed cells by the freezing and thaw method. Briefly, cells were detached by scraping in PBS 1x and collected by centrifugation at 1000 rpm for 15 min at 4°C. The pellet was resuspended in PBS 1x containing protease and phosphatase inhibitors (purchased by SIGMA) and 20 mM Phenyl-Methyl-Sulphonyl-Fluoride (PMSF). Cells were lysed by four repeated passages between liquid nitrogen to freeze and 37°C to thaw. NaCl at a final concentration of 400 mM was subsequently added, to allow extraction of nuclear protein. Samples were incubated 10 min on ice and extract clarified by centrifugation at 14000 rpm for 30 min at 4°C in a table-top centrifuge (Eppendorf). Twenty micrograms of total extract were then separated by SDS-PAGE and transferred to nitrocellulose membrane (Schleicher & Schuell). The membranes were pre-incubated with blocking buffer (5% non-fat dry milk, 20 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.1% Tween 20) for one hour, after which the primary antibodies were added at appropriate dilutions and incubated for two hours; the secondary antibodies conjugated with horseradish peroxidase were then added and incubated for one hour. The bands were revealed using Super Signal West Dura (Pierce). Standard molecular weights (New England Biolabs) were loaded in parallel.

2.9 Experiments with *Xenopus laevis* oocytes, granulosa cells of rats, HeLa cells, Chinese Hamster Ovary (CHO) cells and human fibroblasts (Participant 7)

2.9.1 ELF-EMF-exposure setup

See 2.1.1

2.9.2 ELF-EMF exposure, expression in Xenopus oocytes and RNA preparation of rCx46

The cDNA for rCx46 were subcloned in the SP64T vector for RNA transcription. SP64T contains 250 bp of the non-coding sequence from *Xenopus laevis* b-globin including a poly-A tract that increases translational efficiency. RNA was prepared by using a synthesis kit containing SP6 RNA polymerase and CAP analogue purchased from Ambion (Austin, USA). The Xenopus expressions construct was linearised with XbaI for RNA transcription. The transcript concentration was estimated spectrophotometrically and analysed on agarose gels. The oocytes were isolated from *Xenopus laevis* ovaries and stage V-VI oocytes were collected and defolliculated by collagenase treatment (5 mg/ml, 355 U/mg, 1.5 h; Worthington, Type 2) in Ca^{2+}-free ND96 solution (96 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, Na-HEPES at pH 7.4 and adjusted with sorbitol to 240 mosmol/l). An injection apparatus

(Nanoliter Injector, World Precision Instruments) was used to inject 23 nl of 25 ng/µl of Cx46 cRNA and 23 nl of DNA antisense to the endogenous XenCx38 oligo 5¢-gCT gTG AAA CAT ggC Agg Atg (500 ng/µl) (Tib Molbiol) to eliminate endogenous hemi-channel currents. Oocytes were incubated in ND96 supplemented with antibiotics (100 U/ml penicillin/streptomycin) at 17°C. During the expression period of 14h, 17h and 20h the oocytes were exposed to ELF-EMF of 50 Hz powerline or sham exposed. Field strength of 2.3 mT was either continuously or intermittently (5 min on/10 min off) applied. In a further series of experiments 1.0 mT was applied with the intermittent application protocol. For the cell-to-cell coupling assay of paired oocytes, each cell of a pair of oocytes were injected with 23 nl of DNA antisense to the endogenous XenCx38 in order to suppress endogenous coupling in addition 23 nl of rCx46 cRNA (25 ng/µl)were injected. The expressing oocytes were incubated for 16h in ND96 medium, followed by manual removal of the vitelline layers. Two oocytes were paired at their vegetal poles and incubated with ND96 at 17°C for further 8h in the ELF-EMF setup (Participant 10). During this incubation the oocytes pairs were exposed to ELF-EMF (50 Hz, powerline, 1.0 mT, intermittently (5 min on/10 min off)).

2.9.3 Electrophysiological recordings of single and paired oocytes

The two electrode voltage clamp technique was applied to measure the expressed and conducting rCx46 gap-junctional hemi-channels in single *Xenopus laevis* oocytes using a voltage-clamp amplifier Turbo TEC-10 CD (npi electronic, Tamm, Germany). Voltage protocols were applied by a Pentium 100 MHz Computer linked to an ITC-16 interface (Instrutech. Corp., NY). The following pulse protocol was used throughout the experiments: From a constant holding potential of -90 mV or -80 mV variable test potentials were applied for 15 s in the range from -110 mV to +70 mV in steps of 10 mV after repolarisation of the oocyte at a holding potential of -90 mV or -80 mV. The latter was constantly applied for at least 70s. The current signals were filtered at 1 kHz and were sampled at 0.5 or 0.25 kHz. Data acquisition and analysis were performed by using Pulse/PulseFit (HEKA, Germany), Igor Pro (Wave Metrics, USA), Origin (Microsoft), PatchMaschine (V. Avdonin, University of Iowa, USA). n denotes the numbers of individual oocytes. The data are given as mean ± s.e.m. For electrophysiological recordings on paired oocytes the setup was extended by a second amplifier and a further pair of micromanipulators /electrodes. For the pulse protocol of paired oocytes a holding potential of -40 mV, close to the resting potential, was used for both oocytes. The depolarising test pulses were applied for 5s (10s) in the range from -120 mV to +120 mV and the corresponding holding potential was held for 15s (30s). For the electrophysiological recordings the micropipettes were filled with 3 M KCl resulting in input resistances of 1-1.5 M. During the current recordings the oocytes were continuously superfused with the corresponding solution at a rate of 0.5 ml/min and all recordings were performed at room temperature (20-22°C). The standard bath was a nominal Ca^{2+}- free ND96 solution at pH 7.4. The different Ca^{2+} concentrations of ND96 were obtained by addition of suitable concentrations of $CaCl_2$ to the standard solution. For experiments on single oocytes Ca^{2+}-concentrations of 0.0, 0.25 and 0.5 mM and on paired oocytes 1.8 mM and 5 mM were used.

2.9.4 Voltage-jump current-relaxation and membrane conductance of hemi-channels

The steady-state current amplitudes were leak-subtracted and denoted as Iss. The Iss values are presented as function of driving voltage (V-Vrev). The leak current at the applied test potential V was determined by extrapolation of the corresponding current values in the range of -100 mV to -70 mV, at this voltage the voltage dependent hemi-channels are closed.. The reversal potential (Vrev) of the rCx46-mediated current was calculated by a 4-point interpolation polynom of third order. The corresponding membrane conductance G(V) was calculated from the steady-state current amplitude divided by the driving voltage (V-Vrev) and plotted as function of test potential V. In the absence of a significant time- and voltage-dependent current inactivation the corresponding G(V) values in the range of -110 mV < V < +40 mV could be fitted by a simple Boltzmann distribution according to: $G(V)=(A/(1+\exp(-(V-V1/2)zF/RT))+B)$. R, T, F have their usual meanings. V1/2 denotes the half-activation voltage at which 50% of the maximal membrane conductance is observed. z gives the number of membrane bound equivalent gating charges. The parameter A denotes the maximal membrane conductance Gmax of expressed and conducting rCx46-connexons hemi-channels and B the corresponding leak conductance of the oocyte. B is assumed to be voltage independent. For different experiments G(V) was normalised to the corresponding values

obtained at A=1 and B=0, respectively. A similar subtraction of the leak-current was considered in the experiments on paired oocytes.

2.9.5 Cell cultures

Granulosa cell line GFSHR-17 (rat) (Keren-Tal et al. 1993), HeLa cells (human), Chinese Hamster Ovary (CHO) cells and fibroblasts (human, Participant 3) were cultivated in Dulbecco´s modified Eagle´s medium F-12 Ham (DMEM-F12, Sigma Corp., USA) added with 10% fetal calfserum (FCS, Sigma Corp., USA) and 50 U/ml Penicillin, 50 µg/ml Streptomycin (Gibco BRL, G) (300 mosmol, pH 7.4). Both cell lines were incubated under an atmosphere containing 5% CO_2 at 37°C. The culture dishes (35 mm in diameter) for the measurement of the intercellular free calcium ($[Ca^{2+}]i$) contained six coverslips of 10 mm diameter. Under these conditions the cultured cells were ELF-EMF exposed (50 Hz sinusoidal, 4h - 24h, 1.0 mT, 5 min on/10 min off) or sham exposed. The culture dishes (50 mm in diameter) for the measurement of volume regulation and the Comet assay analysis for contained a coverslip of 25 x 50 mm diameter. Under these conditions the cultured cells were ELF-EMF exposed or sham exposed.

2.9.6 Measurement of [Ca2+]I

Measurement of $[Ca^{2+}]i$ was performed according to Grynkiewicz et al. (1985). For measurement of $[Ca^{2+}]i$ the cells were loaded with fura 2-AM (5 µM and 1% DMSO) for 60 min under ELF-EMF exposure. Fura 2-AM was added to the bath during an off phase of ELF-EMF exposure, respectively. After the indicated time of exposure Fura 2-Amloaded loaded cells grown on a coverslip were transferred to an exposure-free superfusion chamber mounted on an inverted Axiovert (Zeiss, Germany) microscope. The cells were superfused with a bath solution containing (in mM): 145 NaCl, 5 KCl, 2 $CaCl_2$, 1.5 $MgCl_2$, 5 glucose, 10 Hepes, (pH 7.4, adjusted with NaOH; 300 mosm) at 2 ml/min for 3 min to wash-off extracellular fura 2-AM and DMSO at room temperature. The cells were excited at 340 nm and 380 nm using a monochromator polychrome II (T.I.L.L. Photonics GmbH, Planegg; Germany) by a 75 W XBO xenon lamp and the corresponding fluorescence was registered with a digital CCD camera (C4742-95, Hamamatsu Photonics K.K.; Japan). The ratio of excitation at 340 nm to 380 nm was calculated and calibrated to determine $[Ca^{2+}]i$ using the program Aquacosmos (Hamamatsu Photonics K.K.; Japan). Measurement of $[Ca^{2+}]i$ was started about 10 min after completion of ELF-EMF exposure and recorded for 4-8 cells simultaneously. In a further series of experiments two additional stressors were applied after the period of ELF-EMF exposure, respectively. Either 200 µM H_2O_2 was added to the bath or 30 mM NaCl was replaced by KCl. The cells were superfused with the corresponding solution (2 ml/min) for 10 min. Thereafter the solution was replaced by the bath and the recording of $[Ca^{2+}]i$ started.

2.9.7 Comet assay

The granulosa cell line of rat (GFSHR-17), HeLa cells (human) and Chinese Hamster Ovary (CHO) were cultivated as described above. The cells were ELF- and sham- exposed at various frequencies using the exposure parameters 5 min on/10 min off, 1.0 mT applied during 12 to 20 hours. After exposure the Comet assay was performed as described by Ivancsits et al. (2003a, b). The viability of the cells was determined by trypan blue and only slides containing cells with a viability of more than 90% were analysed. For each experiment 3000 nuclei are scored (1000 per slide) for exposed and sham exposed cells and classified into 5 categories according to material and methods of Ivancsits et al. (2002b). The results for DNA damage in the Comet assay are obtained from at least 2 independent exposure experiments. Analysis of exposed and sham-exposed cells was performed in a double-blind approach.

2.9.8 Measurement of cell volume regulation

For measurement of the cell volume the cells were trypsinised (0.25% Trypsin, pH 7.4), collected and centrifuged for 5 min at 500 xg after ELF exposure (18h expression time, 1.0 mT, 50 Hz, 5 min on/10 min off). The pellet was resuspended in 10 ml PBS (in mM: 140 NaCl, 2.7 KCl, 8 Na_2HPO_4, 1.5 KH_2PO_4, 300 mosmol, pH 7.4). For the volume measurements, 1-2 µl of the cell suspension were placed on a cover slip in an exposure-free superfusion chamber mounted on an inverted Axiovert (Zeiss, Germany) microscope. After 5 min, the cells adhered and 2-3 ml of PBS-solution (300 mosmol) were added to the

dish. The cells were superfused with the PBS-solution (300 mosmol) and the cell volume was recorded at time intervals of 30s about 40 minutes. The cell sizes were registered with a digital CCD camera (C4742-95, Hamamatsu Photonics K.K.; Japan). After 30 sec the 300 mosmol PBS-solution was replaced by a 250 mosmol PBS-solution (or 350 mosm solution). This hypotonic (or hypertonic) solution was exchanged after 20 min by PBS-control (300 mosmol), again. The diameter of the cells of spherical shape (breath, height) were measured and the corresponding rotationellipsoid volume determined. The time dependent volume V(t) was subtracted by the basis volume V0, normalised to the maximal volume and the mean calculated.

2.10 Experiments with the human endothelial cell lines EA.hy926 and EA.hy926v1 (Participant 6)

2.10.1 RF-EMF 900 MHz GSM signal exposure system dosimetry

Cells were irradiated with a simulated mobile phone microwave radiation in specially constructed exposure system, which is based on the use of high Q waveguide resonator operating in TE_{10} mode. The irradiation chamber has been placed vertically inside a cell culture incubator with two 55 mm-diameter glass Petri dishes placed so that the E-field vector was parallel to the plane of the culture medium. Temperature controlled water was circulated through a thin (9 mm) rectangular glass-fiber-molded waterbed underneath the Petri dishes. In all experiments reported here, cells were exposed for 1 hour to 900 MHz GSM signal at an average SAR of 2.4 W/kg. SAR values ranged from 1.8 W/kg to 2.5 W/kg depending on the area of the dish, what was caused by the non-uniform distribution of the RF-EMF radiation field. The average SAR level of 2.4 W/kg was selected because it is slightly above of the safety limit for the mobile phone microwave radiation emission as defined by ICNIRP (International Commission on Non-Ionizing Radiation Protection). RF-EMF signal was generated with EDSG-1240 signal generator and modulated with pulse duration 0.577 ms and repetition rate of 4.615 ms to match the GSM signal modulation scheme. Signal was amplified with RF Power Labs R720F amplifier and fed to the exposure waveguide via monopole type feed post. The SAR distribution in the cell culture and the E-field above the cell culture were determined using computer simulations (FDTD method). The simulations were done with commercial XFDTD code (Remcom, USA) with simulation grid size of 3x3x3 mm^3 in the main grid and 1x1x1 mm^3 in sub grid, consisting of the culture dishes and part of the waterbed. The maximum SAR was obtained in the centre of Petri dish, decreasing to about 6 dB at the edges of dish. Simulation results were verified with measurements. Electric field in the air above cell cultures was measured with a calibrated miniature Narda 8021B E-field probe. The measured E-field values differed less than 15% from the corresponding simulated E-fields. The SAR distribution was measured with small, calibrated temperature probes (Luxtron and Vitek) directly from the culture medium. The measurements were done at room temperature outside the incubator with increased culture medium height, in order to reduce the measurement uncertainty at air-medium boundary. The temperature was measured (Vitek probe, BSD Medical, USA) for 10 sec. in order to limit the effect of heat convection and conduction (Moros and Pickard, 1999). The Luxtron probe has lower temperature resolution (0.1°C) compared to Vitek probe (0.001°C) and thus the temperature had to be measured for 1 min. to achieve sufficient temperature rise (1°C). Due to these short measurement times the power fed to the chamber was increased up to 25 W and the resulting SAR value was afterwards scaled down to 1 W of input power. The measured SAR values at the centre of the culture medium (3-mm depth) were 2.5 W/kg (Luxtron, USA) and 5.0 W/kg (Vitek, USA). These values can be compared to the simulated value of 2 W/kg and 3.6 W/kg, respectively, with simulation parameters changed to correspond with the measurement situation. The measured values can be considered as the upper and lower limits of SAR due to measurement uncertainties described above and thus they validate the simulations. Waveguide resonator's water-cooling system was tested with long-term temperature measurements by using Luxtron probe. The temperature was recorded twice a minute over normal 1-hour exposure period at 2 W/kg. The temperature remained at 37 ± 0.3°C during the whole measurement time. Therefore, the reported biological effects are of non-thermal nature. Additionally, human endothelial cells were also exposed to GSM 1800 MHz radiation in talk and cw mode. See also 2.1.

2.10.2 Cell cultures and exposure

EA.hy926 and EA.hy926v1 cells (gift from Dr. Cora-Jean S. Edgell, North Carolina University at Chapel Hill, NC, USA) (Edgell et al. 1983) were grown Dulbecco's MEM, supplemented with antibiotics, 10% fetal bovine serum, L-glutamine and HAT-supplement. For experiments, cells were removed from culture flasks with trypsin, washed and seeded at density of 1.2 x 10^6 cells per 55 mm-diameter glass Petri dish (900 MHz GSM exposure) or seeded at density of 0.4 x 10^6 cells per 35 mm-diameter plastic Petri dish (1800 MHz GSM signal exposure). After overnight culture semi-confluent monolayers of EA.hy926 cells were exposed to sham or RF-EMF radiation. Cell cultures for sham and irradiation were prepared in the same kind of glass dishes, derived from the same batch of cells, were seeded at the same cell density and were grown for the same period of time before experiment. The only difference between irradiated and sham samples was that the irradiated dishes resided for 1-hour in incubation chamber with RF-EMF radiation turned-on whereas sham dishes resided in the irradiation chamber for the same period of time but with irradiation turned off.

2.10.3 ^{32}P-othophosphate metabolic labelling

To determine changes in protein phosphorylation ^{32}P-orthophosphate was present in cell culture during the 1-hour sham or RF-EMF exposure. In experiments where the time-course of hsp27 phosphorylation was determined, ^{32}P-orthophosphate was present in cell cultures during the whole post-exposure incubation period. During the phosphorylation cells were incubated in culture medium consisting of phosphate-free DMEM that was supplemented with dialysed FBS and with ^{32}P- orthophosphate (NEN, Cat no. NEX-053s). Briefly, confluent monolayers of endothelial cells were washed twice with the pre-warmed (37°C) labelling medium that did not contain ^{32}P-orthophosphate, in order to wash away residual phosphates from the cell cultures. Thereafter, pre-warmed ^{32}P-orthophosphate-containing medium (5 mCi) was added to the cells and dishes were irradiated immediately for one hour. Following irradiation petri dishes were placed on ice, labelling medium was aspirated, cells were rinsed with cold PBS supplemented with protease/phosphatase inhibitors (1 mM PMSF; 0,4 mM orthovanadate) and cells were scraped and collected with ice cold PBS. From this point onwards orthovanadate was present in all solutions used to extract phosphoproteins. In experiments where the time-course of hsp27 phosphorylation was determined, ^{32}P-orthophosphate was present in cell cultures during the whole post-exposure incubation period (up to 5 hours).

2.10.4 2D-electrophoresis - for protein phosporylation studies

Cells were harvested, washed once with ice cold PBS containing 1mM PMSF and lysed on ice for 10 minutes in buffer consisting of 9.5 M Urea, 2% CHAPS, 0.8% Pharmalyte pH 3-10 and 1% DTT. Lysates were cleared of debris by centrifugation 42000 xg at +15°C for 1 hour. The pellet containing insoluble debris was discarded and supernatant was collected and its protein concentration was measured with the Bradford method. Proteins in the lysates were separated using standard 2D-electrophoresis method - isoelectrofocusing (IEF) in the first dimension and SDS-PAGE in the second dimension.

1st-dimension isoelectric focusing

The 125 ug protein was applied to the groove of the re-swelling tray that contained 11 cm-long IPG strip with pH range of 3-10 (APBiotech, Sweden). The IPG strips were incubated overnight with the protein lysate solution. The proteins in IPG strips were separated by isoelectrofocusing (IEF) using the programmable power supply with the following protocol:

- 300 V, 1 W, 1 mA for 6 minutes at 20°C
- 3500 V, 1 W, 1 mA for 6 minutes at 20°C
- 3500 V, 1 W, 1 mA for 24 hours at 20°C

After completion of the IEF-separation the strips were equilibrated for 10 minutes on a rocking platform in solution-I consisting of urea (6 M), glycerol (30% w/v), SDS (2% w/v), DTT (100 mg/10 ml) in 50 mM Tris-HCl buffer pH 8.8 with a trace of bromphenol blue (migration marker). Thereafter, the strips were placed in a solution-II that contained iodocetamide (480 mg /10 ml) instead of DTT and equilibrated on a rocking platform for another 10 minutes at room temperature.

2nd-dimension SDS-PAGE

Equilibrated IPG strips were attached on the top of 8% SDS-PAGE gel with melted agarose to ensure firm contact. Gels were run with 40 mA/gel for ca. 2.5 hours at 4°C.

After completion of the electrophoretic separation gels were silver stained using Morrissey's modification of the Merril's method and images for computerised analysis were acquired into PC using the Bio-Rad GS-710 densitometer.

2.10.5 ^{32}P-autoradiography

2D-gels, containing metabolically ^{32}P-labelled phosphoproteins, were dried in gel dryer and used for autoradiography. Images generated on X-ray films were acquired into PC for computerised analysis using the Bio-Rad GS-710 densitometer.

2.10.6 2D-electrophoresis - protein expression screening

Immediately after the end of the exposure to 900 MHz GSM mobile phone radiation-like signal at the average specific absorption rate (SAR) of 2.4 W/kg cells were placed on ice, washed with ice-cold PBS and lysed with buffer consisting of: 7 M urea, 2 M thiourea, 4% chaps, 2% IPG buffer pH 3-10 NL, and 1% dithioreitol (DTT), 1 mM sodium orthovanadate and 1 mM PMSF. Protein concentration in lysates was measured using Bradford-method and 175 µg of total protein was used for 2-DE.

IEF was performed using IPGphor apparatus and non-linear pH 3-10 18 cm long IEF strips (Amersham Biosciences, Sweden). The samples were loaded using in-gel rehydration in a buffer containing 9 M urea, 2% chaps, 0.2% DTT, 0.5% IPG buffer pH 3-10 NL for 12 hours. IEF was run at 20°C using step-and-hold and gradient methods as follows: 30 V - 2 hrs, 100 V - 0.5 hrs, 300 V - 0.5 hrs, 600 V - 0.5 hrs, 1500 V - 0.5 hrs, 8000 V gradient 4 hrs, 8000 V - until the 65000 volt-hours were achieved.

For SDS-PAGE the IEF strips were equilibrated for 15 min with 6 M urea, 30% glycerol, 50 mM Tris-HCl pH 8.8, 2% SDS, and 10 mg/ml DTT for 15 min and then for another 15 min in the same buffer 25 mg/ml iodoacetamide replacing DTT. SDS-PAGE was run in 8% gel using Protean IIxi Multicell apparatus (Bio-Rad, UK) and a constant current of 40 mA/gel at 10°C.

After electrophoresis gels were fixed with 30% ethanol and 0.5% acetic acid overnight, washed with 20% ethanol and ddH_2O, sensitised with sodium thiosulfate (0.2 g/l), incubated in silver nitrate solution (2 g/l) and developed in a solution of potassium anhydride (30 g/l), 37% formaldehyde (0.7 ml/l) and sodium thiosulfate (0.01 g/l). The development was stopped with Tris (50 g/l) and acetic acid (0.05%) solution. Silver stained gels were stored in ddH_2O at 4°C. The gels were scanned using GS-710 densitometer (Bio-Rad, UK).

The MALDI-MS analysis service was purchased from the Protein Chemistry Laboratory of the Institute of Biotechnology at the Helsinki University, Finland. The spots were reduced with DTT and alkylated with iodoacetamide before overnight digestion with trypsin (Sequencing Grade modified Trypsin, promega, USA). The peptide mixture was concentrated and desalted using Millipore ZipTip^{TM} µ-C18 pipette tips. The peptide mass fingerprints were measured with Bruker Biflex^{TM} MALDI-ToF mass spectrometer in a positive ion reflector mode using α-cyano-4-hydroxycinnamic acid as a matrix. The MALDI spectra were internally calibrated with the standard peptides, angiotensin II and adrenocorticotropin-18-39. The database searches were performed using ProFound (http://prowl.rockefeller.edu/cgi-bin/ProFound) and Mascot (http://www.matrixscience.com) searches.

2.10.7 Western blotting

Immediately after the completion of 2D-electrophoretic separation of protein lysates, gels were placed into the transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol and 0.1% SDS) and blotted onto PVDF membrane (Bio-Rad) using Novablot semi-dry blotting apparatus (APBiotech, Sweden). The transfer of proteins on the membrane was performed with current of 0.8 mA/cm^2 for 45 minutes at room temperature. Following transfer, the membranes were blocked overnight at 4^{o}C in Tris-buffered-saline (TBS, pH7.4) containing 5% of non-fat milk proteins. The expression of Hsp27, MAP p38 kinase and protein phosphatase-1α was detected in western blot membranes by ECL method using specific polyclonal antibodies, peroxidase-conjugated second antibody and West Pico ECL kit (Pierce, USA).

2.10.8 Immunoprecipitation

Immunoprecipitation experiments were performed using cells that were metabolically labelled with ^{35}S-methionine (APBiotech, Sweden) as follows. Briefly, confluent EA.hy926 monolayers were washed twice with the pre-warmed (37°C) labelling medium (phosphate-free) to remove phosphates. After completion of washing, the pre-warmed ^{35}S-methionine-containing (2 mCi) labelling medium was added to culture dishes and cells were allowed to incorporate ^{35}S-methionine overnight. Then, ^{35}S-methionine-containing cultures were irradiated for 1 hour. After the end of irradiation dishes were placed on ice, cells scraped in 1 mM PMSF containing PBS and used in immunoprecipitation. In experiments where the time-course of protein expression changes was determined the ^{35}S-methionine was present in the cell cultures during the whole post-exposure incubation period (up to 8 hours). Harvested cells were lysed in ice-cold RIPA buffer. Lysates were centrifuged 10000 xg at 4°C for 10 minutes to remove debris and lysates' protein concentration was measured with the Lowry-Ciocalteau method. Samples containing 230 µg of proteins were placed in eppendorf tubes and pre-cleared with 2.3 µg of non-immune goat IgG (Santa-Cruz, USA; sc-2028) and 20 µl of recombinant-Protein-G-conjugated Sepharose-4B (Zymed, USA, Cat. no. 10-1242) at 4°C on a shaker for 30 minutes. After pre-clearing the Sepharose-beads were removed by centrifugation (1000 xg; 4°C; 10 min) and selected proteins (hsp27, MAP p38 kinase, protein phosphatase-1α) were immunoprecipitated with 2 µg of specific antibody and 20 µl of recombinant-Protein-G-Sepharose-4B-conjugate (overnight at 4°C on a shaker). Thereafter, beads were collected by centrifugation, washed 4 times with RIPA buffer, dispersed in the electrophoresis sample buffer, boiled on water-bath for 3 minutes and proteins released from the beads were resolved using 8% SDS-PAGE gel (40 mA/gel). Gels were stained with coomassie blue, dried between cellophane sheets and exposed with X-ray film for different periods of time to detect the ^{35}S-methionine labelled immunoprecipitated proteins.

2.10.9 cDNA Expression Arrays

Total RNA isolation

For the isolation of total RNA from RF- or sham-exposed cells we used NucleoSpin RNA II kit (Clontech, USA). Briefly, confluent cell cultures we directly lysed on glass culture dishes. RNA, from the cleared cell lysates, was directly immobilised in Spin columns provided by the manufacturer. After DNAse treatment the total RNA was eluted from the columns and analysed for the possible remaining DNA contamination by PCR using b-actin primers against genomic DNA. RNA from several independent experiments were pooled and stored at -80^{o}C for further use.

Probe synthesis and analysis of gene expression

For the synthesis of cDNA probes and differential analysis of gene expression we used Atlas Pure Total RNA Labelling System (Clontech) and Atlas cDNA Expression Arrays (Clontech), respectively. In this system mRNA was enriched from the total RNA by binding it to Streptavidin-biotin-oligo- (dT) coated magnetic beads. After enrichment of the mRNA they were reverse transcribed to radioactive cDNA probes directly when still bound to magnetic particles. Purified probes were hybridised with Atlas filters containing complementary cDNA spots and analysed by autoradiography. AtlasImage 2.0 software was used for the differential gene expression analysis of autoradiograms.

2.10.10 Cell cycle analysis

Cell cycle distribution among the EA.hy926 cells was detected by staining the DNA with propidium iodide followed by flow cytometry analysis. Briefly, cells were collected by centrifugation, washed once with phosphate-buffered saline (PBS) and fixed in 90% methanol on ice for 10 min. After fixation, the cells were washed twice with PBS and suspended in RNAse solution in PBS (100 units/ml) and incubated at 37^{o}C for 30 minutes. At this point the propidium iodide solution (10 mg/ml in PBS) was added to the cells in RNAse solution, and the incubation was continued on ice overnight. Upon termination of incubation, cells were washed once with PBS and analysed by FACScan (Becton Dickinson, USA).

2.10.11 Caspase-3 activity

Caspase-3 activity was detected in non-fixed cells using CaspaTaq Caspase-3 Activity Kit (Intergen, USA). The active caspase-3 molecules are labelled in cells with a green fluorescent probe (FAM-DEVD-FMK) which only binds to the active caspase-3. Dead cells were excluded from the analysis by staining with propidium iodide that, when used with non-fixed cells, labels only cells with permeable membrane (necrotic or late apoptotic cells). The cell cultures were either sham- or RF-EMF-exposed with or without staurosporin (positive control of caspase-3 activation). Activity of caspase-3 was analysed either immediately after the exposure or 4h to 24h after exposure. Fluorescent content of the cells was analysed by flow cytometry with Lysys II software (Becton-Dickinson, USA).

2.10.12 Immunhistochemistry

A standard indirect immunofluorescence method was used for immunohistochemistry. Cells were washed twice with PBS and fixed in cold 3.7% paraformaldehyde in fixing buffer (0.1 M Pipes, 1 mM EGTA, 4% polyethyl glycol 8000, 0.1 M NaOH pH 6.9) overnight at 4°C. After fixing cells were rinsed twice with PBS, permeabilised with 0.5% Triton X-100 in fixing buffer 10 min, rinsed with PBS and permeabilised with 0.1% sodiumborohydride in PBS. After permeabilisation cells were rinsed with PBS and blocked with 5% BSA in PBS for 30 min. The primary antibody (Hsp27 StressGen, Canada) was incubated for an hour as well as the TRITC-labelled secondary antibody (DAKO, Denmark). After antibody incubations cells were rinsed with PBS and stained with Alexa Fluor 488 phalloidin for 30 min. Specimens were observed using a Leitz fluorescense microscope and computerized image acquisition system (Metafer, Germany)

2.10.13 Image analysis

Images of 2D-gels and X-ray films were analysed with the PDQuest 6.1.0/6.2.0 software (Bio-Rad, UK) or Phoretix 1D Advanced 5.0 (Nonlinear Dynamics, USA).

2.10.14 cICAT method

Protein Labelling and Purification

1 mg each of sample (talk and cw exposed) was labelled separately using the acid cleavable isotope-coded affinity tag (cICAT) reagent (Applied Biosystems, USA) following the vendors protocols as has been described (Burlingame). Briefly, the protein samples were separately reduced at cysteine residues using Tris (2-carboxyethyl) phosphine (TCEP, Pierce, USA), and the free sulfhydryl groups labelled using either the normal ($C^{13}(0)$) cICAT reagent, or the isotopically heavy ($C^{13}(9)$) cICAT reagent containing nine C^{13} atoms. The samples were then combined, enzymatically digested using trypsin (Promega, USA) and the resulting peptides were fractionated using strong-cation exchange (SCX) HPLC. The SCX HPLC was carried out on an Integral HPLC system (Applied Biosystems, USA) using a 2.1 mm x 250 mm polysulfoethyl A SCX HPLC column (PolyLC). The A buffer was 5 mM KH_2PO_4/25% acetonitrile pH 3.0 and the B buffer was 5 mM KH_2PO_4/25% acetonitrile pH 3.0 containing 300 mM KCl. The peptides were eluted and collected in one minute fractions using a gradient profile of 0-25% B over 30 minutes, followed by 25-100% B over 20 minutes, followed by washing of the column for 10 minutes at 100% B. Collected peptide fractions were affinity purified using avidin chromatography columns. The purified, labelled cysteine-containing peptides were then subjected to an acid incubation to cleave the biotin affinity tag from the peptides (Burlingame). The cleaved samples were then separated using offline microcapillary reverse-phase liquid chromatography (μLC) with collection in one minute fractions onto a MALDI sample target using a 180 μm x 15 cm reverse phased column home-packed with 5 μm, 300 Å C18 material (Magic, Michrom Inc., USA) and an Ultimate capillary LC system coupled to a Probot sample spotter (Dionex, USA). The MALDI matrix α-cyanohydroxycinnamic acid (CHCA, Agilent, USA) was automatically added to the eluent at each spot on the sample plate.

Automated mass spectrometric analysis

After μLC fractionation to the MALDI sample plate, the samples were analysed using an abundance-ratio dependent methodology on a oMALDI qQTOF mass spectrometer (oMALDI Qstar, MDS-Sciex/Applied

Biosystems). First, a single-stage mass spectrum of each sample spot on the plate is acquired for 30 seconds at each spot. The relative intensity ratios, as well as singlet peaks for all of the detected, cICAT labelled peptides are then automatically calculated using an automated software algorithm. Those peptides showing $C^{13}(0):C^{13}(9)$ relative intensity ratios of >1.7 or <0.6 were outputted to an inclusion list for identification by tandem mass spectrometry (MS/MS). All those peaks identified as singlets (i.e. no matching $C^{13}(0)$ or $C^{13}(9)$ peak) were also selected for MS/MS analysis. The peptide masses contained in the inclusion list at each sample spot were then analysed by MS/MS analysis, using one minute data acquisition time for each peptide.

Sequence database searching and quantitative analysis

The acquired MS/MS data were searched against the human protein sequence database maintained at the National (USA) Cancer Institute using the search program Sequest. For all MS/MS data, the search was run with no enzyme constraint, or amino-acid composition constraint (i.e. only those sequences containing cysteine), and a mass tolerance of 0.1 Da was used for the precursor peptide mass. A differential mass addition to cysteine of 227.13 was indicated in the search parameters for the $C^{13}(0)$ reagent, and 236.16 for the $C^{13}(9)$ reagent. Only matches to peptide sequences containing cysteine were kept after the database search. The results were further statistically scored using a recently described statistical algorithm for validation of sequence database search results. Only those peptides having a confidence score of 0.85 or greater using this tool were considered to be accurate matches. The quantitative $C^{13}(0)/C^{13}(9)$ values determined by the automated software described above were matched to each identified peptide, and these ratios were each checked for accuracy by manual inspection of the raw mass spectral data.

2.11 Effects of ELF-EMF and RF-EMF on gene expression in human cells analysed with the cDNA array (Participant 12)

2.11.1 ELF-EMF and RF-EMF exposure setups

See 2.1

2.11.2 Cell cultures and RNA isolation

See reports of the REFLEX Participants who provided samples for this investigation.

2.11.3 RZPD cDNA arrays

The whole-genome Human Unigene RZPD-2 cDNA array contains about 75,000 cDNA clones (I.M.A.G.E. clone collection), the Mouse Unigene RZPD-1 array about 25,000 clones, each selected from UniGene clusters (group of Bernhard Korn (RZPD), see also: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=unigene). The cDNA products were PCR amplified by M13 forward and reverse standard primers and spotted in duplicates on 22 x 22 cm nylon membranes (mouse: 1 part, human: 3 parts) in a 5 x 5 pattern (group of Uwe Radelof (RZPD), see also Boer et al. (2001) Genome Res. 11, 1861-1870). Each 5 x 5 field contained 11 genes spotted in duplicates as well as the E.coli kanamycin gene (1 spot) and an Arabidopsis gene (2 spots) as “empty” spots for background subtraction during data analysis. For quality control, M13 forward and reverse primers were end labelled with ^{33}P gamma ATP and hybridised to each membrane to control of all filters of the same robot run were spotted even and complete. After quality control, the membranes were stripped and used for complex hybridisation after about 6 weeks. Only filters from the same robot run containing comparable concentrations of PCR products representing single genes or ESTs were used for hybridisation with the different samples to be compared. One individual hybridisation experiment was done on the same filter batch with all 4 samples. Repetitions, however, were performed with different filter batches to exclude biases caused by using filters from only one robot run.

2.11.4 Hybridisation of global cDNA arrays and image analysis

RNA was isolated from exposed and as a control sham-exposed cells from different cell lines (Table 1). RNAs coming from individual experiments were checked separately for degradation (28S/18S rRNA ratio 1.5-2.0) and concentration (at least 1 µg/µl in H2O) with the Bioanalyzer (Agilent). Afterwards, RNAs from 2 individual exposures were pooled for each hybridisation sample in same concentrations. Hybridisation was performed according to Boer et al. (2001) with minor modifications: 10 µg of total RNA per sample was reversely transcribed using (dT)18 primer and 33P alpha-dCTP without amplification (Superscript II reverse transcriptase, Invitrogen) and purified. The labelled cDNA was hybridised with the arrays. The hybridisation solution contained 6 x SSC/5 x Denhardt's, as well as Cot-1 DNA (Invitrogen Co., Germany) and (dA)40 oligonucleotide for blocking. After exposition of the hybridised membranes, the PhosphorImager screens were scanned (Fuji FLA-3000, 100 µm resolution, Fuji BAS-reader software). The primary image analysis (estimation of nVol grey level values for each individual spot) was done by the help of the ArrayVision software package (Interfocus), which had been adjusted to the 5x5 array before. The background was corrected locally in each 5x5 field by subtracting the empty spot signal (average signal of 3 spots, see above). Normalisation was done via the average signal intensity (without empty spots) on the whole membrane. Two independent hybridisations were performed for each experiment (4 data points per gene because of spotting of each gene in duplicates).

2.11.5 Pre-processing (data cleaning) and Modified SAM method (and Selective SAM method)

Original expression profiling data ("control" data *ctrl*, related to gene expression without the applied EMF, and "exposed" *exp*, related to gene expression after EMF exposure) were normalised and the background was removed as mentioned above. For each experiment (Table 1) at least two hybridisations were performed. In a first pre-processing, signals were removed giving a zero or an infinite ratio (*ratio = exp/ctrl*), that could lead to a reduction of the available measurements for some genes.

Table 1. Gene expression profilings on human and mouse global cDNA arrays

Cell line	Exposition	Exposure experiments/ profiling	Array	Participant
ES-1	ELF/EMF: 50 Hz, 1 mT	4	Human	3
human primary fibroblasts	5 min ON, 10 min OFF, 24 h		Unigene RZPD-2	
ES-1	ELF/EMF: 50 Hz, 1 mT	4	Human	3
human primary fibroblasts	5 min ON, 10 min OFF,15 h		Unigene RZPD-2	
SY5Y	ELF/EMF: 2 mT	4	Human	11
human neuroblastoma	5 min ON, 5 min OFF, 16 h		Unigene RZPD-2	
ES	ELF/EMF: 50 Hz powerline	3	Mouse	4
mouse embryonic stem cells	2.3 mT		Unigene RZPD-1	
NB69	RF/EMF: 1800 MHz (GSM Basic)	2	Human	5
human neuroblastoma	SAR 2 W/kg		Unigene RZPD-2	
	5 min ON, 10 min Off, 24h			
EA.hy926	RF/EMF: 900 MHz, GSM	2	Human	6
human endothelial	SAR 1.8-2.5 W/kg, 1h		Unigene RZPD-2	
EA.hy926	RF/EMF: 1800 MHz, GSM	2	Human	6
human endothelial	SAR 1.8-2.5 W/kg, 1h		Unigene RZPD-2	
EA.hy926	RF/EMF: 1800 MHz, GSM	2	Human	6
human endothelial	SAR 1.8-2.5 W/kg, 1h		Unigene RZPD-2	
T-lymphocytes	RF/EMF: 1800 MHz	2	Human	8
human, quiescent	DTX only		Unigene RZPD-2	
from peripheral blood	SAR 1.4 W/kg			
	10 min ON, 20 min OFF, 44 h			
	(RNA prepared in Heidelberg)			
U937	RF/EMF	5	Human	9
human lymphoblastoma	GSM-900 MHz		Unigene RZPD-2	
	2 W/kg, 1 h			
CHME5 (µglie)	RF/EMF	5	Human	9
human microglial	GSM-900 MHz		Unigene RZPD-2	
	2 W/kg, 1 h			
HL-60	RF/EMF: 1800 MHz	3	Human	2
human hematopoietic	DTX		Unigene RZPD-2	
	SAR 1.0 W/kg			
	5 min ON, 5 min OFF, 24 h			
HL-60	RF/EMF: 1800 MHz	3	Human	2
human hematopoietic	DTX		Unigene RZPD-2	
	SAR 1.3 W/kg			
	continuous waves, 24 h			
HL-60	RF/EMF: 1800 MHz	3	Human	2
human hematopoietic	DTX		Unigene RZPD-2	
	SAR 1.3 W/kg			
	continuous waves, 24 h			

2.11.6 Biostatistics (Dr. Daniel Remondini, Participant 8)

The statistical analysis to find those genes that significantly changed their expression level between the ctrl and the exp state was done as follows: In order to increase the statistical significance of the test, we considered only those genes with *NG* = 4 "good" measurements (both for ctrl's and exp's). The genes of each experiment were kept separated in 3 different groups, related to each part (nylon membrane) they belonged to ("Part 1", "Part 2", "Part 3"). Normalisations and analysis were performed on each part separately, in order to avoid possible biases due to different behaviour of the arrays during hybridisation or scanning. Data were processed in order to evaluate and reduce possible artefacts due to the array reading procedure:

- as a first step, data were rescaled by means of a cubic root function in order to gather the data in a smaller interval: $ctrl' = \sqrt[3]{ctrl}$, $\exp' = \sqrt[3]{\exp}$;

- the averages of all 8 measurements for each gene were taken as a "reference" set. A scatter plot was made of each measurement (ctrl or exp) versus the reference set, and the resulting plot was fitted linearly. On the basis of the fit parameters a rescaling was performed on data if they were not on a Y=X curve.

The "interesting" genes were found by calculating a normalised difference *diff(i)* between exp and ctrl values for each gene *i*:

$$diff(i) = \frac{E[\exp(i)] - E[ctrl(i)]}{\sqrt{\frac{\sigma_{ctrl}^{2}}{NG-1} + \frac{\sigma_{\exp}^{2}}{NG-1}} + s_0},$$

where "E[]" denotes "expected value of ", and s_0 is a correction term that removes possible divergences in the denominator, calculated as the median of the σ_i distribution for each gene (ctrl and exp distributions were merged). For a first selection, genes were considered outliers if they exceeded the threshold of 3σ calculated from the resulting distribution of *diff(i)*.

To increase the robustness of our selection, following the bootstrapping procedure as shown in [PNAS 2001, Vol. 98 no. 9, pg. 5116-5121], new datasets were generated as permutations of the original ones, and the same analysis was performed over these datasets, ranking the differences $diff_p(i)$ (*p* referring to the p-th permutation) from larger to smaller. A plot of *diff(i)* vs. $E[diff_p(i)]$ was generated for each group of genes, and the interesting genes were chosen as those that significantly deviated from the y=x line: the distance of *diff(i)* from the y=x line, defined as *dist(i)*, was to be larger than a threshold value Δ=1.2.

Finally, the outlier genes selected for each part in each experiment were chosen from the intersection between the initial outliers ($|\text{diff(i)}| \geq 3\sigma$) and those from the bootstrapping procedure ($|dist(i)| \geq \Delta$). In this way the outlier selection results more selective than just applying the technique as shown in [PNAS 2001, Vol. 98 no. 9, pg. 5116-5121].

2.11.7 Data mining

The ratio of the spot-to-spot comparison was taken for further analysis, without values smaller than 0.001 or bigger than 1,000, respectively, which were eliminated before. Since each PCR fragment was spotted twice on each membrane, and each hybridisation experiment was performed twice, four ratios went into an own database tool (Martin Holst, access database program, Microsoft) to make an analysis in a non-statistical manner: All eight values coming from two hybridisations had to show the same tendency to be taken over in the final list showing clones appearing up- or down-regulated in both experiments. The clones of this list were connected to the gene ontology data via a Stanford database (http://genome-www5.stanford.edu/cgi-bin/SMD/source//sourceBatchSearch) according to IMAGE IDs. With the help of these data and a text query tool (Microsoft Excel, Matthias Schick) regulated genes belonging to certain gene families of interest could be extracted manually. The same procedure was applied on genes extracted by bio-statistical analysis.

3.0 RESULTS

The studies in REFLEX cover a wide range of frequencies within the spectrum of electromagnetic fields (EMF). Based on the assumption that possible biological effects may be generated by EMF in a different way dependent on their frequencies the results of the REFLEX project are reported separately for extremely low frequency electromagnetic fields (ELF-EMF) and radio frequency electromagnetic fields (RF-EMF).

3.1 Results in ELF-EMF research

3.1.1 Genotoxic effects

3.1.1.1 Human fibroblasts, lymphocytes, monocytes, melanocytes and muscle cells and granulosa cells of rats (Participant 3)

Intermittent ELF-EMF exposure, but not continuous ELF-EMF exposure induced DNA strand breaks in human fibroblasts.

In the first set of experiments fibroblasts were continuously exposed to ELF-EMF at 1000 µT for 24h. In these experiments significant differences in DNA-breaks between exposed and sham-exposed cells were observed neither with the alkaline nor with the neutral Comet assay. As Nordenson et al. (1994) reported positive genotoxic effects applying intermittent field exposure, our next experiments concentrated on exposures at different intermittence conditions. Intermittence of 5 min on/5 min off and 15 min on/15 min off revealed an increase of DNA-breaks in both, the alkaline and the neutral Comet assay, compared to sham-exposed cells, whereas at 5 min on field/25 min off field did not enhance the frequency of DNA-breaks (Tables 2, 3).

Table 2. Mean values of alkaline Comet tailfactors at different exposure conditions (n = 2), cell strain IH-9

Alkaline Comet Assay - different exposure conditions				
	exposed		sham	
	Comet tailfactor %	±SD[#]	Comet tailfactor %	±SD[#]
Continuous exposure (24h)	4.29	0.02	4.27	0,03
15'/15' on/off	6.47*	0.14	4.23	0.05
5'/5' on/off	6.98*	0,04	4.41	0.16
5'/10' on/off	7.47*	0.13	4.48	0.05
5'/15' on/off	6.68*	0.17	4.42	0.03
5'/20' on/off	5.90*	0.12	4.38	0.12
5'/25' on/off	4.27	0.04	4.23	0.03
1'/10' on/off	5.89*	0.19	4.21	0.14
3'/10' on/off	6.60*	0.06	4.19	0.22
10'/10' on/off	6.91*	0.07	4.24	0.07
15'/10' on/off	6.56*	0.15	4.11	0.08
25'/10' on/off	5.37*	0.05	4.21	0.04

[#] SD indicates standard deviation

* indicates significant differences ($p < 0.05$) exposed vs. sham

Table 3. Mean values of neutral Comet tailfactors at different exposure conditions (n = 2), cell strain IH-9

Neutral Comet Assay - different exposure conditions				
	exposed		sham	
	Comet tailfactor %	±SD[#]	Comet tailfactor %	±SD[#]
Continuous exposure (24h)	4.20	0.03	4.17	0.05
15'/15' on/off	5.72*	0.01	4.25	0.04
5'/5' on/off	6.09*	0.02	4.31	0.08
5'/10' on/off	6.21*	0.01	4.35	0.07
5'/15' on/off	5.66*	0.06	4.23	0-13
5'/20' on/off	4.52	0.16	4.50	0.21
5'/25' on/off	4.25	0.05	4.34	0.07
1'/10' on/off	4.16	0.15	4.16	0.13
3'/10' on/off	5.94*	0.05	4.20	0.06
10'/10' on/off	6.19*	0.11	4.11	0.11
15'/10' on/off	6.02*	0.03	4.21	0.10
25'/10' on/off	5.44	0.01	4.15	0.01

[#]SD indicates standard deviation
* indicates significant differences ($p < 0.05$) exposed vs. sham

Based on these findings, we tried to find out the optimal exposure conditions for maximal effects on DNA strand break levels. We started with a fixed field-on time of 5 min and varied field-off times from 5 to 25 min. These experiments indicated that DNA strand break levels (SSB and DSB) culminated at an off-time of 10 min and reached control levels at extended off-times (Figure 7). Significant differences ($p< 0.01$) between exposed and sham-exposed cells were found at 5 min on/5 min off, 5 min on/10 min off, 5 min on/15 min off and 5 min on/20 min off intermittence for alkaline Comet assay and at 5 min on/5 min off, 5 min on/10 min off and 5 min on /15 min off intermittence for neutral Comet assay, but not at 5 min on/ 25 min off for both assays.

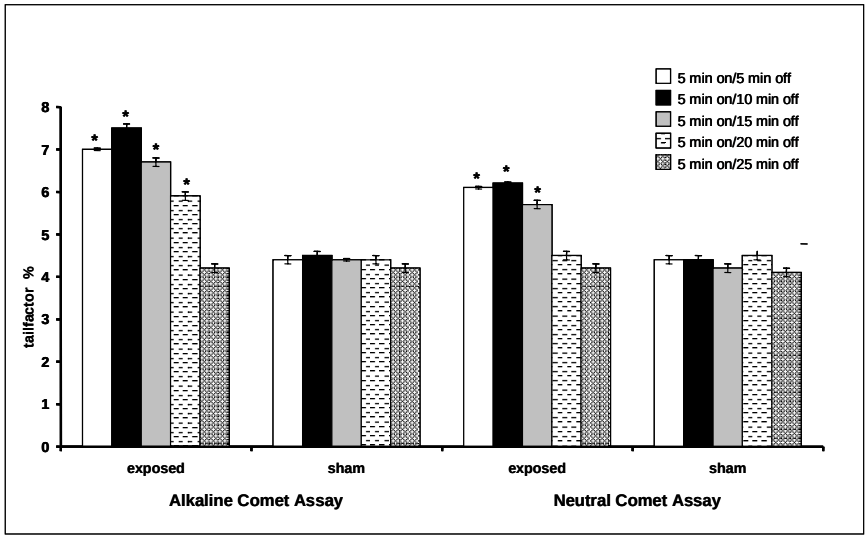


Figure 7. Alkaline and neutral Comet Assay tailfactors of ELF exposed fibroblasts (cell line IH-9, 50 Hz sinus, 24h,1000 µT, intermittent) - variation of off-time. * $p<0.01$ exposed versus sham-exposed

Subsequently, a fixed off-time of 10 min was chosen and on-times have been varied from 1 to 25 min. Again, the highest level of DNA strand breaks was obtained at an intermittence of 5 min on/10 min off (Figure 8). Comet tailfactors of exposed and sham-exposed cells differed significantly at each on-time in

alkaline Comet assay and at 3 to 15 min on in the neutral Comet assay. Solely the alkaline Comet tailfactors of 5 min on/10 min off, 5 min on/25 min off and 25 min on/10 min off-EMF exposed cells differed significantly to the other applied intermittence conditions. Since an intermittence of 5 min on/10 min off was able to induce the highest levels of DNA strand breaks in both alkaline and neutral Comet assay, further experiments were performed at 5 min on/10 min off.

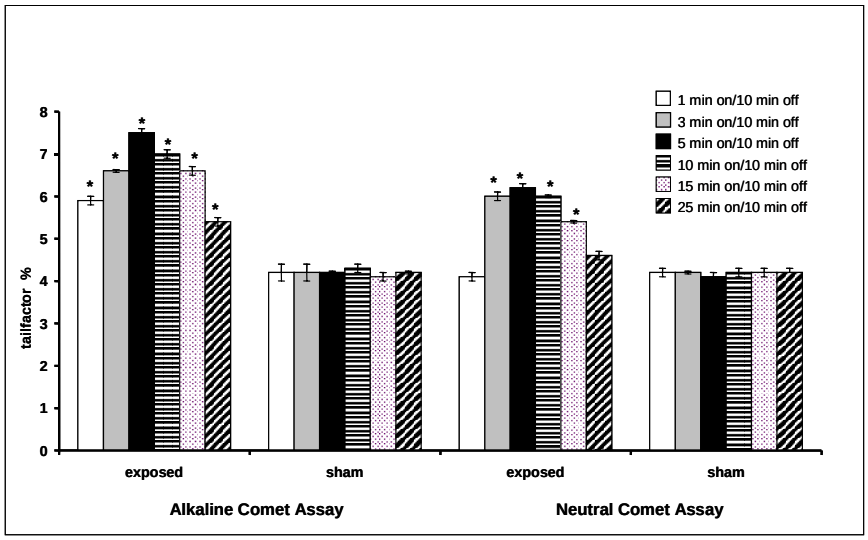


Figure 8. Alkaline and neutral Comet assay tailfactors of ELF exposed fibroblasts (cell line IH-9, 50 Hz sinus, 24 h, 1000 µT, intermittent) - variation of on-time. * $p < 0.01$ exposed versus sham-exposed

ELF-EMF 50 Hz sinus generated a higher rate of DNA strand breaks in human fibroblasts than ELF-EMF powerline.

By comparing 50 Hz sinus to the 50 Hz powerline signal it was found out that at 50 Hz powerline Comet assay tailfactors were significantly lower than at 50 Hz sinusoidal (Figure 9). All further experiments in the ELF-EMF range were, therefore, carried out with 50 Hz sinus.

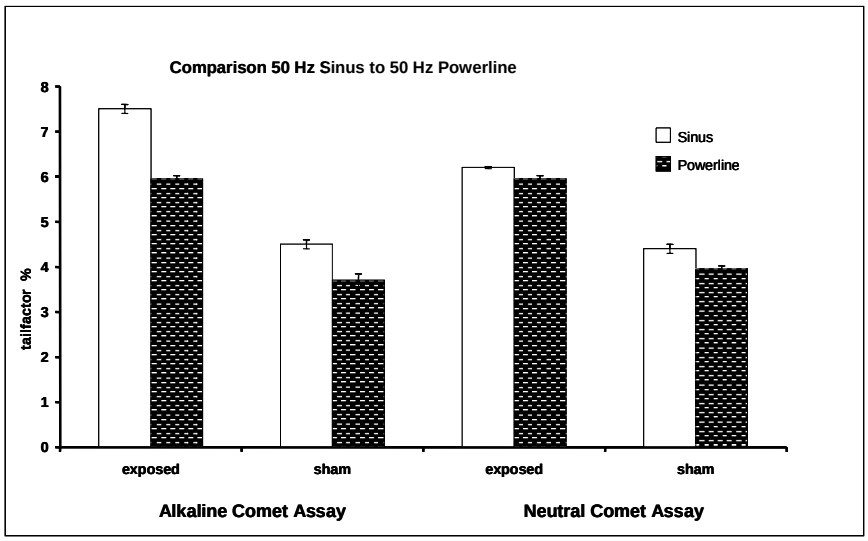


Figure 9. Alkaline and neutral Comet Assay tailfactors of ELF exposed fibroblasts (cell line IH-9, 24 h, 1000 µT, intermittent)

Genotoxic effects were frequency dependent.

In order to investigate the frequency dependence of genotoxic effects of ELF-EMF (1 mT, 5 min on/ 10 min off) cultured human fibroblasts were exposed to different frequencies (3-550 Hz). Exposure time was set to 15 hours. Genotoxic effects were evaluated using the alkaline Comet assay. Figure 10 presents the tailfactors in exposed and sham exposed cells. Significant increases in DNA damage could be found at 3 Hz, 16 2/3 Hz, 30 Hz, 50 Hz, 300 Hz, and 550 Hz. Effects on strand break levels varied with the applied frequencies and could be ranked as follows: 50 Hz > 162/3 Hz > 3 Hz > 300 Hz > 550 Hz > 30 Hz. Quite obviously, the extent of induced DNA damage did not correlate with the applied frequency.

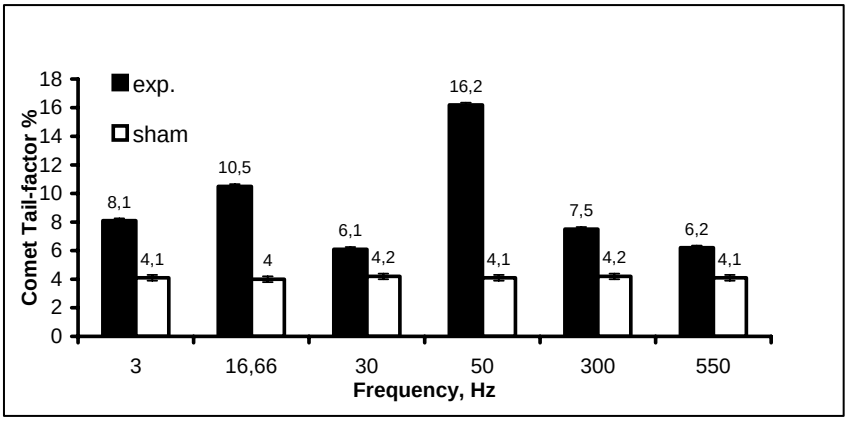


Figure 10. Alkaline Comet Assay tailfactors of ELF-EMF exposed and sham exposed fibroblasts (cell line ES-1, 15 hrs, 1000 µT, intermittent) after variation of exposure frequency (3-550 Hz).

Increase in DNA strand breaks in human fibroblasts after ELF-EMF exposure was dependent on exposure time.

Alkaline and neutral Comet tailfactors increased with exposure time (1-24 hours, 1000 µT, intermittent (5 min on/10 min off)), being largest at 15 hours (Figure 11). Comet assay levels declined thereafter, but did not return to basal levels.

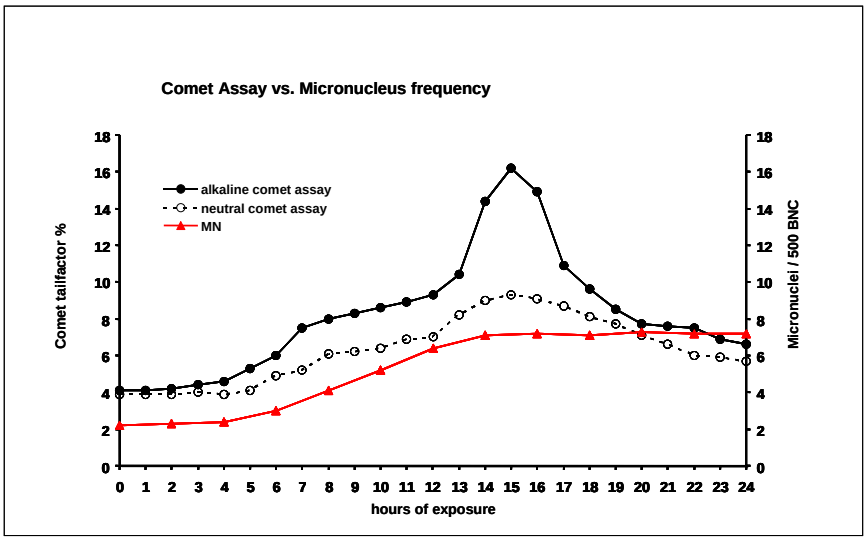


Figure 11. Influence of exposure time on formation of DNA single and double strand breaks and micronuclei in human fibroblasts (cell strain ES-1, 1 mT, 5 min on/10 min off cycles).

Increase in DNA strand breaks in human fibroblasts after ELF-EMF exposure was dependent on the age of the donors.

Fibroblasts from six healthy donors (ES1, male, 6 years old; AN2, female, 14 years old; IH9, female, 28 years old; KE1, male, 43 years old; HN3, female, 56 years old; WW3, male, 81 years old) revealed differences in response to ELF-EMF exposure (Table 4). Cells from older individuals exhibited a higher rate of single and double strand breaks and their break levels started to decline later than in cells from younger donors (Figures 12, 13).

Table 4. Alkaline and neutral Comet assay tailfactors of donors with different age (ES-1: 6, AN2: 14, IH9: 28, KE1: 43, HN3: 56, WW3: 81 years of age) - variation of exposure duration (basal-, maximum-, and end-levels)

			Alkaline Comet Assay		Neutral Comet Assay	
	cell strain	hours exposure duration	Comet tailfactor %	±SD#	Comet tailfactor %	±SD#
basal levels	ES1	0	4.112	0.018	3.901	0.006
	AN2	0	4.077	0.064	3.900	0.035
	IH9	0	4.223	0.047	4.161	0.148
	KE1	0	6.227	0.044	5.224	0.013
	HN3	0	6.802	0.018	6.313	0.064
	WW3	0	7.101	0.064	6.816	0.023
maximum levels	ES1	15	16.155	0.184	9.305	0.057
	AN2	15-16	16.501	0.004	9.394	0.134
	IH9	16	16.707	0.040	9.716	0.054
	KE1	18	17.300	0.064	10.462	0.277
	HN3	18-19	18.311	0.078	11.364	0.122
	WW3	19	18.517	0.069	12.822	0.076
and levels	ES1	24	6.611	0.017	5.742	0.023
	AN2	24	7.210	0.062	5.824	0.030
	IH9	24	7.511	0.017	6.127	0.054
	KE1	24	8.242	0.038	6.738	0.023
	HN3	24	8.718	0.008	6.761	0.006
	WW3	24	9.229	0.037	8.010	0.063

#SD .standard deviation

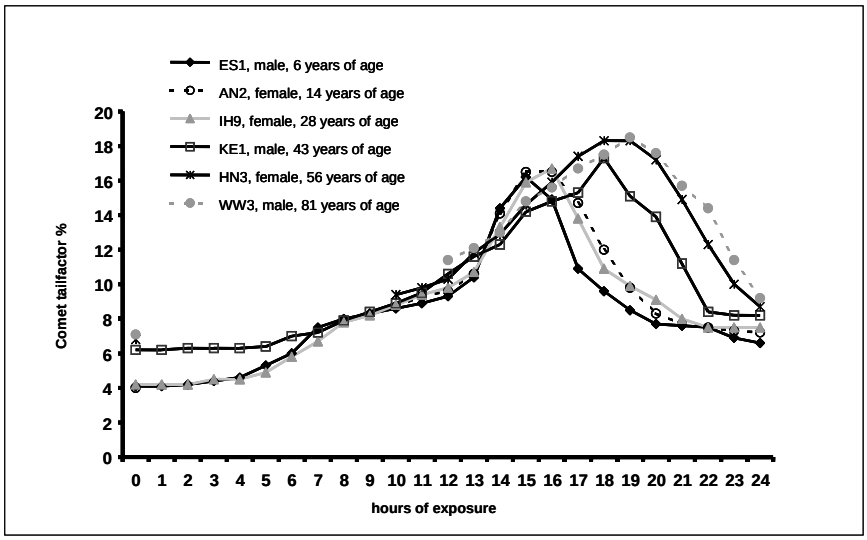


Figure 12. Alkaline Comet tailfactors of human diploid fibroblasts of donors with different years of age exposed to ELF-EMF (1 mT, intermittent 5 min on/10 min off) for 1-24 hours

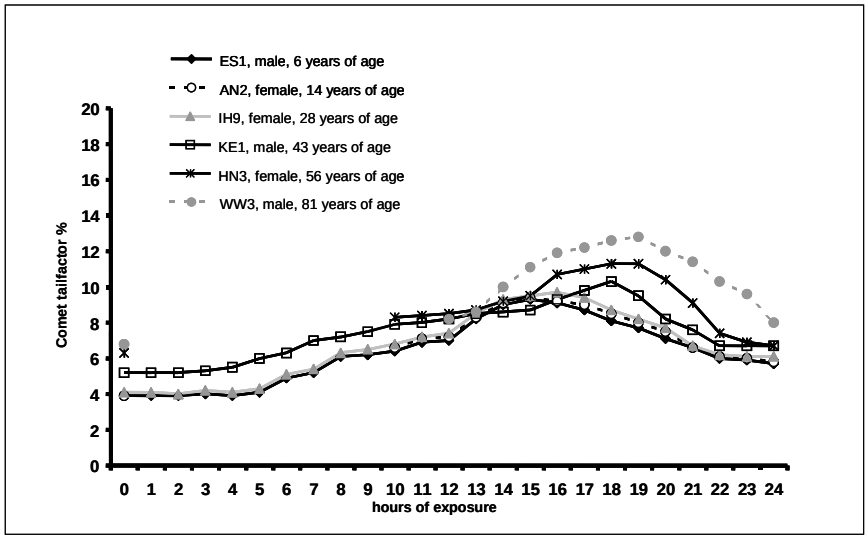


Figure 13. Neutral Comet tailfactors of human diploid fibroblasts of donors with different years of age exposed to ELF-EMF (1 mT, intermittent 5 min on/10 min off) for 1-24 hours

Increase in DNA strand breaks in human fibroblasts after ELF-EMF exposure was accompanied by a rise in micronuclei frequencies.

In addition, variation of exposure time from 2 to 24 hours revealed a time dependent increase in micronucleus frequencies. As shown in Figure 11, this increase became significant ($p<0.05$) at 10 hours of ELF-EMF exposure. Thereafter, micronucleus frequencies reached a constant level, which was about 3-fold as compared to the basal levels (Figure 14).

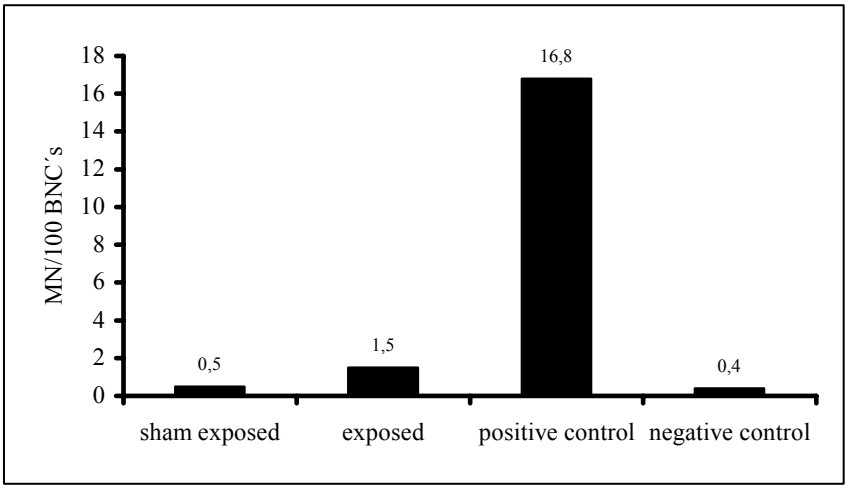


Figure 14. Micronucleus frequencies of ELF-EMF exposed (50 Hz, 1 mT, 15 h, 5 min on/10 min off) cultured human fibroblasts and controls (Vienna). Bleomycin 10µg/ml was used as a positive control.

ELF-EMF exposure did not diminish the number of fibroblasts in culture.

No differences in cell count between exposed and sham exposed cells at any exposure duration could be detected. Therefore, an elimination of cells by apoptosis and cell death during ELF-EMF exposure can probably be ruled out.

ELF-EMF exposure induced DNA strand breaks in human fibroblasts in a dose dependent way.

When magnetic flux density increased from 20 to 1,000 µT, a dose dependent rise in Comet assay tailfactors could be observed. At an exposure time of 24 hours a magnetic flux density as low as 70 µT produced significantly elevated ($p < 0.01$) alkaline and neutral Comet assay levels as compared to sham-exposed controls (Figure 13). At 15 hours of exposure genotoxic effects already occurred at 35 µT (Table 5, Figure 12). Using regression analysis, a significant correlation between Comet tailfactors and applied magnetic field (alkaline Comet assay: $r = 0.843$, $p = 0.004$; neutral Comet assay: $r = 0.908$, $p = 0.0007$), as well as between alkaline and neutral Comet assay could be found ($r = 0.974$, $p = 0.00001$).

Table 5. Mean values of alkaline and neutral Comet tailfactors at intermittent ELF exposure (5/10 on/off, 1000 µT, 24 h) (n = 2) dose response, cell line ES-1

	Alkaline Comet Assay				Neutral Comet Assay			
	exposed		sham		exposed		sham	
µT magnetic flux density	tailfactor %	±SD#	tailfactor %	±SD#	tailfactor %	±SD#	tailfactor %	±SD#
20	4.16	0.02	4.21	0.13	3.63	0.01	3.60	0.08
50	4.16	0.06	4.20	0.12	3.70	0.16	3.72	0.03
70	4.87*	0.03	4.28	0.02	3.99*	0.01	3.71	0.01
100	5.25*	0.06	4.28	0.05	4.32*	0.00	3.73	0.04
250	5.31*	0.02	4.25	0.07	4.24*	0.06	3.60	0.02
500	5.52*	0.01	4.22	0.01	4.48*	0.02	3.79	0.05
750	6.17*	0.08	4.26	0.11	5.08*	0.08	3.67	0.10
1000	6.50*	0.18	4.27	0.10	5.71*	0.01	3.79	0.16
2000	6.62	0.01	4.13	0.04	5.79*	0.05	3.70	0.01

SD indicates standard deviation
• indicates significant differences ($p < 0.05$) exposed vs. sham

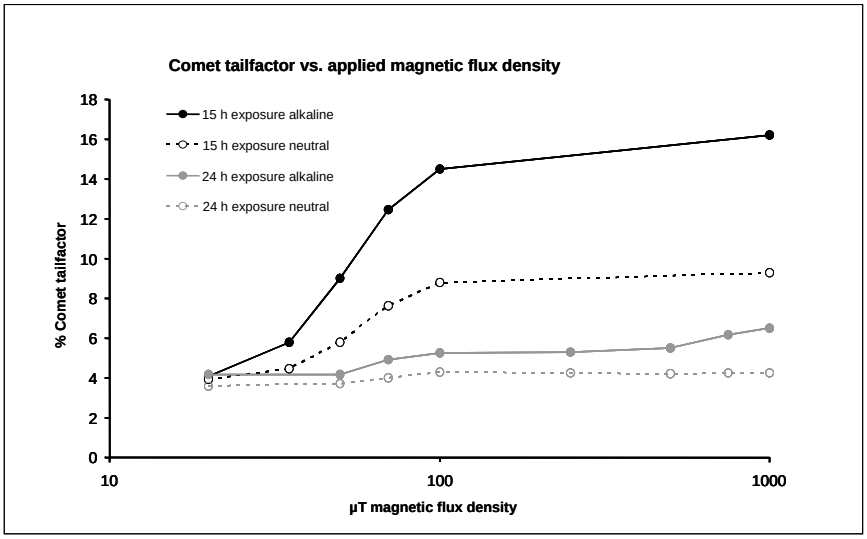


Figure 15. Dose dependent formation of DNA single and double strand breaks determined with Comet assay under alkaline and neutral conditions with cell strain ES-1 (exposure time 15 and 24 hours, 5 min on/10 min off cycles)

DNA strand breaks in human fibroblasts after ELF-EMF exposure were rapidly repaired.

After having demonstrated a time dependent relationship between alkaline and neutral Comet assay tailfactors and ELF-EMF exposure, the next aim was to find an explanation for the declining of the Comet assay levels after reaching the peak value. When exposure was terminated after 12 or 15 hours the Comet tailfactors returned to basal levels after a repair time of 7 to 9 hours (Figures 15, 16), comprising in a fast repair rate of DNA single strand breaks (< 1 hour) and a slow repair rate of DNA double strand breaks (> 7 hours). The marked Comet peak value between 12-17 hours disappeared when the Comet assay was performed at pH 12.1 instead of pH >13, thereby eliminating the cleavage of alkali labile sites in the DNA (Figure 18). The decline of Comet tailfactors after 15-20 hours of exposure could be prevented, when the cells were exposed in the presence of 10 µg/ml cycloheximide, an inhibitor of protein synthesis (Figure 18.

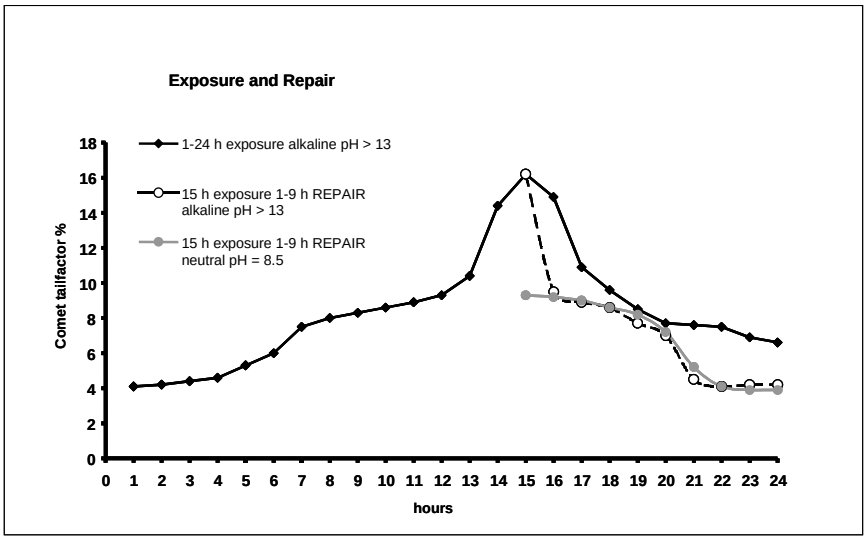


Figure 16. Repair kinetics of DNA single and double strand breaks in human fibroblasts (cell strain ES-1) after termination of ELF-EMF exposure (cell strain ES-1, 1 mT, 5 min on/10 min off cycles) using alkaline and neutral Comet assay - repair after 15 h ELF-EMF exposure

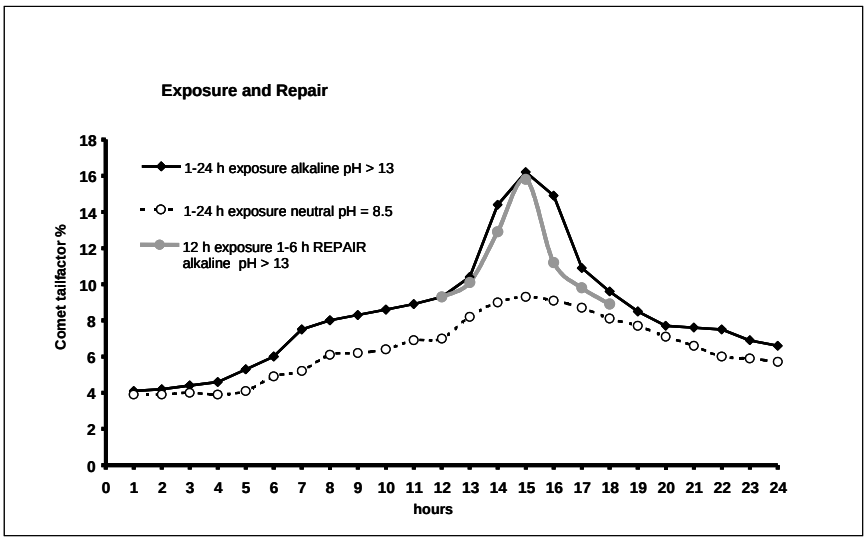


Figure 17. Repair kinetics of DNA single and double strand breaks in human fibroblasts (cell strain ES-1) after termination of ELF-EMF exposure (cell strain ES-1, 1 mT, 5 min on/10 min off cycles) using alkaline and neutral Comet assay - repair after 12 hours ELF-EMF exposure

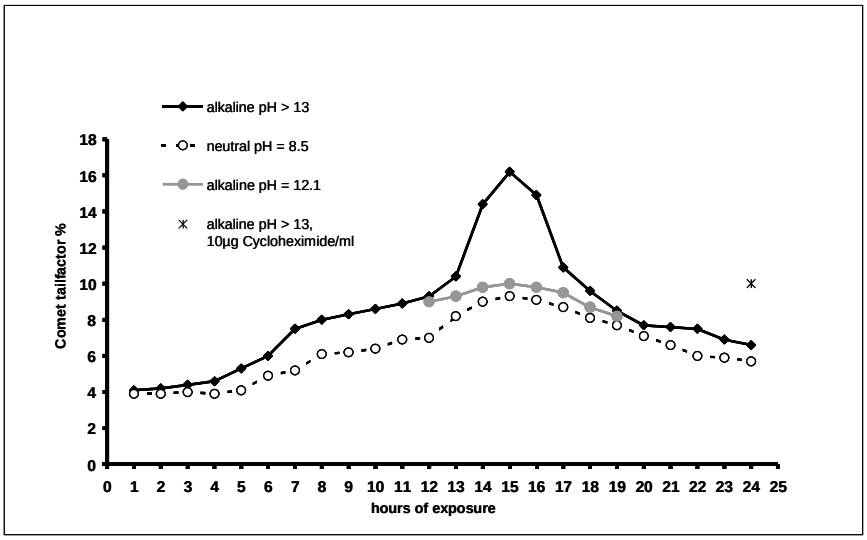


Figure 18. Comet assay of exposed human fibroblasts was performed at different pH (1 mT, intermittent 5 min on/10 min off)

DNA repair deficient cells react differently to ELF-EMF exposure.

Diploid human fibroblasts from patients with the genetically determined DNA repair defects Cockayne Syndrome, Ataxia Teleangiectatica, and Bloom Syndrome were obtained from Coriell Cell Repository (Camden, New Jersey, USA). The cells were cultured under standardized conditions and exposed (24 hours, 1 mT, 5 min on/10 min off) or sham exposed, and alkaline Comet assay was performed as described. As a result the Cockayne and Bloom Syndrome fibroblasts exhibited a similar pattern of genotoxicity as normal control fibroblasts, whereas the cells from a patient with Ataxia Teleangiectatica showed an almost threefold increased ELF-EMF induced Comet tailfactor as compare to normal cells (Figure 19).

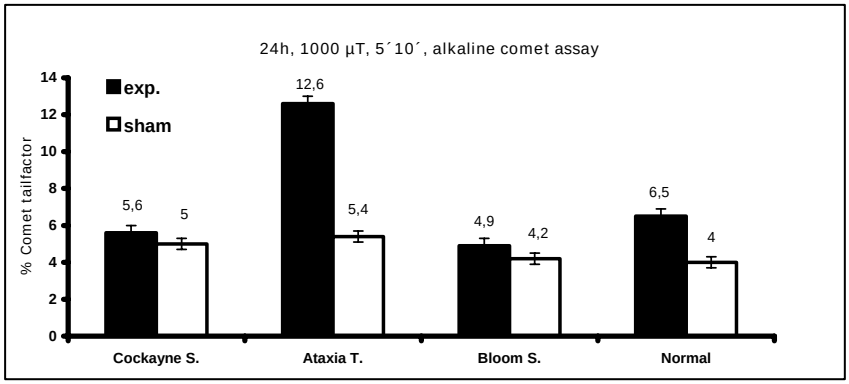


Figure 19. Alkaline Comet assay tailfactors of ELF-EMF exposed human fibroblasts from patients with various genetically determined DNA repair defects and normal controls.

Generation of DNA strand breaks through ELF/EMF was cell type specific.

ELF-EMF exposure (50 Hz sinusoidal, 1 mT, 5 min on/10 min off, 1-24 hours) of different human cell types (melanocytes, skeletal muscle cells, fibroblasts, monocytes, stimulated and quiescent lymphocytes) and of SV40 transformed rat granulosa cells revealed differences in induced DNA damage. Rat granulosa cells exhibited the highest DNA strand break levels and seemed to be most sensitive to intermittent ELF-

EMF exposure (Figures 20, 21). Human melanocytes also reacted, but not as strong as fibroblasts or rat granulosa cells. In contrast, stimulated or non-stimulated lymphocytes, monocytes and skeletal muscle cells did not respond at all.

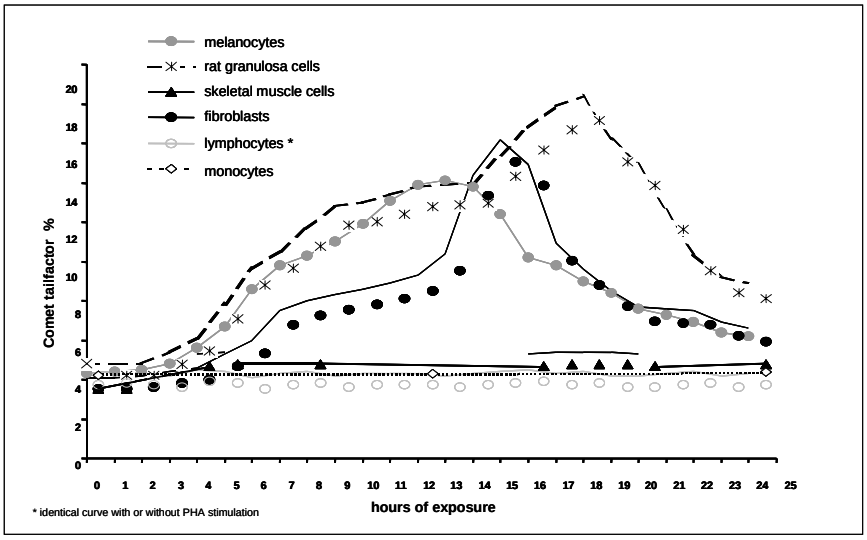


Figure 20. Alkaline Comet tailfactors of different human cell types (fibroblasts, melanocytes, monocytes, lymphocytes, skeletal muscle cells) and SV 40 transformed rat granulosa cells exposed to ELF-EMF (50 Hz sinusoidal, 1 mT, intermittent 5 min on/10 min off) for 1 to 24 hours.

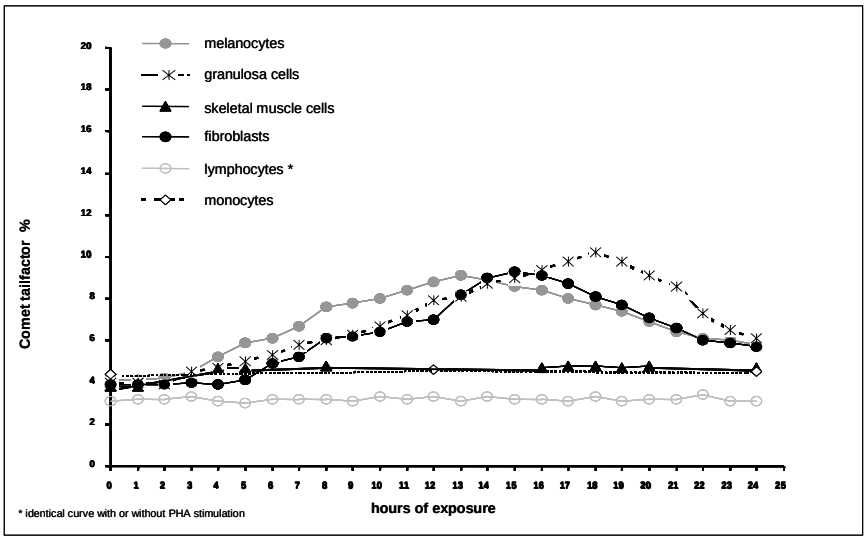


Figure 21. Neutral Comet tailfactors of different human cell types (fibroblasts, melanocytes, monocytes, lymphocytes, skeletal muscle cells) and SV 40 transformed rat granulosa cells exposed to ELF-EMF (50 Hz sinusoidal, 1 mT, intermittent 5 min on/10 min off) for 1 to 24 hours.

Generation of DNA strand breaks in human fibroblasts through ELF-EMF and their repair were modified by UVC or heat stress.

To test a possible impact of ELF-EMF exposure on DNA repair, cells were subjected to combined exposures to ELF + UVC or ELF-EMF + heat stress. In the first set of experiments fibroblasts were pre-exposed to UVC (10 min., 1.2 kJ/m^2). Subsequently, ELF-EMF exposure (50 Hz, sinus, 1000 µT) was varied from 1-24 hours. Results of the alkaline Comet assay showed that DNA damage caused by UVC could be removed within 7 hours of ELF-EMF exposure (Figure 22). UV/ELF-EMF exposed cells

resulted in 50 % higher Comet assay levels than UV/sham exposed cells after 1 hour of ELF-EMF exposure. In UV/ELF-exposed cells DNA-damage was repaired very slowly, but the maximum at 15 hours ELF-EMF-exposure could not be detected any more. The results were similar with the neutral Comet assay, but DNA damage (DNA double strand breaks) was repaired within a shorter time (Figure 23)

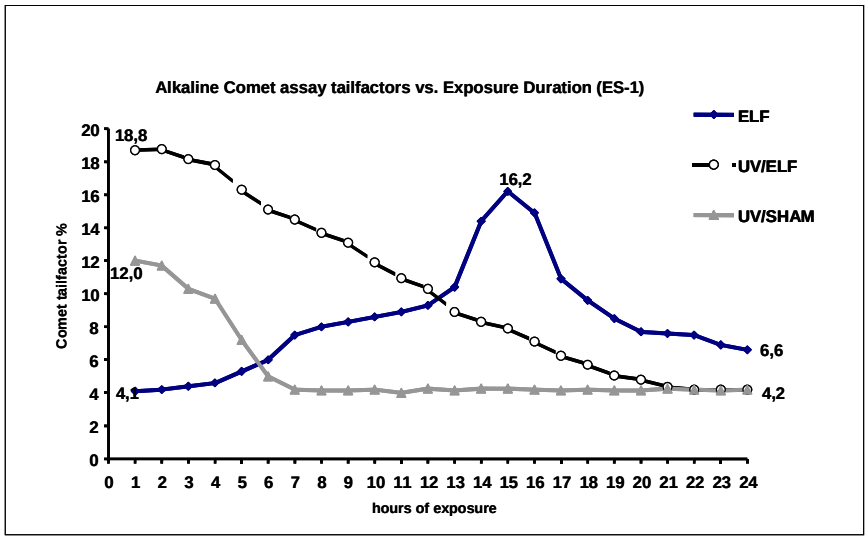


Figure 22. Repair kinetics of DNA single and double strand breaks in human fibroblasts (cell strain ES-1) after exposure with UV-C, ELF-EMF or UV C + ELF-EMF (cell strain ES-1, 1 mT, 5 min on/10 min off cycles) using alkaline Comet assay

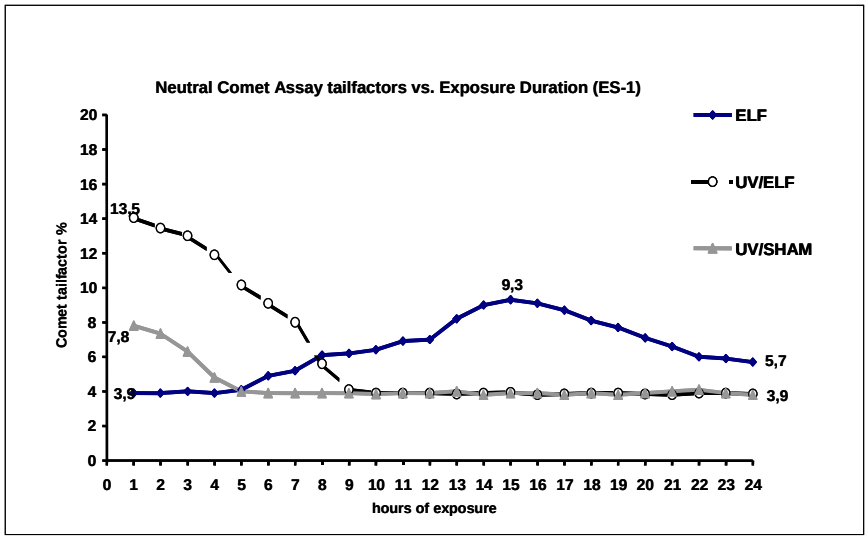


Figure 23. Repair kinetics of DNA single and double strand breaks in human fibroblasts (cell strain ES-1) after exposure with UV-C, ELF-EMF or UV C + ELF-EMF (cell strain ES-1, 1 mT, 5 min on/10 min off cycles) using neutral Comet assay

Based on the results with human fibroblasts, which suggest an induction of DNA repair upon intermittent ELF-EMF exposure, we concluded that pre-exposure to ELF-EMF would have a protective effect and diminish effects of additional exposures from other genotoxic factors. To check this assumption, fibroblasts were pre-exposed to ELF-EMF (50 Hz sinusoidal, 5 min on/10 min off, 1 mT) for 20 hours to ensure maximum induction of DNA repair. Subsequently, cells were either exposed to UVC (254 nm, 4.5

kJ/m², 30 min) or to mild heat stress (38.5°C, 4 h). Recovery of DNA damage was evaluated using alkaline and neutral Comet assay. UVC-exposure produced 50 % higher DNA strand break levels than ELF-EMFs alone and DNA damage was completely repaired after 3 hours (Figure 24). DNA damage induced by mild thermal stress was even higher and persisted longer than 6 hours after exposure termination. Pre-exposure to ELF-EMF intensified and elongated UVC or temperature induced DNA damage. After 24 hours of recovery time ELF-EMF pre-exposed cells still exhibited higher DNA strand break levels and just about 50% of the initially induced DNA damage had been repaired after this time. The results were similar with the neutral Comet assay, indicating induction and repair of DNA double strand breaks (Figure 25).

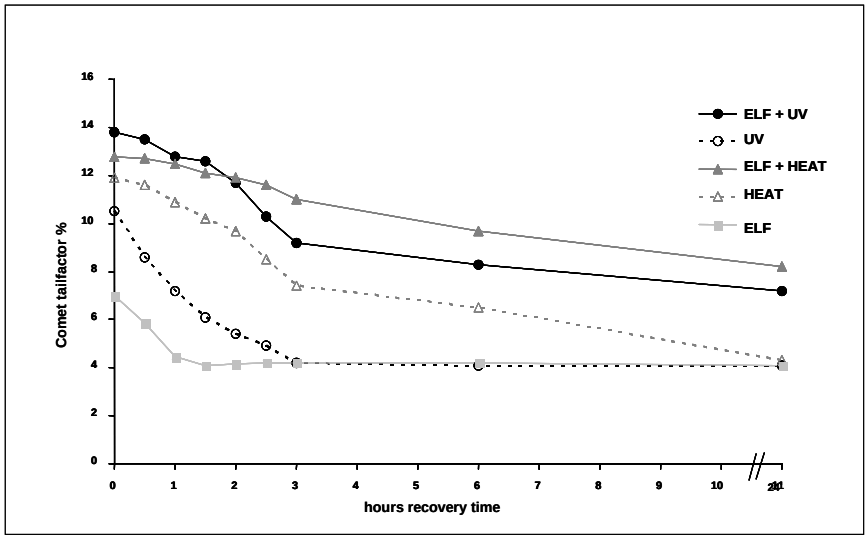


Figure 24. DNA damage and repair of cultured human fibroblasts pre-exposed to ELF-EMF (50 Hz sinusoidal, 5 min field-on/10 min field-off, 1 mT, 20 hours) and additionally exposed to UVC (254 nm, 30 min, 4.5 kJ/m²) or mild thermal stress (38.5°C, 4 hours) evaluated using alkaline Comet assay.

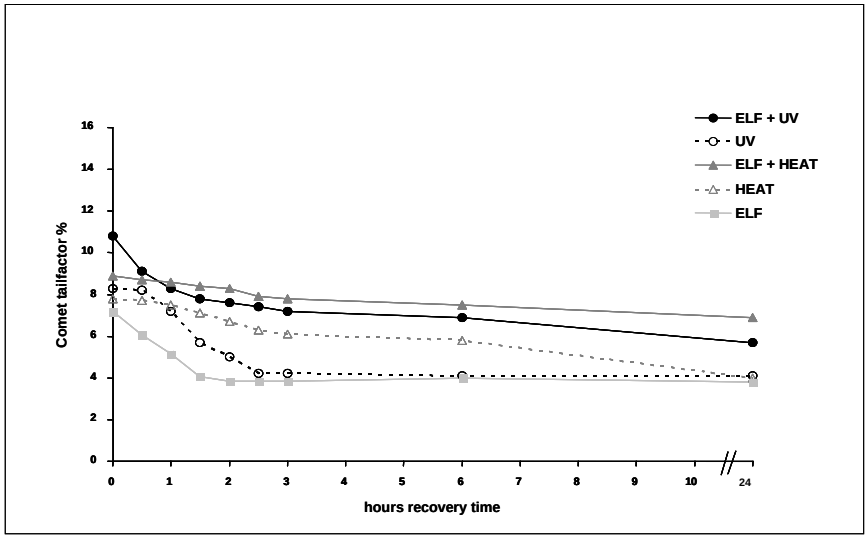


Figure 25. DNA damage and repair of cultured human fibroblasts pre-exposed to ELF-EMF (50 Hz sinusoidal, 5 min field-on/10 min field-off, 1 mT, 20 hours) and additionally exposed to UVC (254 nm, 30 min, 4.5 kJ/m²) or mild thermal stress (38.5°C, 4 hours) evaluated using neutral Comet assay.

ELF-EMF generated chromosomal aberrations in human fibroblasts.

Chromosomal aberrations were evaluated at exposure conditions producing maximum effects in the Comet assay and in the micronucleus test (15 h, 1 mT, 5min on/10 min off). Five different types of aberrations were separately scored (gaps, breaks, rings, dicentric chromosomes, acentric fragments). Significant increases ($p < 0.05$) between exposed and sham exposed cells could be demonstrated for all types of aberrations (Table 6). Gaps were 4-fold increased, breaks 2-fold, and dicentric chromosomes and acentric fragments 10-fold. Translocations were evaluated using the fluorescence in situ hybridisation (FISH) technique. 1,000 metaphases were scored for each specifically labeled chromosome (1-22, X and Y) after ELF-EMF exposure (50 Hz, 24h, 5 min on/10 min off, 1 mT). No stable translocation in any of the 24,000 metaphases of ELF-EMF exposed cells could be detected (data not shown).

Table 6. Percentage of chromosomal aberrations induced by ELF-EMF exposure (50 Hz, 5´field-on/10´field-off, 1 mT, 15 h) in cultured human fibroblasts.

Types of aberrations	ELF-exposed (% ± SD)	sham-exposed (% ± SD)	p-value*
chromosome gaps	24.3 ± 1 %	5.5 ± 0.7 %	< 0.001
chromosome breaks	2.2 ± 0.3 %	1.3 ± 0.3 %	0.0015
ring chromosomes	0.1 ± 0.07 %	---	0.0133
dicentric chromosomes	0.4 ± 0.1 %	0.06 ± 0.05 %	< 0.001
acentric chromosomes	0.3 ± 0.07 %	0.02 ± 0.04 %	< 0.001

[a] A number of 1,000 metaphases were scored in each of five independent experiments. Results are expressed as percentage chromosomal aberrations per cell.
[b] Significant differences ($p < 0.05$) as compared to sham-exposed controls using Student's t-test for independent samples

ELF-EMF did not alter the mitochondrial membrane potential in human fibroblasts.

The experimental settings in the present tests were based on conditions, which resulted in the highest inducible frequencies of these DNA strand breaks in human fibroblasts. Evaluating changes in the mitochondrial membrane potential after ELF exposure (50 Hz, 15 hours, 1 mT, 5 min on/10 min off) using JC-1, revealed no significant differences between exposed and sham-exposed fibroblasts.

3.1.1.2 Granulosa cells of rats, Chinese hamster ovary cells (CHO) and HeLa cells (Participant 7)

ELF-EMF exposure caused a significant increase of DNA strand breaks in cultured rat granulosa cells, CHO cells and HeLa cells.

The effect of ELF-EMF was analysed on the genomic level by use of the Comet assay. Especially the dependence on exposure time and frequency was analysed. Figure 26 shows that exposure to ELF-EMF at 16 2/3 Hz (5 min on/10 min off, 1.0 mT) caused a significant increase in single and double DNA strand breaks in cultured granulosa cells. The same result was obtained with CHO and HeLa cells (not shown). The data presented in Figure 26 indicate that the genotoxic effect at 16 2/3 Hz is time dependent with a maximum after about 18 hours of exposure, which resembles the results obtained at 50 Hz by Participant 3 (Ivancsits et al., 2003).

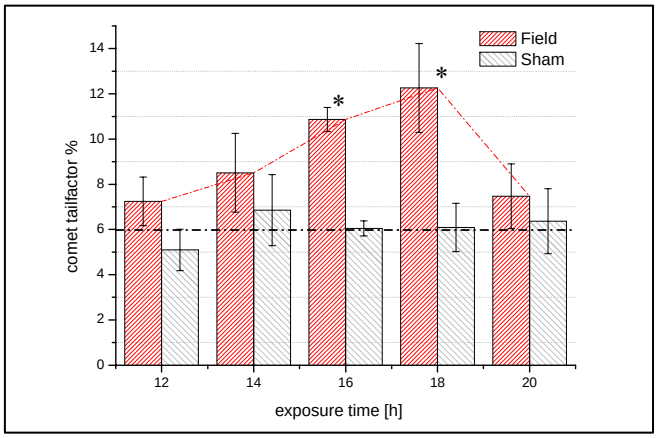


Figure 26. DNA damage of rat granulosa cells after exposure for 12 to 20 hours to ELF-EMF (16 2/3 Hz, 5 min on/10 min off, 1 mT) evaluated using alkaline Comet assay. For values of n see Material and Methods. (* p< 0.05)

To investigate the frequency dependence of the genotoxic effect of ELF-EMF (5 min on/10 min off) exposure of rat granulosa cells a constant exposure time of 18h was selected. Figures 27 and 28 present the tailfactors of exposed and sham exposed granulosa cells using the alkaline and neutral Comet assay. At the applied frequencies within the range of 8 Hz to 1000 Hz a significant frequency dependence was not observed for the rate of double DNA strand breaks as derived from the neutral Comet assay (Figure 28). The corresponding results of the alkaline Comet assay are presented in Figure 27. At 8 Hz, 16 2/3 Hz and 50 Hz an intensity of 1 mT could be applied (Figure 27a). A significant increase of DNA strand breaks was found at 16 2/3 Hz. Surprisingly, especially at 50 Hz the s.e.m. data of sham and ELF-EMF exposed cells differ significantly. The large error could be caused by a variable time dependent location of the maximum and/or the influence of the specific cell passage. Further experiments are under analysis to confirm the data presented in Figure 27a. At 1000 Hz the recorded DNA damage is significantly lower than observed at 16 2/3 Hz (Figure 27b). But it has to be noted that the maximal applied flux density was limited to 0.6 mT due to the used exposure system (Participant 10). Furthermore, DNA damage was measured at 20 µT, which approximately corresponds to the maximal acceptable magnetic flux density as recommended by the 26. BlmSchV[1]. Again a significant increase of DNA strand breaks was observed (Figure 27b).

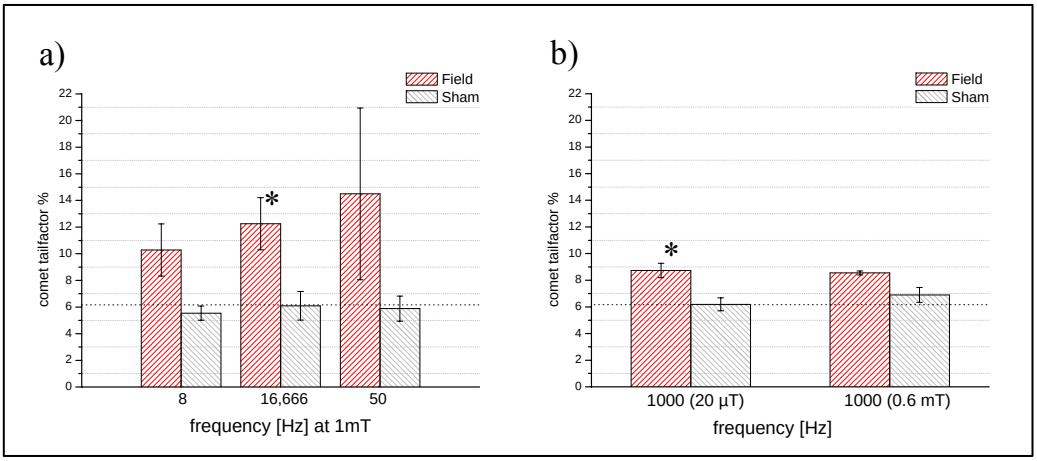


Figure 27. DNA damage of rat granulosa cells as function of frequency of ELF-EMF (5 min on/10 min off) after an exposure for 18 hours as derived by the alkaline Comet assay. **a)** In the frequency range of 8 to 50 Hz the flux density was set to 1 mT. **b)** At 1000 Hz the flux density was adjusted to 20 µT and 0.6 mT (for further explanation, see text). For values of n see Material and Methods. (* p< 0.05)

[1] 26. Verordnung zur Durchführung des Bundes-Immissionschutzgesetzes (Verordnung über elektromagnetische Felder – 26. BlmSchV)

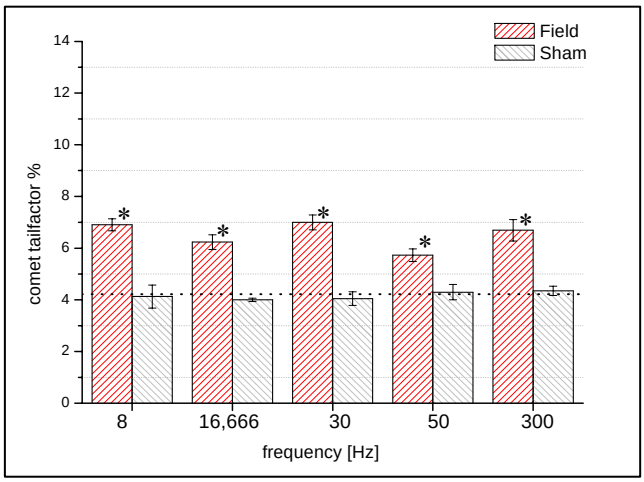


Figure 28. Double DNA strand breaks of rat granulosa cells as function of frequency of ELF-EMF (5 min on/10 min, 1 mT) after an exposure time of 18 hours as derived by the neutral Comet assay. For values of n see Material and Methods. (* $p < 0.05$)

3.1.1.3 Embryonic stem cells (ES) of mice (Participant 4)

Lack of effects on single and double strand break induction 0, 18, 24 and 48 hours after completion of a 6 or 48 hours ELF-EMF exposure.

The effects of ELF-EMF on the integrity of DNA strands in differentiating ES cell from EB outgrowths were studied. Two schemes were applied: (1) For ELF-EMF exposure (50 Hz Powerline, 2.0 mT, 5 min on/30 min off, 6 hours) the percentage of primary DNA damage was measured immediately after ELF-EMF exposure at the stage of neural differentiation (4+4d - 4+6d) and 18 hours after exposure using the alkaline and neutral Comet assay. (2). In the second set of experiments, the same ELF-EMF exposure conditions were applied for 48 hours instead of 6 hours, and the alkaline Comet assay was done immediately after exposure, while the neutral Comet assay was done 24 or 48 hours post exposure. No significant differences were observed in the induction of single or double DNA strand breaks between sham-exposed or ELF-EMF exposed neural progenitors.

3.1.1.4 Summary (Participant 1)

Our data indicate a genotoxic action of ELF-EMF in various cell systems. This conclusion is based on the following findings:

- Intermittent exposure to 50 Hz ELF-EMFs generated DNA single and double strand breaks in various cell systems such as human fibroblasts, melanocytes, granulosa cells of rats, Chinese hamster ovary cells (CHO) and HeLa cells, but not in human lymphocytes, monocytes, myelocytes and neural progenitors from mouse embryonic stem cells (see 3.1.1.1, 3.1.1.2 and 3.1.1.3).
- DNA damage generated by ELF-EMF in human fibroblasts was dependent on time and dose of exposure, on the age of the donors the cells derived from, and on the genetic background of the cells. A flux density of 35 µT was high enough to significantly increase the number of DNA strand breaks (see 3.1.1.1)
- The increase in DNA strand breaks in human fibroblasts due to ELF-EMF exposure was accompanied by an enhanced formation of micronuclei which was also dependent on the exposure time (see 3.1.1.1).
- The DNA repair system in human fibroblasts which was strongly activated by ELF-EMF during exposure did not work error-free as shown by a significant increase of different types of chromosomal aberrations (see 3.1.1.1).
- Genotoxic effects were frequency dependent. Significant increases in DNA strand breaks were found, when an ELF-EMF of 3 Hz, 16 2/3 Hz, 30 Hz, 50 Hz, 300 Hz, 550 Hz and 1000 Hz was applied. The

effect was strongest with 50 Hz ELF-EMF and second strongest with 16 2/3 Hz (see 3.1.1.1 and 3.1.1.2).

3.1.2 Cell proliferation and differentiation

3.1.2.1 Human neuroblastoma cell line NB69 (Participant 5)

ELF-EMF promoted the growth rate of NB69 neuroblastoma cells.

Immunocytochemical staining using antibodies against phenotype-specific antigens revealed that NB69 cells contain the neuroblast-specific protein ßIII-tubulin. However, these cells do not contain the neuroepithelial marker nestin, which is present in immature progenitors and in some neuroblastoma cells, nor the astrocyte-specific antigen GFAP. The cells remained in an undifferentiated state throughout the experimental period. Only the treatment with retinoic acid induced neurite extension accompanied by cell growth reduction.

Two series of experiments were carried out to analyse the cell growth response of NB69 cells to ELF-EMF. In the first series, the ELF-EMF administrated alone (42 hours) provoked a modest, though significant increase in the number of cells at day 5 postplating (5 dpp), both at 10-µT (12% over controls, **p<0.01) and 100-µT MFD (17 % increase over controls, ***p<0.001) as shown in Figure 29A). Retinol (ROL) alone or in combination with ELF-EMF did not change significantly the cell growth (data not shown). The ELF-EMF exposure also provoked modest increases in the total DNA levels, the effect being statistically significant at 10 µT (8 % over controls, p<0.05, Figure 29B). However no significant changes were observed in the protein or protein/DNA contents in the ELF-EMF exposed samples. Taken as a whole, these results indicate that exposure to 50 Hz ELF-EMF at 10 or 100 µT promote cell growth in the NB69 human neuroblastoma cell line.

In the second series of experiments, the 42-hours exposure to ELF-EMF at a flux density of 100 µT significantly increased cell growth (11 % over controls, ***p<0.001, Figure 30A). This result confirms the growth-promoting response obtained in the first experimental series. However, such an effect was not observed, when the ELF-EMF exposure was maintained for a longer period of time, i.e. 90 hours (Figure 30B). The treatment with retinoic acid (RA) alone significantly reduced the cell number, both at 42 and 90 hours, when compared to untreated controls. Also, RA-treated cells did show the growth-promoting effects of a 100 µT ELF-EMF, these samples demonstrating reduced growth rates when compared to unexposed controls: 35% reduction at the end of 42 hours-treatment (p<0.0001) and 57% reduction at the end of 90 hours-treatment (p<0.0001).

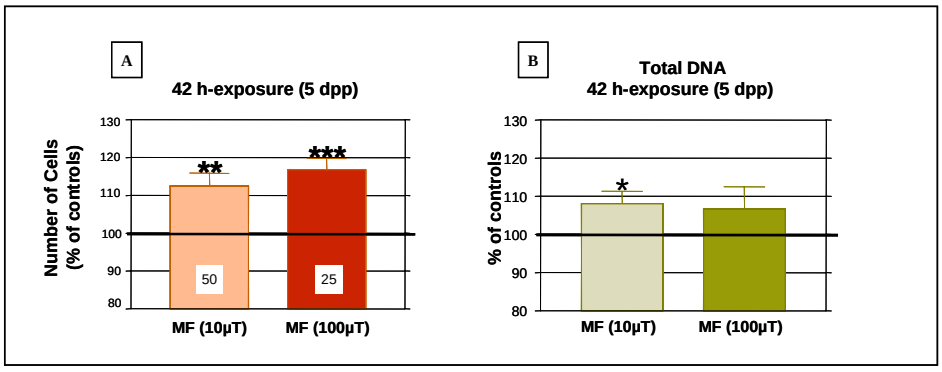


Figure 29. First series of experiments. A-Cell growth estimated by cell counting (Trypan blue exclusion) and B-Total DNA estimated by spectrophotometry.

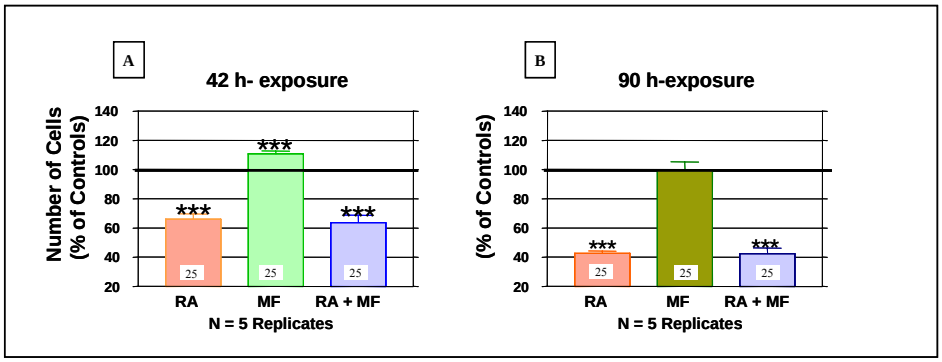


Figure 30. Second series of experiments: A-Number of cells at day 5 postplating (after 42 hours-exposure) and B-Number of cells at day 7 postplating (after 90 hours-exposure).

No experiment could be performed at a flux density of 2.0 mT following the 3 hours on/3 hours off exposure protocol, since the ohmic heat in the coils due to the electric current could not be compensated by the incubator resulting in an unstable ambient temperature. When the samples were exposed to 2 mT ELF-EMF in a 5min on/30 min off cycle, no effect was observed on the cell growth. Similarly, the cells did not respond to a 5 min on/30 min off cyclic exposure to a 100 µT ELF-EMF. It is possible that the NB69 cell line requires a longer exposure cycle to show significant changes in the cell growth.

A growth-promoting effect of ELF-EMF in NB69 neuroblastoma cells was not observed after an extended exposure period.

As described above, the growth promoting effect of a 100-µT EMF was not observed when the exposure was maintained for a longer period of time, i.e. 90 hours (Figure 30B). In NB69 cultures kept in control conditions, the number of cells peaks at day 6 and then decays (Figure 31). In the present experiments, long-term cultures (7 days postplating) reached a confluence stage close to saturation. This physiological condition could be the cause of the lack of response to ELF-EMF after long-term exposures between 3 and 7 days postplating.

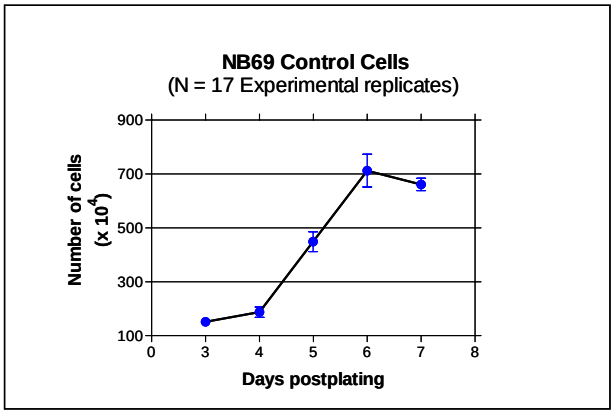


Figure 31. Growth pattern of NB69 cells: On day 7 the number of cells is reduced when compared to that at day 6. Long-term cultures (7 days postplating), reached a confluence stage close to saturation.

ELF-EMF did not counteract the retinoic acid-induced inhibition of cell proliferation in NB69 neuroblastoma cells.

To better characterise the potential ELF-EMF effects on the proliferation/differentiation rate, NB69 cells were treated with a chemical agent that inhibits proliferation and induces differentiation. All trans retinoic

acid (RA) promotes differentiation in NB69 cells, inducing outgrowth of long neurite-like processes and driving of the cell morphology along a neural pathway. As shown in Figure 30AB, the cell growth decreases after treatment with RA (2 µM) for 5 or 7 days. This response to RA remained unchanged after exposure to the ELF-EMF during 42 or 90 hours.

ELF-MF enhanced the cellular proliferation rate NB69 neuroblastoma cells as revealed through analysis of cell proliferation markers (PCNA).

Studies of expression of cell proliferation markers (PCNA) reinforce the described effects of ELF-EMF on cell growth: To determine whether the above growth-promoting effect detected by Trypan Blue exclusion involves changes in cell proliferation, we searched for changes in the proliferating cell nuclear antigen (PCNA). PCNA immunolabelling shows that exposure to 10 µT ELF-EMF significantly increases the proportion of PCNA-positive cells (24% increase; Figure 32). This effect was associated with an increase in the number of cells (15% increase), showing a significant linear correlation between both of the parameters, PCNA positive cells and total number of cells, at the end of 42 hours exposure ($p<0.01$). These results confirm and reinforce the previous observations that a 42 hours exposure to ELF-EMF at a flux density of 10 µT can modulate cell growth in NB69 cells. The mean value of PCNA positive cells in controls is 32 % ± 1,3).

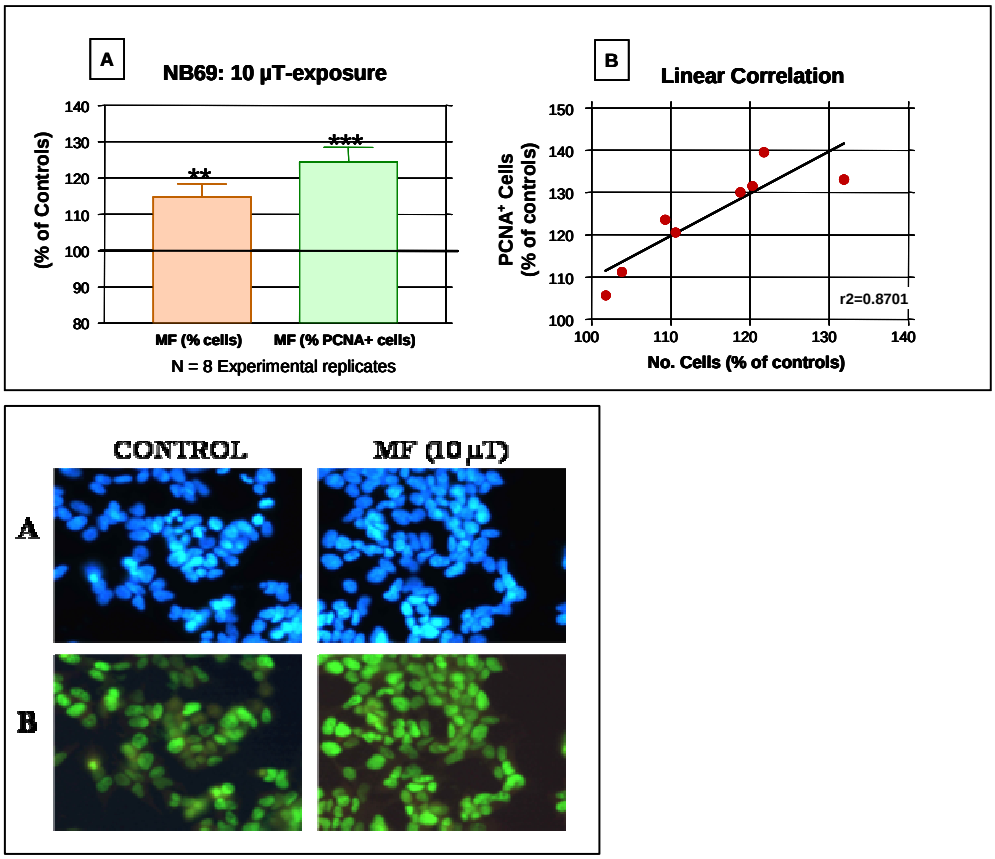


Figure 32. A) Growth response of NB69 to 10 µT after a 42 hours exposure as revealed by Trypan (brown) and PCNA content (green). Student T' test: **p<0.01; ***p<0.001. B) Linear correlation between these two parameters. Photomicrographs of NB69 cells. A: Hoechst-marked nuclei. B: PCNA labelling. The ELF-EMF exposure induced an increase in the number of cells expressing PCNA.

Neuroblastoma cell cultures contain two different phenotypes. One type is characterised by flattened, "sail-like" morphology and shows a strong adherence to the culture flask. These cells are called S-cells. The second phenotype corresponds to much smaller, triangular cells that adhere to the culture flask loosely. These cells, called N cells have a neuroblastic phenotype, are clonigenic and tumorigenic and

grow rapidly in the culture flask. We have observed that the relative proportion of both phenotypes evolves along the successive subcultures, which seems to significantly influence the response of the culture, as a whole, to the field exposure. In fact, young passages, having a high proportion of N cells, are particularly sensitive to ELF-EMF, whereas older passages, very rich in S-cells and with virtually no N cells, are not responsive to ELF-EMF exposure. Consequently, we conducted experiments with young passages, where the cells were exposed to a 100-µT ELF-EMF during 63 hours (day 6 post-plating). A significant increase in the number of cells was observed in the exposed samples (9.7% over controls $p<0.0001$, Figure 33A). The mean cell number in controls was 621.689 ± 62.314 ($x10^4$). The increase in the number of cells was found to be associated with significant increases in the proportion of PCNA-positive cells. Figure 33B shows the percent of PCNA positive cells at days 5 and 6 post-plating (dpp). Only at 6 dpp significant changes in the number of PCNA positive cells were observed (31.7% over controls, $p<0.01$, N = 3 experimental replicates). Those changes do not represent an ELF-EMF-induced increase in PCNA labelling, since the percent of PCNA positive cells in the control cultures spontaneously decreased between days 5 and 6 post-plating (Student T'test *, $p<0.05$). Such a decrease did not occur in the exposed cultures. The present results indicate that the regulation of the kinetics of the cell cycle could be altered by ELF-EMF at 100 µT. Provided that in the proliferating cell the PCNA levels are maximal at late G1 and S phases, it is possible that such phases of the cell cycle are implicated in the above described responses.

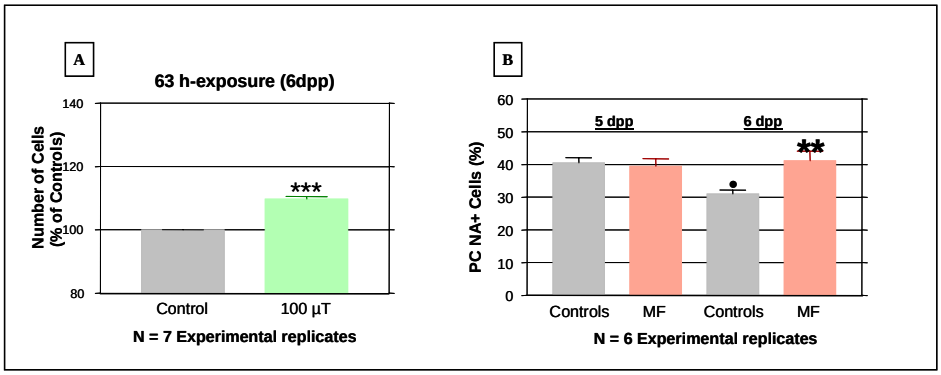


Figure 33. A) Percent of cells at the end of 63-hours exposure and/or incubation (6 day post-plating). B) Percent of PCNA positive cells at 5-6 days post-plating (dpp). In controls the percent of PCNA positive cells significantly decreases between the days 5 and 6 post-plating (Student T'test ●, $p<0.05$), whereas in exposed cells at 100 µT this decrease did not occur).

ELF-EMF increased the DNA synthesis in NB69 neuroblastoma cells.

We also tested the BrdU incorporation into DNA. As shown in Figure 31, at the end of a 42-hours exposure (5 pp) to the 100 µT ELF-EMF a significant increase of BrdU- positive cells was observed in the treated cultures (41 % over controls, Student's T test $p<0.01$). Such an effect was followed (63-hours exposure, 6 pp) by a subsequent increase in the number of cells (9.7% over controls, $p<0.001$, Figure 34). This response was accompanied by a significant reduction in the percent of spontaneously apoptotic cells (58.5 % of that in controls, $p<0.05$).

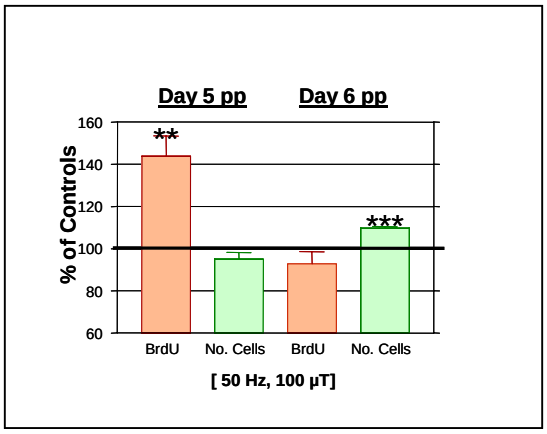


Figure 34. Cell growth in a total of 18 experiments: ELF-EMF effects at 5 days post-plating (42 hours exposure) or at 6 days post-plating (63 hours exposure): Red, percent of BrdU-positive cells; Green, Number of Cells analysed by Trypan blue exclusion. Student T'test: **p< 0.01; ***p< 0.001.

ELF-EMF affected the cell cycle in NB69 neuroblastoma cells.

The experiments performed to test the DNA content and the cell cycle distribution by flow cytometry showed that at the end of day 5 post-plating (Figure 35A), the 42-hours exposure to 100 µT ELF-EMF induces increases in the number of cells in G2-M phase of the cell cycle (28% over controls; N= 3 experimental replicates). The exposure also provoked a modest reduction of cells in G0-G1. However, no significant changes were observed in the number of cells in S-phase and in the number of total cells (Trypan Blue exclusion, Figure 352A). The flow-cytometry assay at the end of day 6 post-plating did not reveal EMF-induced changes in the cell cycle (G0-G1; S and G2-M), even thought a significant increase in the number of alive cells was observed (16.7% over controls, p<0.05; N= 5 experimental replicates, Figure 35B). These results confirm and reinforce our previous observations using other techniques, that 100 µT 50-Hz ELF-EMF can promote cell growth in the NB69 cell line from a human neuroblastoma.

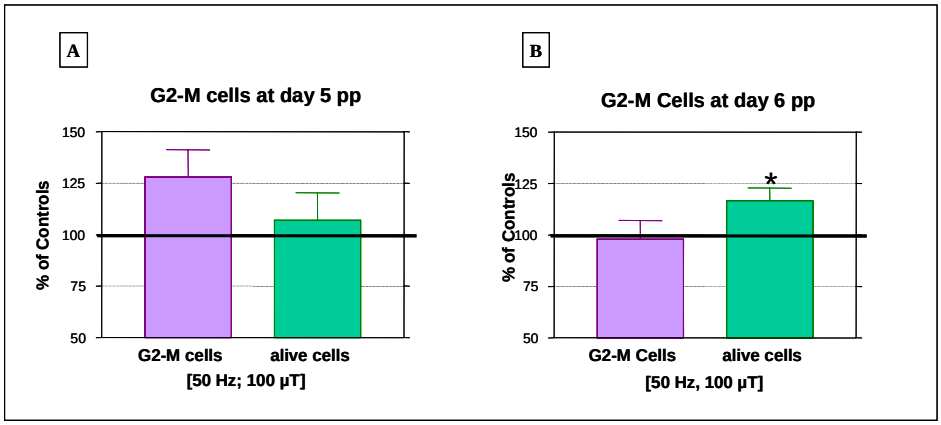


Figure 35. A) A 42 hours-exposure to a 100-µT field provokes an increase in the percent of G2-M cells at day five postplating (5 pp). B) This increase was not observed one day later, after 63 hours- exposure, however, a significant increase in the number of alive cells occurred.

ELF-EMF diminished the spontaneous apoptosis in NB69 neuroblastoma cells.

In order to investigate the potential influence of 50 Hz ELF-EMF on apoptosis the percent of apoptotic cells was estimated with TUNEL-labelling after 63 hours of exposure. Also, the number of cells was

quantified by Trypan Blue exclusion. The results (Figure 36A) indicate that ELF-EMF of 50 Hz at a flux density of 100 μT (3 hours on/3 hours off) induces a significant reduction in the spontaneous apoptosis of the NB69 cell line. This response was associated with an increase in the number of cells (9.7% over controls, $p<0.001$, Figure 36B as we previously observed in experiments described above. Apoptosis was also determined through flow cytometry analysis; the results confirming a reduction at the end of 63 hours-exposure (data not shown).

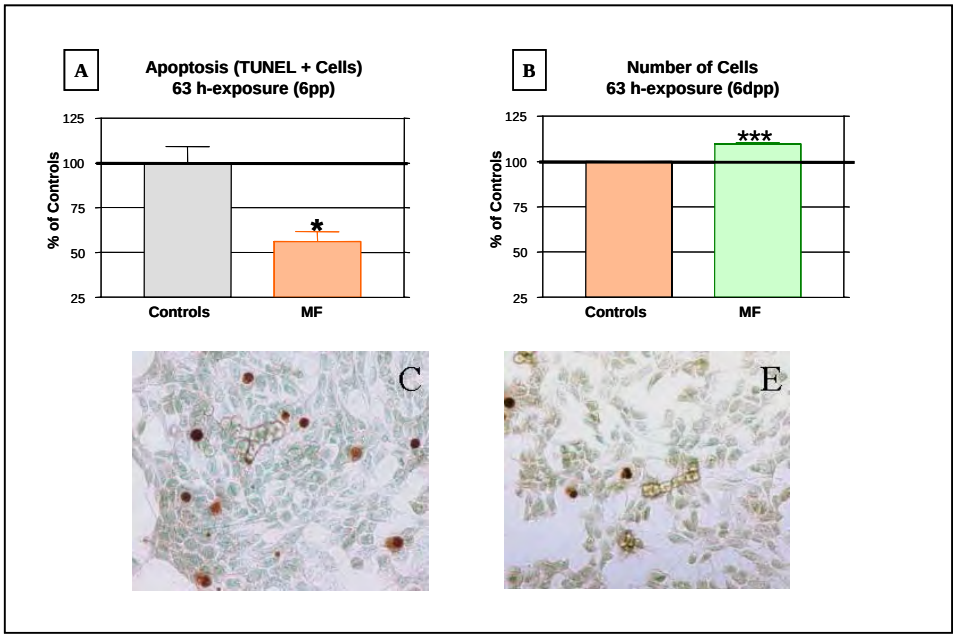


Figure 36. A) ELF-EMF (50 Hz, 100 μT) induce a significant reduction in the spontaneous apoptosis of the NB69 cell line. B) This described response is associated with an increase in the number of cells. (* $p< 0.05$, ***$p<0.001$, Student T´ test, three independent experimental replicates).Photomicrographs showing TUNEL-positive cells in controls (C) and exposed (E) samples.

ELF-EMF altered the activation of the phosphorylated cyclic adenosine monophosphate response-element binding protein (p-CREB).

The results of a total of 8 experiments show that both, the labelling/cell in NB69 cells and the proportion of phospho-CREB positive cells increase after 60 min of exposure (35.4% over controls, $p<0.01$, Figure 37 and photomicrograph). The percent of p-CREB positive cells in controls after 60 min of exposure was 32%. However, no differences were observed between ELF-EMF-exposed and controls samples after 30 or 120 min of exposure. These preliminary results suggest that the activation of p-CREB is involved in the previously described effects of 50 Hz 100 μT ELF-EMF on cell growth/apoptosis. In additional experiments the analysis of Western confirms that the ELF-MF induced a short-time dependent activation of the transcriptional factor CREB, with a peak at 60 min followed by a recovering of the basal levels at 120 minutes of exposure (data not shown).

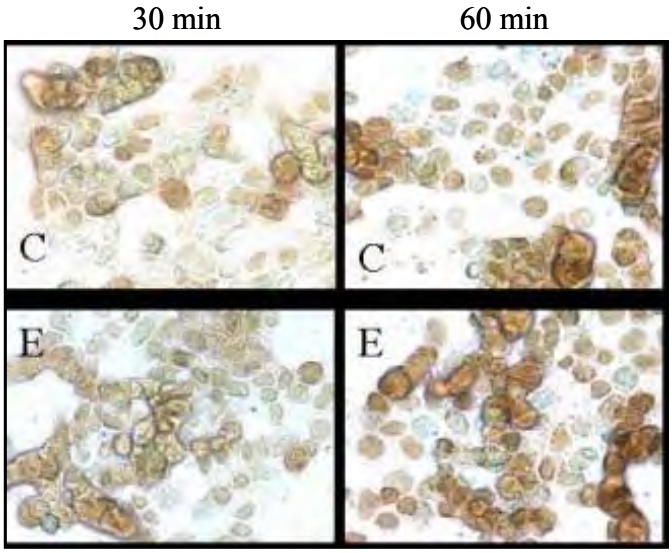


Figure 37. Immunocytochemistry for phospho-CREB. Changes in p-CREB positive cells showed a time-dependent response in the presence of the 100μT ELF-EMF. Photomicrograph showing the p-CREB labelling in brown and the counterstaining in green(methylgreen).

3.1.2.2 Embryonic stem cells of mice during cardiac differentiation (Participant 8)

ELF-EMF accelerated the cardiac differentiation of embryonic stem cells through enhanced expression of cardiac genes.

See 3.1.4.3

3.1.2.3 Human lymphocytes (Participant 8)

ELF-EMF exposure did not have any influence on proliferation, cell cycle and functionality of human lymphocytes.

The experiments with ELF-EMF (50 Hz) were performed at 50 μT magnetic field intensity on cells from 20 donors. Cell proliferation, cell cycle together with membrane activation markers were studied on lymphocytes from young donors. Data obtained by all the experiments performed indicated that no significant differences exist on cell proliferation or DNA synthesis at any time during the continuous exposure up to 6 days, as well as on cell cycle during the continuous exposure up to 96 hours. Cell activation phase was studied on CD3+HLA-DR+ T lymphocytes and CD4+CD25+ T helper lymphocyte subpopulation, but also in this case no differences were found between cells exposed and not exposed.

3.1.2.4 Embryonic stem cells of mice (Participant 4)

ELF-EMF did not have any influence on the growth and neuronal differentiation of embryonic stem cells of mice.

See 3.1.4.1

3.1.2.5 Summary (Participant 1)

Our data show some influence of ELF-EMF on proliferation and differentiation of some, but not all cell systems investigated. This conclusion is based on the following findings:

- ELF-EMF at a flux density of 0.1 mT significantly increased the proliferation of neuroblastoma cells (NB69 cell line) after exposure for 42- and 63-hrs (see 3.1.2.1).
- ELF-EMF at a flux density of 0.8 mT accelerated the cardiac differentiation of embryonic stem cells through enhanced expression of cardiac genes (see 3.1.2.2 and 3.1.4.3)

- ELF-EMF at a flux density of 2 mT did not have any influence on the growth and neuronal differentiation of embryonic stem cells of mice (see 3.1.2.4 and 3.1.4.1)
- ELF-EMF at a flux density of 0.8 mT did not have any influence on proliferation, cell cycle and activation of lymphocytes, either (see 3.1.2.3).

3.1.3 Apoptosis

3.1.3.1 Embryonic stem cells of mice (Participant 4)

ELF-EMF at a flux density of 2 mT up-regulated the transcript levels of the anti-apoptotic gene bcl2 and the growth arrest and DNA damage inducible gene GADD45 and down-regulated bax in ES cell-derived neural progenitor cells. This may indirectly influence the apoptotic process in neural progenitor cells.

See 3.1.4.1

3.1.3.2 Neuroblastoma cell line NB69 (Participant 5)

ELF-EMF at a flux density of 100 µT inhibited the spontaneous apoptosis in NB69 neuroblastoma cells.

See 3.1.2.1

3.1.3.3 Human fibroblasts (Participant 3)

No differences in cell count between ELF-EMF exposed and sham exposed human fibroblasts at any exposure duration could be detected. Therefore a possible elimination of cells by apoptosis and cell death can probably be ruled out.

See 3.1.1.1

3.1.3.4 Summary (Participant 1)

Our data indicate that ELF-EMF may have some indirect effect on apoptosis in certain, but not all cell systems investigated. This conclusion is based on the following findings:

- ELF-EMF at a flux density of 2 mT up-regulated in neural progenitor cells the transcript levels of the GADD45 gene and down-regulated the transcript levels of the bax gene by which the apoptotic process may be modulated (see 3.1.3.1 and 3.1.4.1).
- ELF-EMF at a flux density of 0.1 mT inhibited the spontaneous apoptosis in neuroblastoma cells in a way which is at present not well understood (see 3.1.3.2 and 3.1.2.1).
- ELF-EMF at a flux density of 1 mT did neither measurably affect the apoptotic process nor could a cytotoxic effect be detected in human fibroblasts in the course of a 24h exposure (see 3.1.1.1 and 3.1.3.3).

3.1.4 Gene and protein expression

3.1.4.1 Embryonic stem cells of mice (Participant 4)

ELF-MF exposure resulted in up-regulation of egr-1, c-jun and p21 transcript levels in p53-deficient, but not in wild type ES cells.

To analyse the effects of ELF-EMF, undifferentiated wild type (wt) and p53-deficient ES cells were exposed at different intermittence schemes and flux densities of 0.1, 1,0 and 2.3 mT for 6 and 48 hours, respectively (Table. 7). The exposition of ES cells to 5 min on followed by 30 min off cycles applied at

the high flux density of 2.3 mT resulted in a statistically significant up-regulation of egr-1, p21 and c-jun mRNA levels in p53-deficient ES cells (Figure 38A and C), whereas wild type cells showed no variations in transcript levels compared to sham exposure and control cells (Figure 38A). In contrast, low flux densities of 0.1 and 1 mT ELF-EMF applied at 5 min on/30 min off intermittence cycles induced no significant effects on transcript levels indicating that a high flux density of ELF-EMF signals is necessary to affect mRNA levels of regulatory genes (Table 7).

Table 7. Conditions of the exposure of p53-proficient and deficient pluripotent embryonic stem cells embryonic stem cells to ELF-EMF and summary of the effects on transcript levels of regulatory genes.

Intermittent exposure (5min on/30 min off)			
6 hours ELF-EMF exposure ; wt, $p53^{-/-}$		48 hours ELF-EMF exposure; wt, $p53^{-/-}$	
0.1 mT	no ELF-EMF effect (n=3)	0.1 mT	no ELF-EMF effect (n=3)
1.0 mT	no ELF-EMF effect (n=3)	1.0 mT	no ELF-EMF effect (n=3)
2.3 mT	up-regulation of egr-1, p21 and c-jun in $p53^{-/-}$ cells (without recovery time, RT); no ELF-EMF effect after 18 h RT (n=6)	2.3 mT	no ELF-EMF effect (n=3)

Intermittent exposure (5min on/10 min off)			
6 hours ELF-EMF exposure; wt, $p53^{-/-}$; without RT		6 hours ELF-EMF exposure; wt, $p53^{-/-}$; 18h RT	
2.3 mT	no ELF-EMF effect (n=6)	2.3 mT	no ELF-EMF effect (n=6)

Continuous exposure			
6 hours ELF-EMF exposure; wt, $p53^{-/-}$		48 hours ELF-EMF exposure; wt, $p53^{-/-}$	
0.1 mT	no ELF-EMF effect (n=3)	0.1 mT	no ELF-EMF effect (n=3)
1.0 mT	no ELF-EMF effect (n=3)	1.0 mT	no ELF-EMF effect (n=3)

ELF-MF exposure of p53-deficient cells induced only short-term and transient effects on gene expression levels.

To elucidate, whether ELF-EMF induce short- or long-term responses, p53-deficient and wt ES cells were exposed to intermittent 5 min on/30 min off ELF-EMF signals for 6 hours. In parallel, the cells were analysed after a recovery time of 18 hours. No statistically significant effects could be seen after 18 hours recovery in control, sham- and field-exposed variants suggesting that ELF-EMF induced only an immediate transient response in p53-deficient cells (Figure 38B).These observations correlated with the results of the 48 hours ELF-EMF exposure to p53-deficient ES cells at early differentiation stage, where no ELF-EMF effects on transcript levels were found (data not shown, see Table 7).

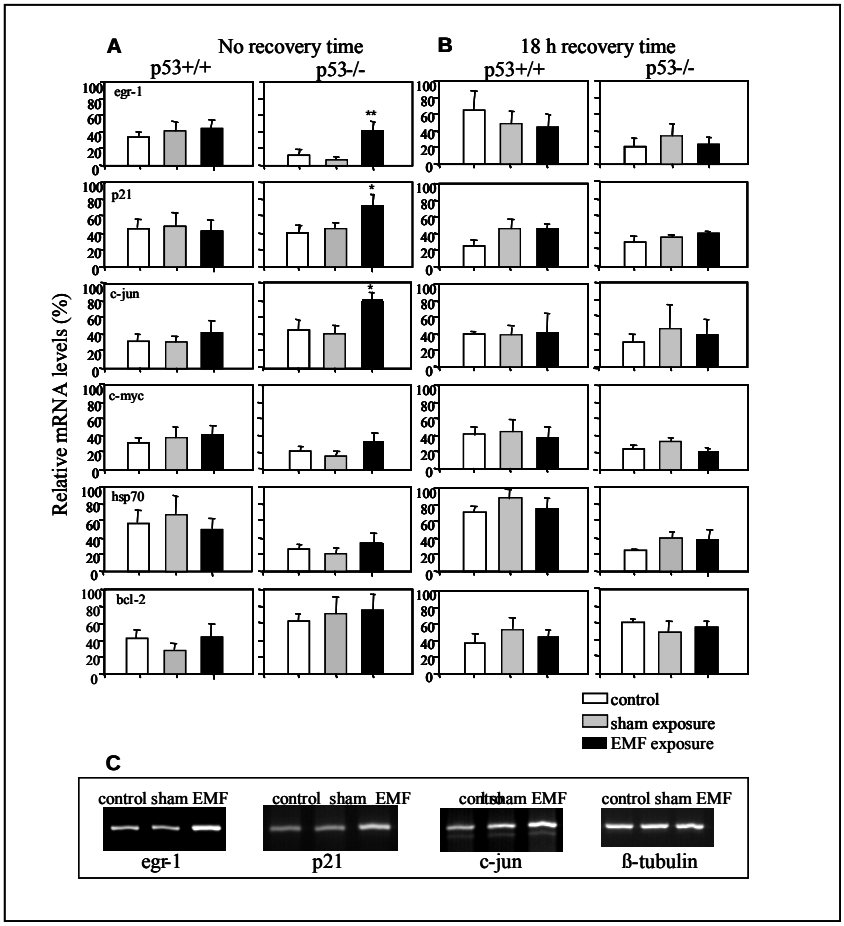


Figure 38. Relative mRNA levels of genes encoding egr-1, p21, c-jun, c-myc, hsp70 and bcl-2 in p53-deficient ($p53^{-/-}$) EC cell-derived embyoid bodies (EB) compared to wild-type (wt, $p53^{+/+}$) D3 cells immediately after 6h ELF-EMF (2.3 mT, intermittency 5 min on / 30 min off cycles) exposure (A) and after 18h recovery time (B) analysed by semi-quantitative RT-PCR. ELF-EMF exposure resulted in a significant, but transient up-regulation of egr-1, c-jun and p21mRNA levels in undifferentiated p53-deficient (C), but not wt ES cells. Statistical significance was tested by Studentt's t-test for significance levels of 1% and 5% (**p<0.01; *p<0.05)

ELF-MF effects on transcript levels of regulatory genes in p53-deficient cells were dependent on intermittence cycles (on/ off cycle duration).

In addition, we analysed in wt and p53-deficient ES cells the influence of ELF-EMF signals applied at another intermittence scheme of 5 min on/10 min off for 6 hours with a flux density of 2.3 mT. We found that 5 min on/10 min off ELF-EMF signals with or without recovery time had no effects on the transcript levels of the investigated regulatory genes in both, wt and p53-deficient cells. Further, continuous ELF-EMF signals at flux densities of 0.1 and 1 mT were applied to wt and p53-deficient ES cells. We found no influence of continuous ELF-EMF on the mRNA levels of the regulatory genes included in the study (data not shown). Experiments with the highest flux density (2.3 mT) could not be performed with the continuous exposure protocol, because the generated ohmic heat of the coils could not be compensated by the incubator and would have resulted in an unstable ambient temperature.

ELF-EMF exposure up-regulated the transcript levels of bcl-2, the growth arrest and DNA damage inducible gene (GADD45) and down-regulates bax in ES cell-derived neural progenitor cells.

Elf-EMF (50 Hz-Power line, 2 mT, 5 min.on/30min. off, Table 8) was applied for 6 or 48 hours on neural progenitors (Table 8). Semi-quantitative RT-PCR analysis revealed no effect of ELF-EMF on transcript levels of genes involved in neuronal differentiation (nurr1, en-1) and on markers of differentiating

(nestin) or differentiated neuronal (TH) or astrocytic (GFAP) cells. In addition, we studied the effect of ELF-EMF on transcript levels of genes involved in the regulation of cell homeostasis (hsp70), cell cycle (p21) and anti-apoptosis (bcl-2). RT-PCR analysis revealed that, whereas transcript levels of p21 and hsp70 remain similar in sham and ELF-EMF exposed variants, a significant up-regulation of the growth arrest inducible gene GADD45 was observed at stage 4d+11d. (Figure 39). The quantitative RT-PCR (Q-RT-PCR) with specific primers and TaqMan probes showed that bcl-2 was first down-regulated at stage 4+7d ($p<0.05$), then up-regulated in the intermediate stage 4+11d ($p<0.01$) and at the terminal stage 4+23d ($p<0.05$). GADD45 was significantly up-regulated at stage 4+11d, then down-regulated at the terminal stage 4+23d (Figure 40). These studies were further substantiated by immunofluorescence analyses of neuronal markers. However, by immunofluorescence analysis, no changes in intracellular distribution and number of cells expressing neuronal markers (ßIII-tubulin, TH, GFAP) were observed (data not shown).

Table 8. Conditions of the exposure of neuronal progenitor cells to ELF-EMF and summary of the effects on transcript abundance, neural differentiation induction and DNA break induction.

Intermittent exposure (5min on/30 min off)			
48 hours, ELF-EMF (Power line, 50Hz)		6 hours, ELF-EMF (Power line, 50Hz)	
2.0 mT	up-regulation followed by down-regulation of GADD45 up-regulation of bcl-2 down-regulation of bax no effect on neural differentiation no effect on DNA break induction (n=6)	2.0 mT	no effect on DNA break induction (n=3)

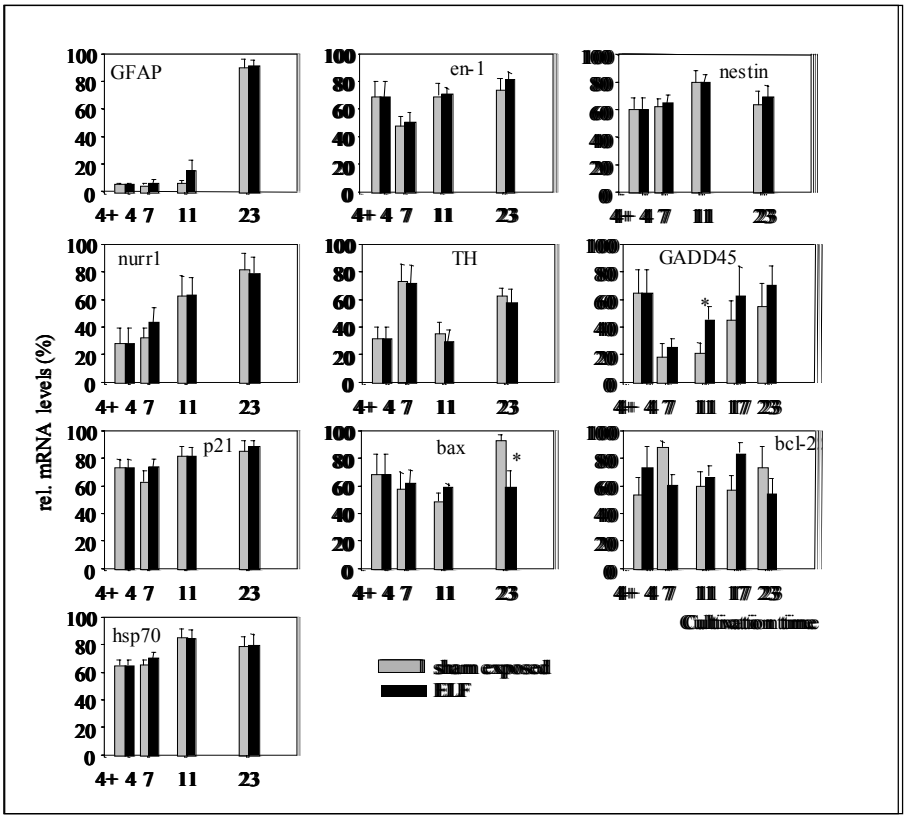


Figure 39. Relative mRNA levels of genes encoding the regulatory genes bcl-2, bax, p21, hsp70 and the genes involved in neuronal differentiation en-1, nurr1, TH, GFAP and nestin in ES-derived neural progenitors after 48 hours ELF (50Hz Powerline) EMF exposure (2.0 mT, intermittence 5 min ON/30 min OFF), at stage 4+4d - 4+6d. EMF exposure resulted in a significant transcript up-regulation of GADD45 and down-regulation of bax. Error bars represent standard deviations. Statistical significance was tested by the Student's t-test for a significance level of 5% (*, p£ 0.05).

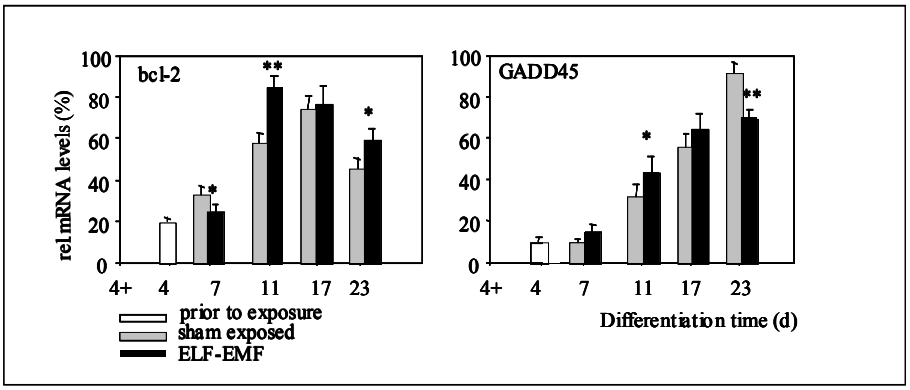


Figure 40.: Quantitative RT-PCR for estimation of relative mRNA levels of genes encoding the regulatory genes bcl-2 and GADD45 in ES-derived neural progenitors after 48 hours ELF (50Hz Powerline) EMF exposure (2.0 mT, intermittence 5 min on/30 min off), at stage 4+4d - 4+6d. EMF exposure resulted in a significant transcript down-regulation followed by up-regulation of bcl-2, which correlated with up-regulation followed by down-regulation of GADD45. Error bars represent standard deviations. Statistical significance was tested by the Student's t-test for a significance level of 5% and 1% (*, p< 0.05 ;**, p< 0.01).

3.1.4.2 Human neuroblastoma cell line SY5Y (Participant 11)

In order to obtain cellular models to study ELF-EMF, we have characterised some neuroblastoma cell lines for their ability to express nAchRs and evaluate whether ELF-EMF can interfere with the expression of alpha3, alpha5 and alpha7 nAchR subunits, as well as with that of Phox2a, Phox2b and dopamine-beta-hydroxylase (DβH).

Three human neuroblastoma cell lines (SH-SY5Y, SK-N-BE and IMR32) have been analysed, by means of northern blot analysis, for the expression of neuronal acetylcholine receptor subunits. Due to the high degree of homology between different subunits, the experiments have been carried out with probes derived from the cytoplasmic portion, the least conserved region of nAchR subunits, in order to avoid cross-contamination. The results showed that these cells express the ganglionic type of nAchRs (alpha3, alpha5 and alpha7), but not alpha 4, mainly expressed in the CNS (data not shown and Fornasari 1997; Flora 2000). Furthermore only human neuroblastoma cell lines SY5Y and IMR-32 appeared to express either Phox2a, Phox2b or DβH (Flora, 2001 and data not shown), although with differences in the level of expression. As the SY5Y lineage shows higher expression of the three genes, we decided to use this as a model in all the experiments.

ELF-EMF did not affect the expression of nicotinic acetylcholine receptors (nAchRs) which represent the neuronal nicotinic system in human neuroblastoma cells.

At the beginning of our experiment we decided to use field strengths which are larger than the maximum real-world exposure and eventually scale-down, in the case of measurable effect, establishing the minimum threshold level to which ELF-EMF do not represent a risk to human health. Neuroblastoma cell line SY5Y was then exposed to ELF-EMF (50 Hz, powerline signal) continuously for 16 hours at flux densities of 2 mT and 1 mT and the expression level of humaṇ alpha 3,alpha 5 and alpha 7 nAchRs subunits analysed by means of Northern blotting. Figure 41 (panel A) shows the results obtained by three independent exposures at 2 mT (lanes 1, 4 and 5) together with that of sham-exposed cells (lanes 2, 3 and 6). The densitometric quantification of the mRNA level, however, has shown no effect on the expression level of nAchR subunits as compared to that of the sham-exposed cells set as 100%, when cells were exposed either at 2 mT or 1 mT (Figure 41), panel B and C respectively). We then decided to explore whether an intermittent exposure might be influent on the expression of the nAchR subunits tested. Exposing the cells to intermittent magnetic field (5 min on/5 min off), 2 mT and 1 mT flux density, for 16 hours did not affect the expression of the alpha3, alpha5 and alpha7 nAchR subunit genes (Figure 42, panel A and B respectively).

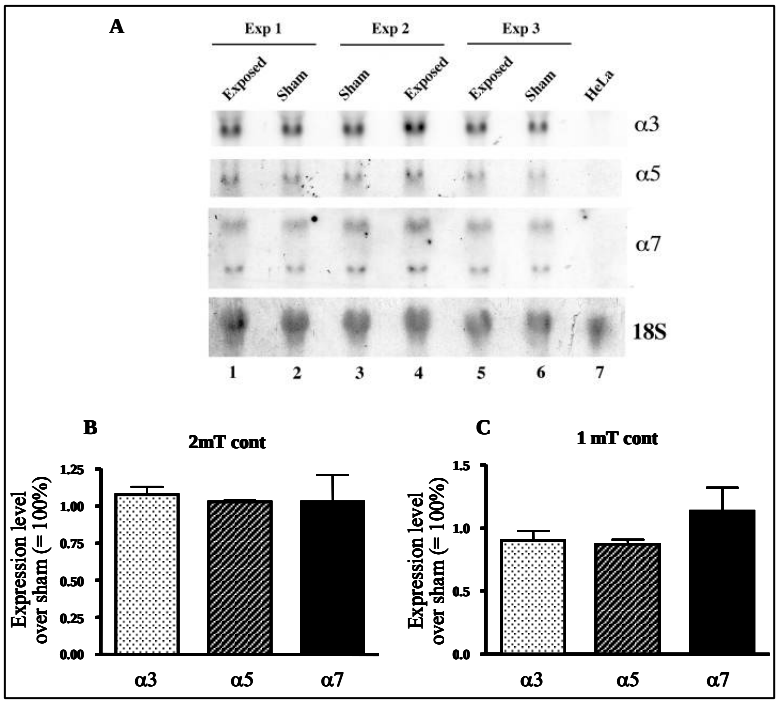


Figure 41. nAchR subunits expression upon exposure to 16 hours continuous ELF-EMF. 20 µg of total RNA extracted from SY5Y cells exposed to 2mT and 1 mT continuous 50 Hz magnetic field, for 16 hours, was hybridised to cDNA probes corresponding to the human alpha3, alpha5 and alpha7 nAchR subunits. The expression level was normalised to that of 18S RNA. A, Northern blot analysis of total RNA extracted upon exposure to 2mT ELF-EMF. Here reported are the results of three independent experiments (Exp1, lanes 1-2; Exp 2, lanes 3-4; Exp 3, lanes 5-6). Lane 7, HeLa total RNA has been used as a negative control. B and C, Densitometric quantification of the expression level of nAchR subunits upon exposure to 2 mT and 1 mT continuous ELF-EMF, respectively. The data are the mean of three independent experiments ± S.E., expressed as a percentage of the sham-exposed sample set equal to 100%.

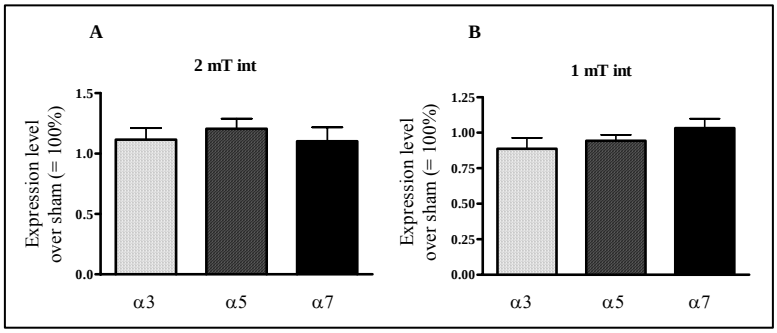


Figure. 42. nAchR subunits expression upon exposure to 16 hours intermittent ELF-EMF. 20 µg of total RNA extracted from SY5Y cells exposed to 2mT and 1 mT continuous 50 Hz magnetic field, for 16 hours, was hybridised to cDNA probes corresponding to the human alpha3, alpha5 and alpha7 nAchR subunits. The expression level was normalised to that of 18S RNA. The data are the mean of three independent experiments ± S.E., expressed as a percentage of the sham-exposed sample set equal to 100%. A, Densitometric quantification of the expression level of nAchR subunits upon exposure to 2 mT intermittent ELF-EMF. B, Densitometric quantification of the expression level of nAchR subunits upon exposure to 1 mT intermittent ELF-EMF.

As we were not able to measure any effect at the mRNA level, we wondered whether the exposure to ELF-EMF might have an effect at the level of receptor proteins. To this purpose we carried out radioligand assays on cells exposed to either continuous or intermittent 50 Hz ELF-EMF, flux densities of 1 mT and 2 mT for 16 hours, to assess the amount of protein functionally assembled in the receptors. The

binding was performed in the presence of radiolabelled ligands, ^{3}H-Epibatidine to quantitate alpha3-containing receptor and ^{125}I-alpha-bungarotoxin to quantitate alpha7-containing receptor. However, as shown in Figure 43, no change in the amount of either alpha3- or alpha7-containing receptor was detected under the same conditions used in Northern blot analysis of Figures 41 and 42, as compared to that of the sham-exposed cells set as 100%.

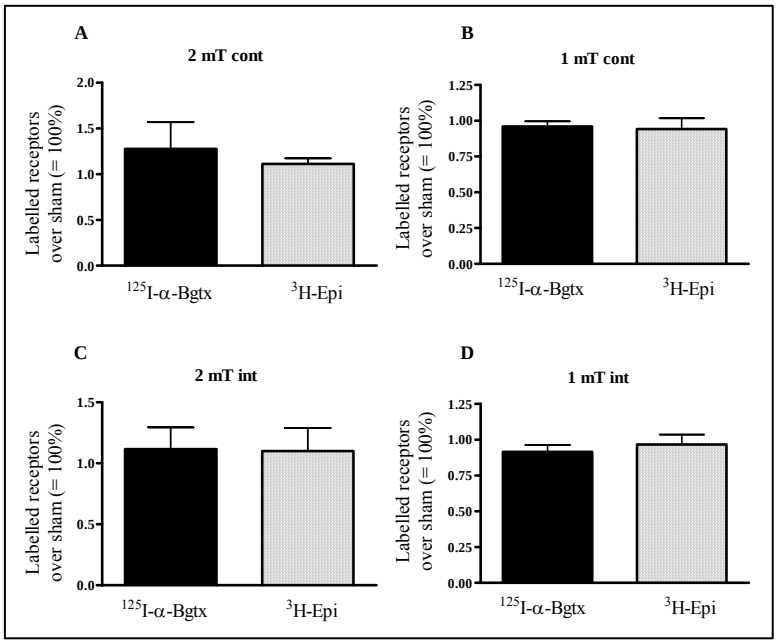


Figure 43. Quantitative analysis of the alpha3- and alpha7-containing receptors upon continuous and intermittent ELF-EMF exposure for 16 hours

The amount of labelled receptors were obtained from the binding of saturating concentration of ^{3}H-Epibatidine (grey bars) and ^{125}I-alpha-bungarotoxin (black bars) to the cell homogenates, performed in quadruplicate. The values are the mean of three independent experiments ± and S.E. are expressed as percentage of labelled receptors in the exposed samples with respect to the sham-exposed cells set as 100%. A and B, continuous exposure to 1 mT and 2 mT ELF-EMF, respectively. C and D, intermittent (5 min on/5 min off) exposure to 1 mT and 2 mT ELF-EMF.

The experiments carried out until now have showed that the ELF-EMF does not influence the expression of nAchRs upon exposure of the cells to magnetic field with flux densities of either 1 mT or 2 mT for a relatively short period of time (16 hours). We then investigated whether the duration time of the exposure of SY5Y cells to ELF-EMF might affect the expression of the genes encoding the nAchR subunits, and especially, whether longer exposure to ELF-EMF might affect the expression of some of the genes. To answer this question two different exposure protocols have been used: a) 50 Hz powerline signal, flux density 1 mT, continuous exposure, duration 48 hours. The RNA or proteins were extracted immediately after the end of exposure (immediate recovery); b) 50 Hz powerline signal, flux density 1 mT, continuous exposure, duration 48 hours. The RNA or proteins were extracted 48 hours after the end of exposure (delayed recovery).

As shown in Figure 44, panel A, the expression of nAchR subunits, as measured at mRNA level, was again not affected by a prolonged exposure to the ELF-EMF followed by an immediate recovery of the cells (protocol a). Furthermore, no effect was detected at the level of receptor proteins (Figure 44, panel B). We then wondered whether the effect could be a delayed one, that is mediated by the activation of a cascade of second messengers that results in a change of gene expression. To test this hypothesis, cells were collected for RNA and protein analysis 48 hours after the end of the exposure (protocol b). The

results shown in Figure 44 seemed to rule out an indirect effect as neither the level of mRNA (Figure 45, panel A) nor of the receptor proteins (Figure 45, panel B) changed under these experimental conditions.

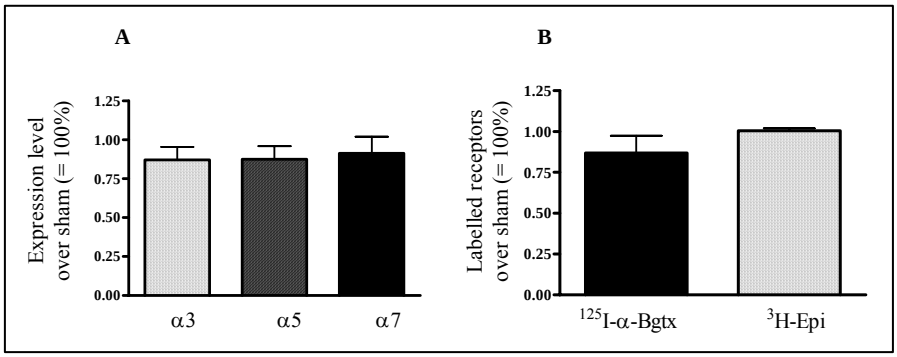


Figure 44. nAchR subunits expression and quantitative analysis of the alpha3- and alpha7 containing receptors upon continuous exposure to 1 mT ELF-EMF for 48 hours: Immediate recovery

Cells were exposed to 1 mT ELF-EMF for 48 hours and recovered immediately after the end of the exposure. A, Densitometric quantification of the expression level of nAchR subunits after Northern blot analysis. The data are the mean of five independent experiments ± S.E., expressed as a percentage of the sham-exposed sample set as 100%. B, quantification of the alpha3- (grey bars) and alpha7-containing (black bars) receptors. The values are the mean of three independent experiments ± and S.E. are expressed as percentage of labelled receptors in the exposed samples with respect to the sham-exposed cells set as 100%.

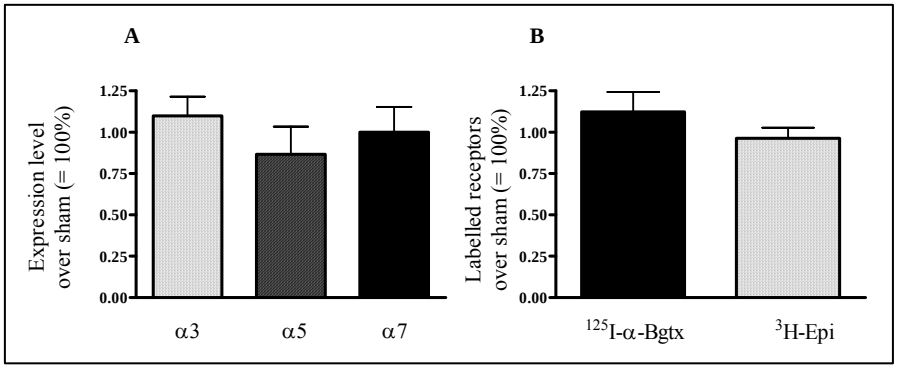


Figure 45. nAchR subunits expression and quantitative analysis of the alpha3- and alpha7 containing receptors upon continuous exposure to 1 mT ELF-EMF for 48 hours: Delayed recovery

Cells were exposed to 1 mT ELF-EMF for 48 hours and recovered 48 hours after the end of the exposure. A, Densitometric quantification of the expression level of nAchR subunits after Northern blot analysis. The data are the mean of five independent experiments ± S.E., expressed as a percentage of the sham-exposed sample set as 100%. B, quantification of the alpha3- (grey bars) and alpha7-containing (black bars) receptors. The values are the mean of three independent experiments ± and S.E. are expressed as percentage of labelled receptors in the exposed samples with respect to the sham-exposed cells set as 100%.

ELF-EMF did not affect the expression of markers of the cathecolaminergic system in neuroblastoma cells.

In collaboration with Participant 1 we decided to investigate the influence of ELF-EMF on neurotransmitter release. In particular, the activity of the dopamine-beta-hydroxylase (DβH) which is a key enzyme in the synthesis of noradrenaline has been studied. Furthermore, we investigated possible modifications on the expression of two related homeo-domain transcription factors, Phox2a and Phox2b, that are relevant for the specification of the autonomic nervous system. Moreover, in noradrenergic cells, they are directly involved in the determination of the neurotransmitter phenotype by regulating the expression of DβH. As protocols, we applied the same exposure conditions used for the analysis of the human nAchR subunits. As shown in Figure 46, panels A and B, continuous exposure of SY5Y neuroblastoma cells to 1 mT and 2 mT 50 Hz ELF-EMF did not affect the expression level of either Phox2a, Phox2b and DβH genes, as compared to that of the sham-exposed cells set to 100%. Again we asked whether an intermittent exposure might have an effect on gene expression of these proteins. We then measured the mRNA level upon intermittent exposure (5 min on/5 min off) to 1 mT ELF-EMF, but no change was observed (Figure 47). Previous experiments have shown that an exposure of SY5Y cells for 48 hours at 1 mT flux density reduced the amount of mRNA of Phox2a, but not of Phox2b and DβH. We then exposed the cells under these conditions up to nine times independently.

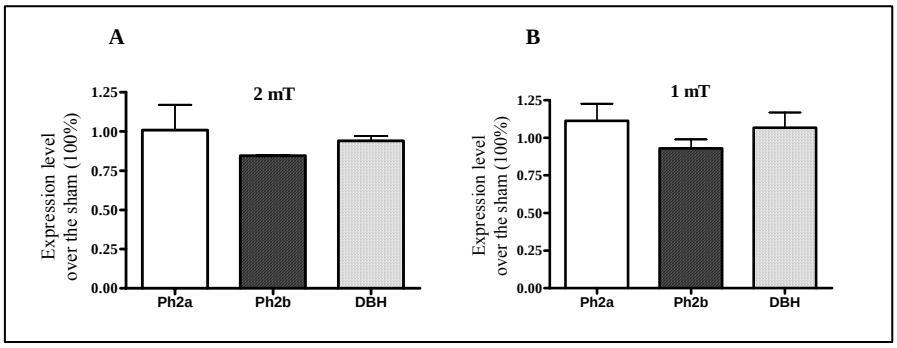


Figure 46. Noradrenergic phenotype specifying genes expression upon continuous exposure to ELF-EMF for 16 hours

20 µg of total RNA extracted from SY5Y cells continuously exposed to 1mT and 2 mT 50 Hz ELF-EMF for 16 hours was hybridised to cDNA probes corresponding to the human Phox2a, Phox2b and DβH genes. The expression level was normalised to that of 18S RNA. A Densitometric quantification of the expression level of the three genes upon continuous exposure to 2 mT ELF-EMF. B, Densitometric quantification of the expression level of the three genes upon continuous exposure to 1 mT ELF-EMF. The data are the mean of five independent experiments ± S.E., expressed as a percentage of the sham-exposed sample set as 100%.

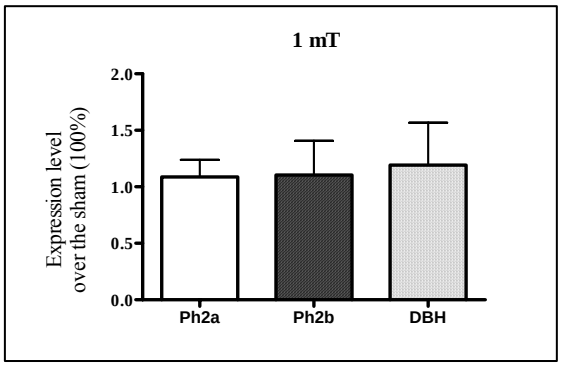


Figure 47. Noradrenergic phenotype specifying genes expression upon intermittent exposure (5 min on/5 min off) to ELF-EMF for 16 hours.

Densitometric quantification of the expression level of the three genes upon intermittent exposure to 1 mT ELF-EMF, after Northern blot analysis. The data are the mean of five independent experiments ± S.E., expressed as a percentage of the sham-exposed sample set as 100%.

Statistical analysis has ruled out that, upon this exposure protocol, the expression of the noradrenergic specifying genes was affected, as shown in Figure 48, either harvesting the cells immediately after the end of the exposure (Figure 48, panel A) or 48 hours later (Figure 48, panel B). As no change was seen at the level of mRNA we asked whether the exposure to ELF-EMF might affect the expression of Phox2a and Phox2b at protein level. To this purpose we decided to measure, by western blot analysis, the protein level upon continuous exposure to 50 Hz ELF-EMF, flux density 1 mT, for 16 and 48 hours (Figure 49). Cells were harvested immediately after the end of the exposure (Figure 49, lanes 10-13 and lanes 6-9, respectively). Also protein extract from cells harvested 48 hours after the end of the continuous 48 hours exposure was tested (Figure 49, lanes 2-5). Densitometric analysis of the signal obtained for Phox2a (Figure 49, panel A) and Phox2b (Figure 49, panel B), normalised to that of the beta-tubulin, revealed that exposure of SY5Y cells to relatively short or longer period of time did not affect the expression of Phox2a and 2b, at protein level, as compared to that of the sham-exposed cells (Figure 49, panel C).

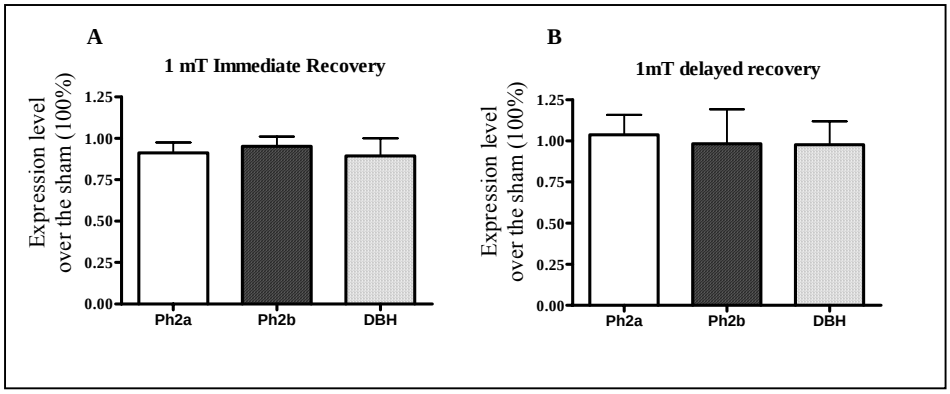


Figure 48. Noradrenergic phenotype specifying genes expression upon continuous exposure to ELF-EMF for 48 hours

SY5Y cells were continuously exposed to 1 mT 50 Hz ELF-EMF for 48 hours and collected either immediately (panel A) or 48 hours (panel B) after the end of the exposure. After northern blot analysis, the expression level of Phox2a, Phox2b and DβH genes was quantified by densitometric scanning of the autoradiogram. The data are the mean of six independent experiments ± S.E. (Phox2b and DβH) and nine independent experiments (Phox2a), expressed as a percentage of the sham-exposed sample set equal to 100%.

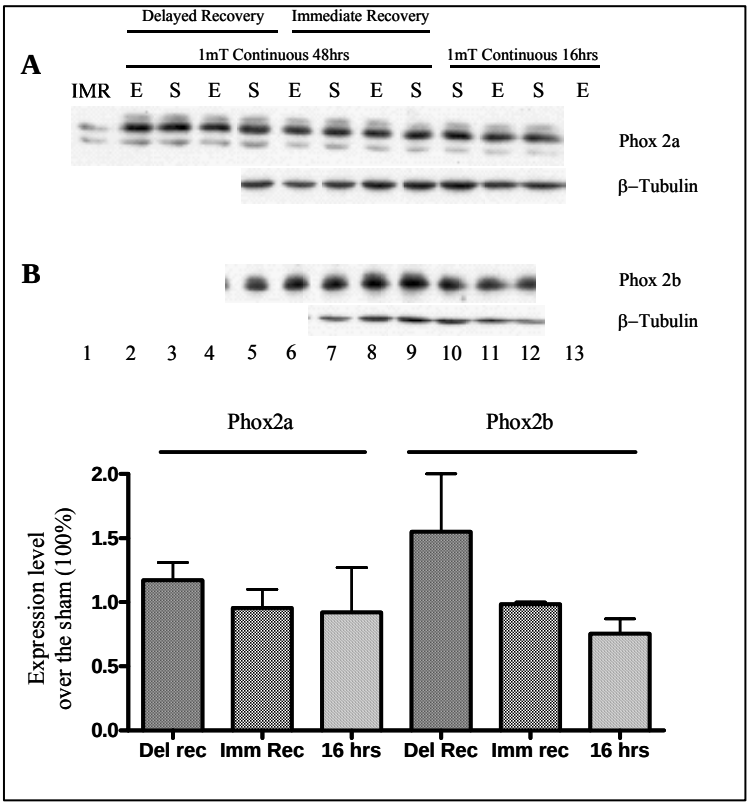


Figure 49. Western blot analysis of Phox2a and Phox2b expression upon continuous exposure to 1 mT ELF-EMF.20 µg of total protein extract were size-fractionated by SDS-PAGE and transferred to nitrocellulose membrane. The expression of Phox2a and Phox2b was detected by incubation with anti-Phox2a (panel A) and anti-Phox2b antibodies (panel B). Lanes 2-9, samples from continuous exposure to 1 mT ELF-EMF for 48 hours recovered 48 hours (lanes 2-5) or immediately (lanes 6-9) after the end of the exposure. Lanes 10-13, samples from 1 mT continuous exposure for 16 hours. Lane 1, IMR 32 neuroblastoma cells nuclear extract used as a control. E= exposed, S= sham-exposed. The expression of Phox2a and Phox2b was normalised to that of β-tubulin. C, quantification of the expression level of Phox2a and Phox2b. The data are the mean of two independent exposures ± S.E. and are expressed as percentage of the sham-exposed samples set as 100%. Del Rec = recovery 48 hours after the end of the exposure; Imm rec = recovery immediately after the end of the exposure; 16 hours = samples were exposed for 16 hours to 1 mT continuous ELF-EMF.

3.1.4.3 Embryonic stem cells of mice during cardiac differentiation (Participant 8)

In higher vertebrates, heart formation is a complex phenomenon that starts at early stages of embryogenesis, prior to the end of gastrulation, with commitment of anterior lateral plat mesoderm cells to cardiogenic lineage. Studies in different organ systems have shown that tissue-specific transcription factors which control the expression of differentiation markers are also regulators of cellular differentiation. Basic helix-loop-helix proteins such as the myogenic factor are key regulators of skeletal muscle differentiation, while the erythroid cell-specific zinc finger protein GATA-1 is crucial for erythroid cell differentiation. It is now becoming evident that inactivation of the mouse homologue of the Drosophila melanogaster homeobox gene tinman, the homeobox gene Nkx2.5 or Csx affects heart morphogenesis (Biben 1997, Lints 1993). Moreover, the GATA-4 protein, a member of the GATA family of transcription factors, has been found to be restricted to the hearts and to characterise very early stages of heart formation during embryonic development (Grepin 1995).

ELF-EMF affected the expression of cardiogenic genes in murine embryonic stem cells (GTR1).

In the first step of our study we looked at the effects of ELF-EMF (0.8 mT, 50 Hz sinusoidal) on the expression of cardiogenic genes in mouse embryonic carcinoma (EC) cells (P19 cells). Despite the encouraging results obtained in our pilot experiments, in a subsequent set of ten separate experiments P19

cells exposed to ELF-EMF desultorily underwent a gene program of cardiogenesis and revealed structural and functional cardiomyocyte features. Only in 2 experiments, exposure to ELF-EMF primed the expression of both GATA-4 and Nkx-2.5 genes, and led to the appearance of alpha-myosin heavy chain (MHC) and myosin light chain-2V (MLC), two cardiospecific transcripts. A representative RT-PCR analysis of cardiogenic and cardiac specific gene expression from ELF-EMF responsive cells is shown in Figure 50).

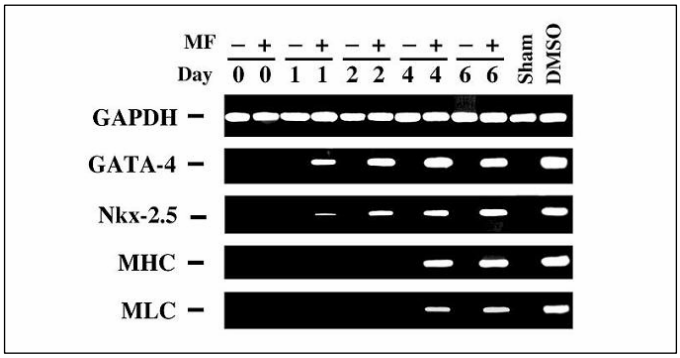


Figure 50. RT-PCR analysis of cardiogenic and cardiospecific transcripts in P19 cells exposed to ELF-EMF (continuous exposure, 4 days). MHC, alpha-myosin heavy chain. ML*C,* myosin light chain-2V.

We reasoned that the lack of data reproducibility of these results could be due to the consistent dilution of the myocardial phenotype within multiple non-myocardial cells encompassed by the P19 model of cell differentiation. To circumvent this problem, we decided to change the biological model, by using a line of pluripotent embryonic stem (ES) cells engineered for a gene trapping selection of a virtually pure population of ES-derived cardiomyocytes. RT-PCR analysis of targeted transcripts in unexposed cells indicated that, differently from undifferentiated LIF-supplemented cells, EBs expressed both GATA-4 and Nkx-2.5 mRNA (Figure 51). The expression of these cardiogenic genes resulted to be further enhanced in puromycin selected, ES-derived cardiomyocytes (Figure 52). Figures 51 and 52 show that ELF-EMF exposure remarkably increased GATA-4 and Nkx-2.5 gene expression in both EBs and cardiomyocytes, as compared to unexposed GTR1 cells. Interestingly, in both groups of cells ELF-EMF also increased the expression of the prodynorphin gene, an endorphin gene that has been recently shown to play a major role in orchestrating ES cell cardiogenesis (Ventura 2003(a), 2003(b), 2000). These responses were further inferred by the quantitative analysis of mRNA levels as shown in RNase protection experiments (Figure 53). Interestingly, nuclear run-off analyses of GATA-4 gene transcription indicated that the ELF-EMF action occurred at the transcriptional level (Figure 54). The activation of a program of cardiogenic gene transcription was also associated with the appearance of the cardiac specific transcripts alpha-myosin heavy chain and myosin light chain-2V (Figure 55).

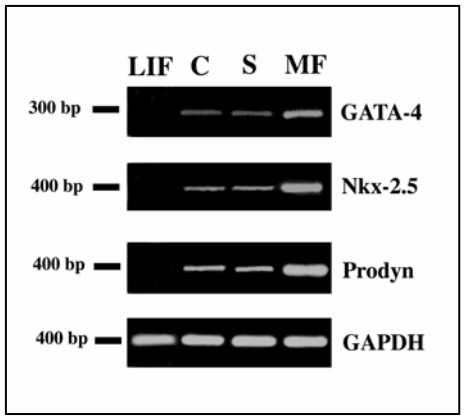


Figure 51. ELF-EMF was applied from the time of LIF removal and EBs were collected after 3 additional days. C: control EBs; S: sham. (Ethidium bromide-stained agarose gels, representative of 4 separate experiments).

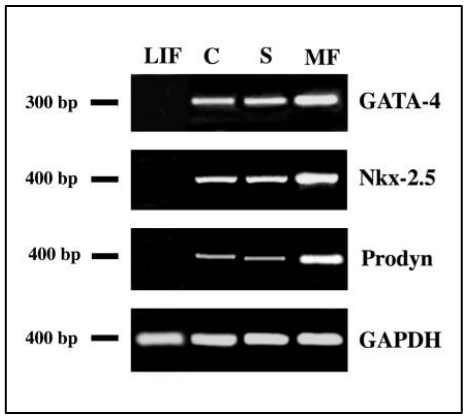


Figure 52. Effect of ELF-EMF (MF) on Cardiogenic gene expression in puromycin-selected cells. ELF-EMF was applied from the time of LIF removal throughout puromycin selection. Four days after puromycin addition (10 days from LIF withdrawal), ES-derived cardiomyocytes were processed gene expression analyses. C: control puromycin-selected cardiomyocytes; S: sham. (Ethidium bromide-stained agarose gels, representative of 4 separate experiments).

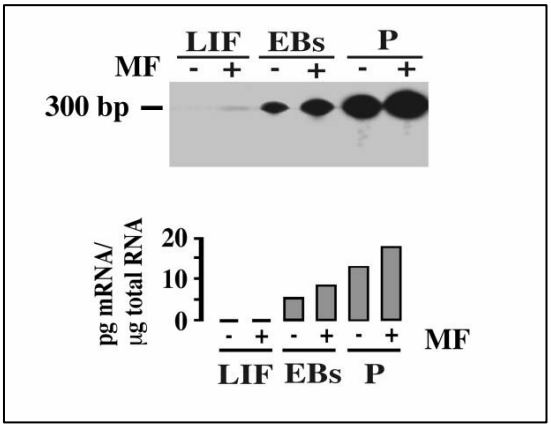


Figure 53. RNase protection analysis of GATA-4 mRNA expression in GTR1 ES cells cultured in the absence or presence of ELF-EMF.LIF, undifferentiated GTR1 cells. EBs, embryoid bodies collected 5 days after LIF removal. *P*, puromycin-selected cardiomyocytes: puromycin was added at day 8 following LIF removal. Each group of cells was cultured in the absence (-) or presence (+) of ELF-EMF. Autoradiograms are representative of 3 separate experiments.

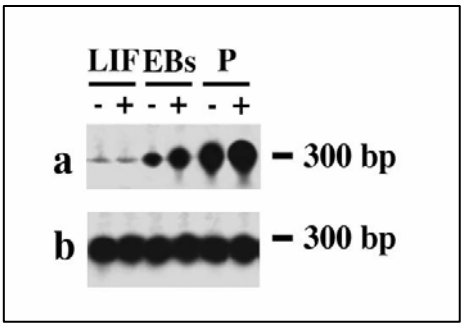


Figure 54. Nuclear run-off analysis of GATA-4 gene transcription in isolated ES cell nuclei. Nuclei were isolated from undifferentiated GTR1 cells (LIF), from EBs collected 5 days after LIF removal (EBs) or from puromycin-selected cardiomyocytes (P): puromycin was added at day 8 following LIF removal. Each group of cells was exposed in the absence (-) or presence (+) of ELF-EMF (MF). Puromycin was added at day 8 following LIF removal. Row a, GATA-4 gene transcription. Row b, cyclophilin gene transcription (cyclophilin was assessed as a constant gene for control). Autoradiograms are representative of 3 separate experiments.

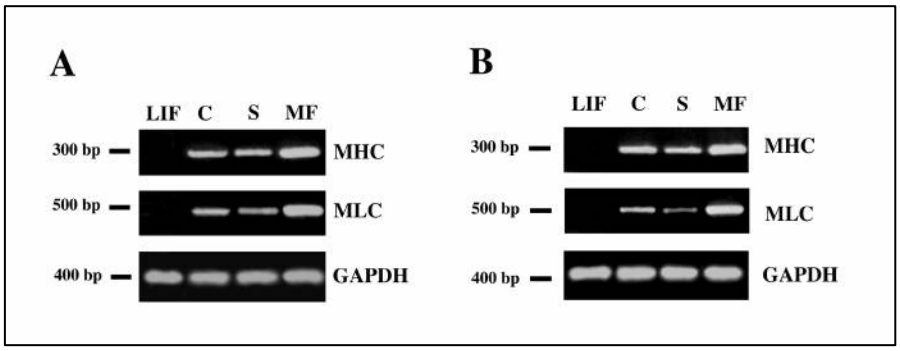


Figure 55: Effect of MF on the expression of cardiac specific genes.A: ELF-EMF (MF) was applied from the time of LIF removal and EBs were collected after additional 3 days. B: MF was applied from the time of LIF removal throughout puromycin selection. Four days after puromycin addition (10 days from LIF withdrawal), ES-derived cardiomyocytes were processed gene expression analyses. C: control cells; S: sham. (Ethidium bromide-stained agarose gels, representative of 4 separate experiments).

Exposure of GTR1 ES cells to ELF-EMF after LIF removal and throughout 4 days of puromycin selection for an overall period of 10 days from LIF withdrawal was able to increase the yield of ES-derived cardiomyocytes: the number of beating colonies reached 170.44 ± 28.0 % of the control value, estimated in cardiomyocytes selected from untreated cells (mean ± SEM of 4 separate experiments).

We finally investigated whether the transcriptional responses evoked by ELF-EMF may encompass genes that are essential for the specification of non-myocardial lineages. Noteworthy, the expression of MyoD, a gene involved in skeletal myogenesis was not affected in both EBs and puromycin selected cells (Figure 56), while the expression of neurogenin1, a neuronal specification gene, was slightly enhanced only in EBs (Figure 56).

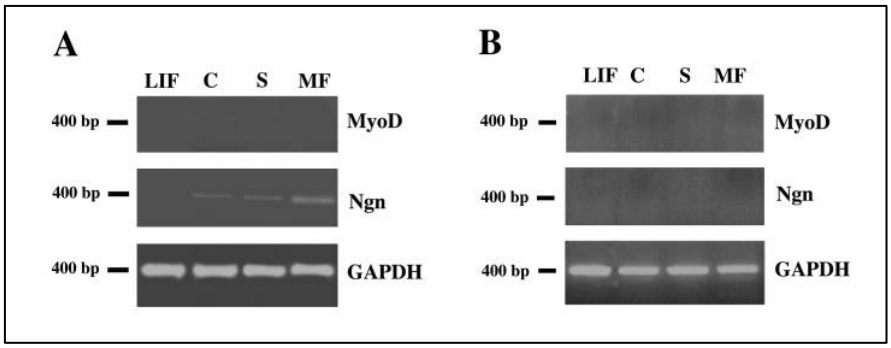


Figure 56. Effect of ELF-EMF (MF) on the expression of genes promoting non-myocardial lineages. A: MF was applied from the time of LIF removal and EBs were collected after additional 3 days. B: MF was applied from the time of LIF removal throughout puromycin selection. Four days after puromycin addition (10 days from LIF withdrawal), ES-derived cardiomyocytes were processed gene expression analyses. C: control cells; S: sham. MyoD and neurogenin1 (Ngn) are skeletal muscle and neuronal specification genes, respectively. (Ethidium bromide-stained agarose gels, representative of 4 separate experiments).

3.1.4.4 Membrane currents of oocytes of *Xenopus laevis* expressing rCx46 (Participant 7)

ELF-EMF did not significantly affect the leak-current of oocytes of Xenopus laevis expressing hemi-channels of rCx46.

During expression of hemi-channels, composed of the connexin rCx46, the oocytes were exposed to ELF-EMF (50 Hz). As suitable parameter for functional integrity of an oocyte the leak-current was selected which was electrophysiologically measured at voltage-clamp. A representative experiment of a sham exposed oocyte is shown in Figure 57. Figure 57a) shows membrane currents recorded at depolarising test potentials starting from a holding potential of –90 mV. The figure indicates that the rCx46-mediated current becomes activated by depolarising test potentials above about –10 mV. Figure 57b) shows the corresponding leak subtracted steady-state current values (I_{ss}) as function of driving voltage ($V\text{-}V_{rev}$) where V_{rev} denotes the corresponding reversal potential. The corresponding steady-state current values were derived at the end of the applied test pulse.

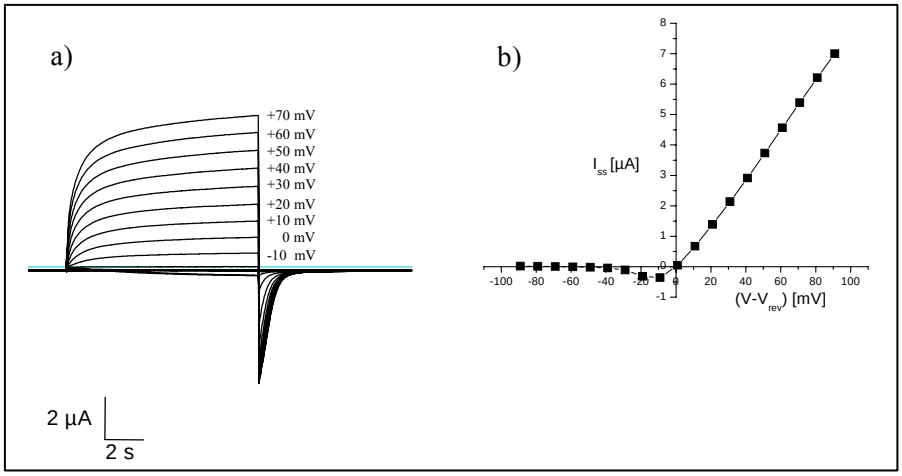


Figure 57. Voltage-dependent current activation of sham exposed *Xenopus* oocytes expressing rCx46 after an expression time of 16 hours.**a)** Representative voltage-jump current-relaxations at given test potentials. The common holding potential was set to -90 mV. The dotted line denotes the zero current level. **b)** Leak subtracted steady-state current amplitudes (I_{ss}, , ■) derived from the data in a) as function of driving voltage ($V\text{-}V_{rev}$)

The leak-current was derived at test potentials in the range of -70 mV to -100 mV using a constant holding potential of -90 mV. A comparison of leak-currents for exposed and sham exposed oocytes is given in Figure 58. Figure 58a shows the leak-current for oocytes which were continuously exposed for 16 hours at 2.3 mT and Figure 55b) the corresponding leak-current after an intermittent exposure (5 min on/10 min off) at 1.0 mT for 16h. A significant influence of ELF-EMF exposure on the leak current could not be observed for the different exposure conditions.

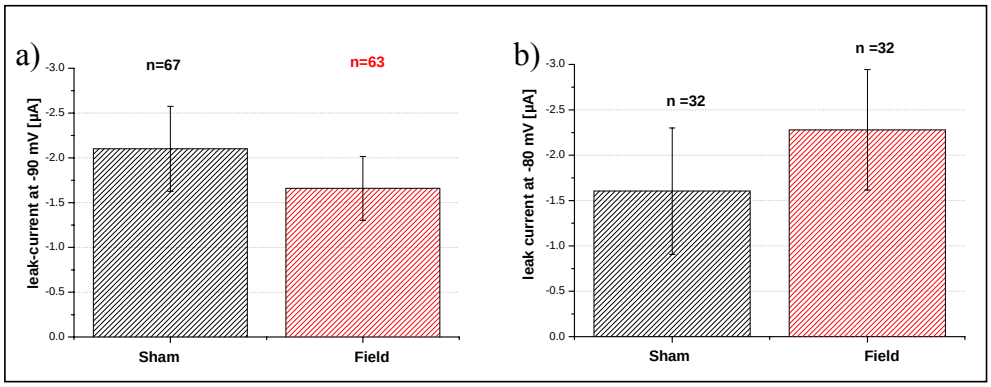


Figure 58. Leak-currents of single oocytes after expression of rCx46- hemi-channels at different holding potential.**a**) Leak current amplitudes at holding potential –90 mV of sham and ELF-EMF (2.3 mT, 16 hours) continuously exposed oocytes. **b**) Leak-currents at holding potential –80 mV of sham and ELF-EMF (1.0 mT, 16 hours, 5 min on/10 min off) exposed oocytes. Data are given as mean ± s.e.m. n denotes the number of different oocytes.

No significant influence of ELF-EMF on the number of expressed and conducting hemi-channels composed of rCx46 in oocytes.

The expression level of hemi-channels composed of rCx46 was estimated from the number of conducting hemi-channels which corresponds to the mean steady-state current amplitude (I_{ss}) and/or the maximal membrane conductance G_{max} at depolarising test potentials. Expression of endogenous hemi-channels was suppressed by injection of the corresponding anti-sense. Figures 59a, 60a and 61a show the relationship I_{ss} vs ($V-V_{rev}$) for different oocytes for the selected exposure condition. For clearer presentation G(V) was normalised to a maximal value of G(V) which is obtained at V = +50 mV (Figures 59b, 60 and 61b). A significant influence of ELF-EMF exposure on the number of expressed and conducting hemi-channels of rCx46 could not be read from the analysed data. This finding is also reflected in the frequency distribution of G_{max} for sham and exposed oocytes (Figure 59c).

No significant influence of ELF-EMF on the voltage-dependent gating properties of rCx46 expressing oocytes

A possible effect of ELF-EMF exposure on the voltage dependent gating properties of conducting hemi-channels of rCx46 was analysed. I_{ss} vs ($V-V_{rev}$) was measured and the corresponding relation G(V) vs ($V-V_{rev}$) derived. The latter relationship could be fitted by a simple Boltzmann equation. The fit yields as essential parameter the number of apparent equivalent voltage gating charges z. z was determined for the different exposure conditions. As can be read from Figure 59a) a significant effect of ELF-EMF exposure on the voltage-dependent gating which is reflected in the apparent number of equivalent charges z (Figure 59b) was not observed.

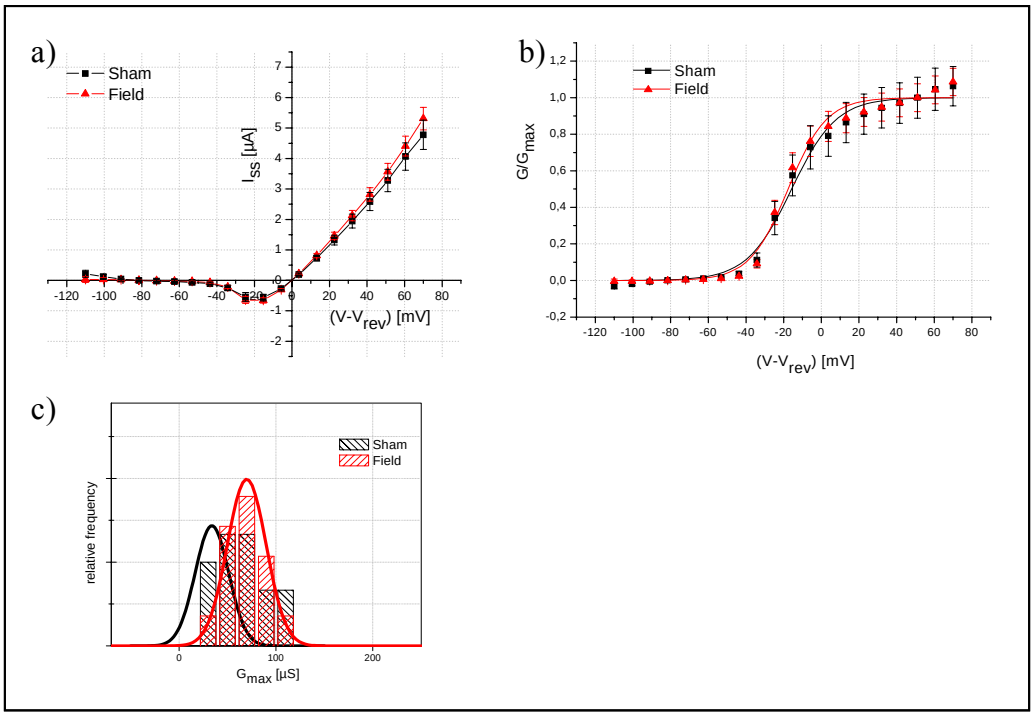


Figure 59. Voltage dependence of macroscopic rCx46-mediated membrane current and corresponding conductance after continuous ELF-EMF exposure for 16 hours at 2.3 mT. **a)** Mean ± s.e.m. of leak subtracted steady-state current amplitudes (I_{ss}) as function of (V-V_{rev}) in the absence and presence of ELF-EMF exposure (sham ■, n = 14; field ▲, n=15). **b**) Mean ± s.e.m. of corresponding normalised membrane conductance G/G_{max} = G(V)/G(V=+50 mV) as function of (V-V_{rev}) in the absence (■, n = 14) and presence of ELF (▲, n = 15). The solid lines show fits of the data by a simple Boltzmann function (for details see Materials and Methods). The derived parameters are z(sham) = 2.11 ± 0.17; z(field) = 2.45 ± 0.23; $V_{1/2}$(sham) = (-15.76 ± 1.06) mV; $V_{1/2}$(field) = (-17.66 ± 1.07) mV. **c**) Distribution of relative frequency of G_{max} = G(V=+50 mV) in the absence (n = 14) and presence (n = 15) of ELF-EMF. Solid lines present the Gauss distribution with the parameters: $G_{max, mean}$(sham)=64.2µS, corresponding standard deviation (sd) sd(sham) = 7.8 µS and $G_{max, mean}$(field) = 69.8 µS, sd(field) = 20.0 µS

To investigate an influence of field intensity on the results presented above, the experiments were repeated at 1.0 mT and the EMF-ELF was intermittently applied (5 min on/10 min off) for 16 hours. The corresponding results are given in Figure 60. Again, a significant effect of ELF-EMF on the number of expressed hemi-channels of rCx46 (Figure 59a) as well as their voltage dependent gating properties was not observed (Figure 60b).

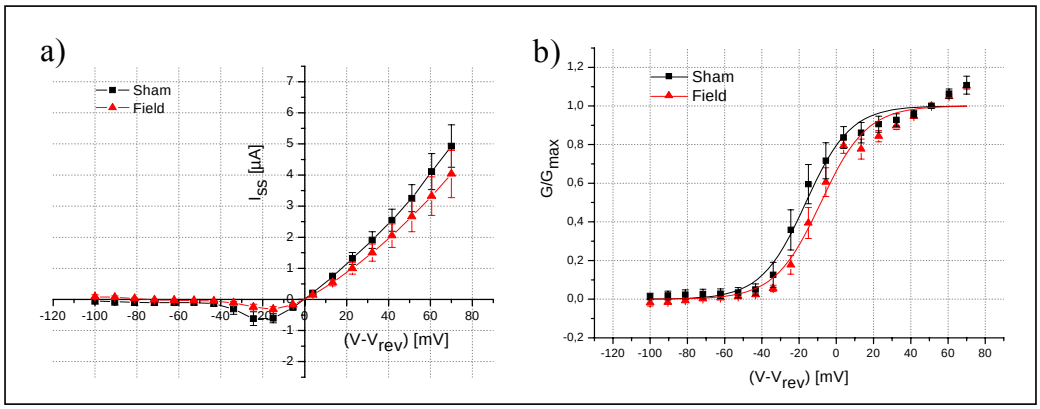


Figure 60. Voltage dependence of rCx46-mediated membrane currents and corresponding membrane conductance after intermittent ELF-EMF exposure (5 min on/10 min off) for 16 hours at 1.0 mT. **a)** Mean ± s.e.m. of leak subtracted steady-state current amplitudes (I_{ss}) as function of (V-V_{rev}) in the absence and presence of ELF-EMF exposure (sham ■, n = 5; field ▲, n = 5). **b)** Mean ± s.e.m. of normalised membrane conductance G/G_{max} = G(V)/G(V = +50 mV) as function of driving voltage (V-V_{rev}) in the absence (■, n = 5) and presence of ELF-EMF (▲, n = 5). The solid lines present fits of the data by a simple Boltzmann function, respectively (for details see Materials and Methods). The derived parameters are z(sham) = 2.08 ± 0.19; z(field) = 2.02 ± 0.20; $V_{1/2}$(sham) = (-16.39 ± 1.29) mV; $V_{1/2}$(field) = (-8.32 ± 1.38) mV

In a further series of experiments ELF-EMF at an intensity of 2.3 mT was intermittently (5 min on/10 min off) applied for 16 hours. The corresponding results are given in Figure 61. A significant effect of ELF-EMF exposure on the number of expressed hemi-channels of rCx46 (Figure 61a) was not observed. The data indicate a decrease of z after ELF-EMF exposure which appears not be significant (see legend of Figure 61b).

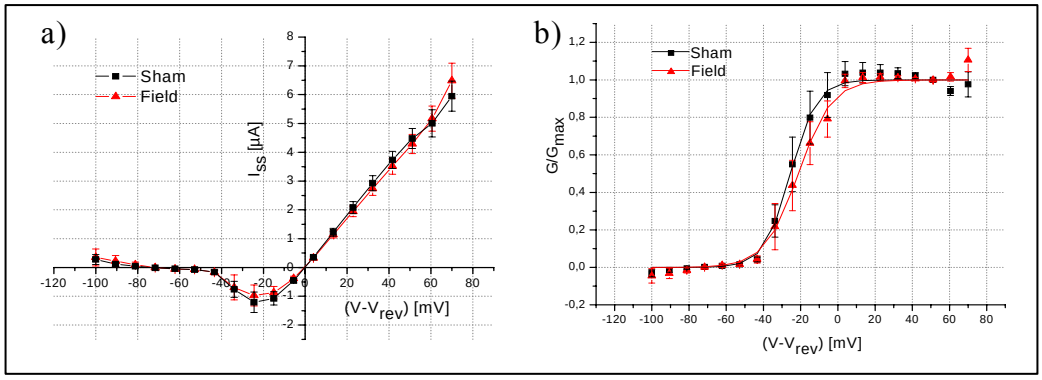


Figure 61. Voltage dependence of macroscopic rCx46-currents (I_{ss}) and corresponding membrane conductance (G) after intermittent exposure (5 min on/10 min off) for 16 hours at 2.3 mT. **a)** Mean ± s.e.m. of leak subtracted steady-state current amplitudes (I_{SS}) as function of (V-V_{rev}) in the absence and presence of ELF-exposure (sham ■, n = 4; field ▲, n = 5). **b)** Mean ± s.e.m. of normalised membrane conductance G/G_{max} = G(V)/G(V = +50 mV) as function of (V-V_{rev}) in the absence (■, n = 4) and presence of ELF-EMF(▲, n = 5). The solid lines show fits of the data by a simple Boltzmann function (see Material and Methods). The derived parameters are z(sham) = 3.54 ± 0.26; z(field) = 2.77 ± 0.24; $V_{1/2}$(sham) = (-25.51 ± 0.59) mV; $V_{1/2}$(field) = (-21.31 ± 0.88) mV

For a more detailed analysis of the voltage dependent gating properties the kinetics of rCx46- mediated current activation was considered. The time dependent current activation could be described by a sum of two exponential functions: $I(t) = a_0 + a_1*\exp(1-\exp(-t/\tau_1)) + a_2*\exp(1-\exp(-t/\tau_2))$. The corresponding time constants of activation τ_1 and τ_2 were obtained from corresponding fits to the experimental data and the

results are presented in Figures 62a-c. The figure indicates that ELF-EMF exposure does not influence the voltage dependent time constants of channel activation significantly.

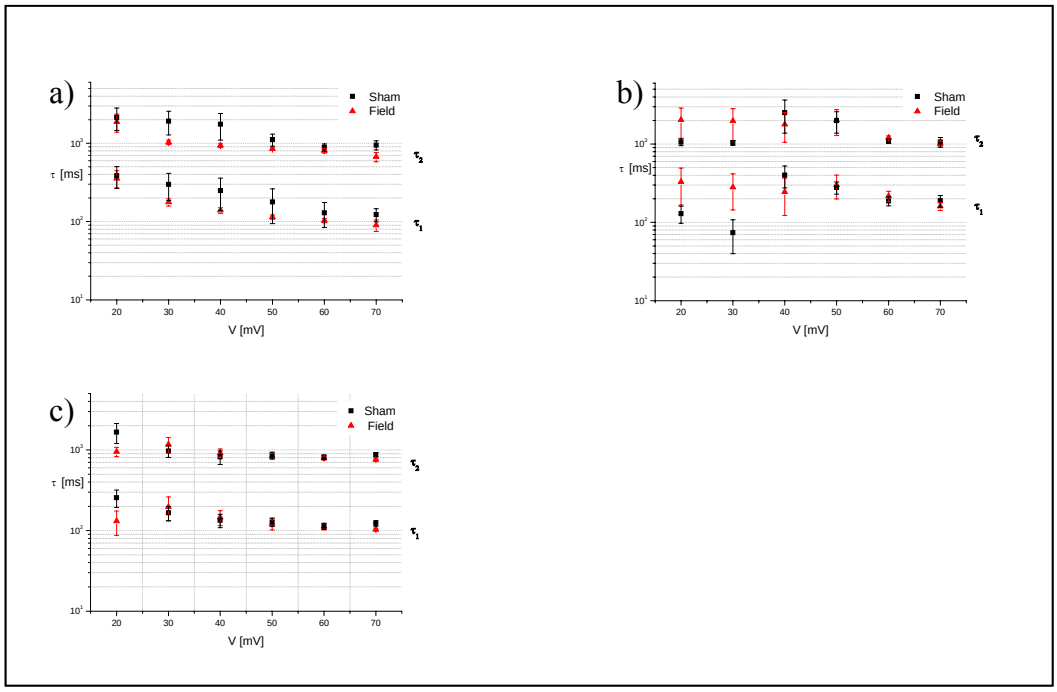


Figure 62. Time constants of voltage-dependent current activation. Time constants of current activation were plotted as function of voltage. Each point represents mean ± s.e.m of five different oocytes. The time constants of activation were obtained by fitting the time course of current activation by a sum of two exponential functions (see text). **a**), **b)** and **c)** present the time constants τ_1 and τ_2 at different exposure conditions: **a)** 2.3 mT, 16 hours continuous, **b)** 2.3 mT, 16 hours intermittent (5 min on/10 min off) and **c)** 1.0 mT, 16 hours intermittent (5 min on/10 min off).

No significant influence of ELF-EMF on the reversal potential of rCx46-mediated membrane current in oocytes.

Finally, the reversal potential V_{rev} of the rCx46-mediated membrane current was considered at different exposure conditions (Figure 63). The reversal potential is mainly determined by the expressed and conducting hemi-channels composed of rCx46, but also includes the contribution of all electrogenic transport systems. A field induced shift of the reversal potential would indicate a change of the intrinsic voltage sensor of the channel of by variation of the intracellular ion composition. No significant effect on the reversal potential of rCx46-mediated membrane current could be observed.

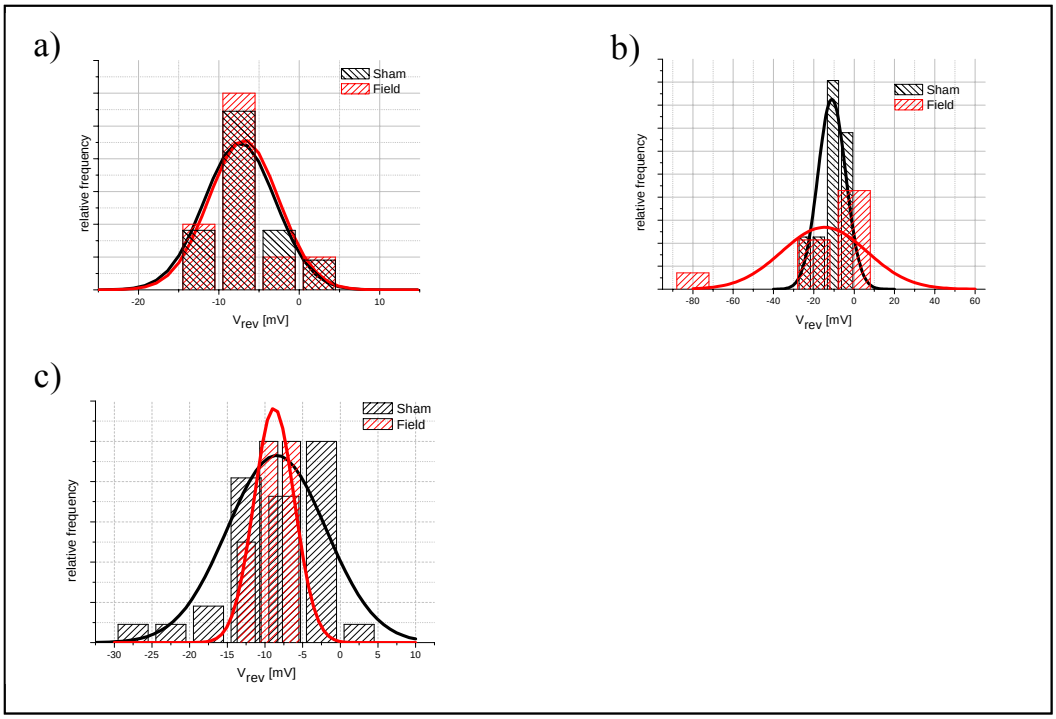


Figure 63. Relative frequency of reversal potential (V_{rev}) of conducting hemi-channels composed of rCx46 for different exposure conditions during an expression period of 16 h. The data were derived from the corresponding experiments given in Fig. 59. **a)** 1.0 mT, intermittent exposure: n = 11 for sham exposed and n = 10 for ELF-EMF exposed oocytes; **b)** 2.3 mT, permanent exposure: n = 18 for sham exposed and n = 20 for ELF-EMF exposed oocytes; **c)** 2.3 mT intermittent exposure: n =5 for sham exposed and n = 4 for ELF-EMF exposed oocytes. Solid lines present the corresponding Gauss distributions using the parameters mean of relative frequency ($V_{rev,\,mean}$) and standard deviation (sd): **a)** $V_{rev,\,mean}$(sham) = -6.9 mV, sd(sham) = 4.4 mV, $V_{rev,\,mean}$ (field) = -7.4 mV, sd(field) = 4.4 mV; **b)** $V_{rev,\,mean}$(sham) = -11.1 mV, sd(sham) = 6.9 mV, $V_{rev,\,mean}$(field) = -14.8 mV, sd(field) = 2.1 mV; **c)** $V_{rev,\,mean}$(sham) = -8.8 mV, sd(sham) = 2.1 mV, $V_{rev,\,mean}$(field) = -8.5 mV, sd(field) = 0.7 mV

A slight but not significant influence of ELF-EMF on the gating properties of hemi-channels expressed in Xenopus oocytes dependent on the external calcium concentration was observed.

The expression level of rCx46 in single were characterised by detailed biophysical analysis of corresponding voltage-jump current relaxation experiments. In parallel the gating by external Ca^{2+} concentration was characterised. A significant influence on the rCx46 mediated membrane conductance, the corresponding half-activation voltage ($V_{1/2}$) and the number of apparent equivalent gating charges (z) of the rCx46-hemi-channels in exposed and sham-exposed *Xenopus laevis* oocytes could not be observed for intermittently applied ELF-EMF at 50 Hz powerline signal (1.0 mT, 5 min on/10 min off) after an exposure time of 14 hours, 17 hours and 20 hours, respectively. Since it is known that external calcium significantly modulates the voltage dependent gating behaviour of expressed hemi-channels composed of rCx46, the experiments were repeated at various external calcium concentrations. The results indicate an influence by ELF-EMF exposure, but the differences are not significant (Figure 64). The membrane conductance and the gating parameters of exposed oocytes expressing rCx46 are smaller than those of sham exposed cells after an exposure time of 14 hours and 20 hours, respectively.

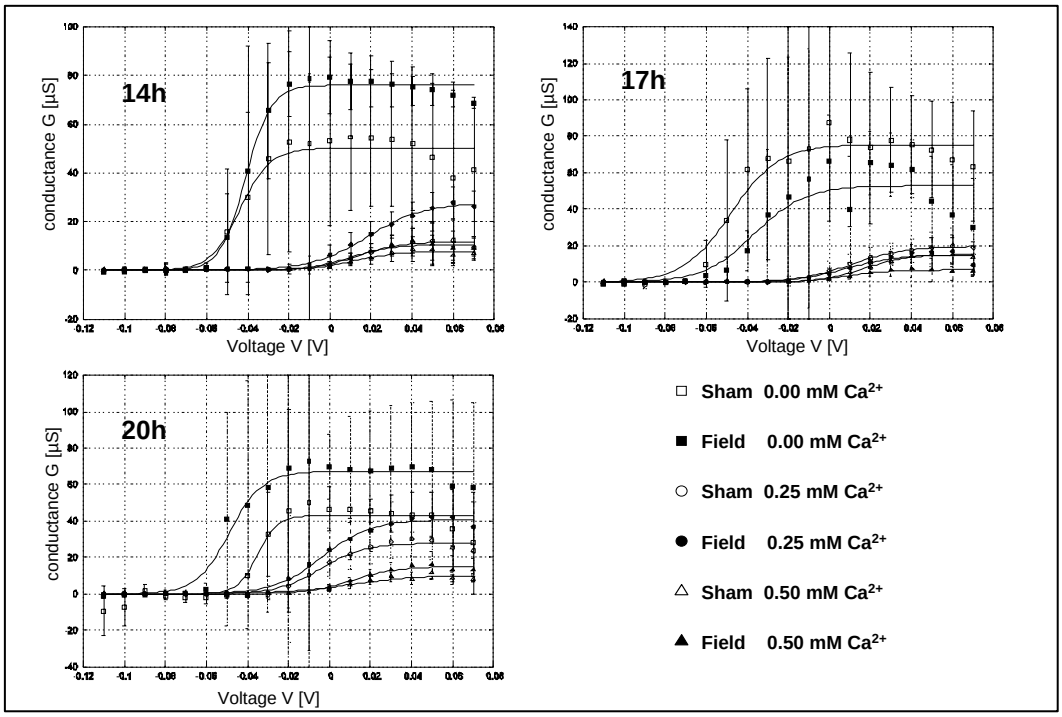
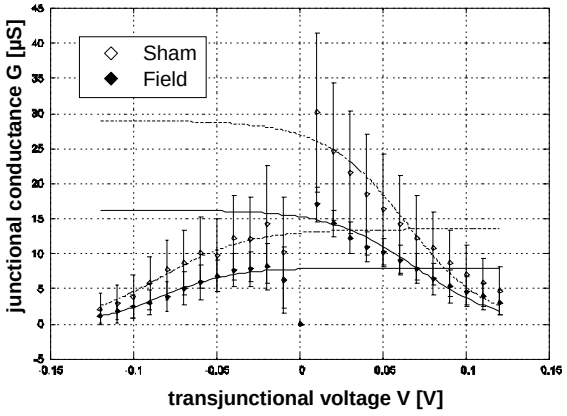


Figure 64. Conductance of hemi-channels composed of rCx46 expressed in oocytes after intermittent exposure (5 min on/10 min off) for 14 hours, 17 hours and 20 hours at 1.0 mT in the presence of 0.0 mM (n = 2-6), 0.25 mM (n = 2-4) and 0.5 mM (n = 2-5) Ca^{2+} in the bath. Closed symbols denote results of exposed oocytes and open symbols those of sham exposed oocytes.

ELF-EMF did not significantly affect the results of electrophysiological recordings of paired Xenopus oocytes.

The voltage-clamp experiments were repeated using paired oocytes. Paired oocytes expressing rCx46 form cell-to-cell channels (gap junctions) by head-to-head association of two hemi-channels which results in an increase of transjunctional conductance (G) between paired oocytes. Paired oocytes were intermittently ELF-EMF exposed (5 min on/10 min off) for 8 hours at 50 Hz powerline signal of 1.0 mT. The half-activation voltage ($V_{1/2}$) and the number of apparent equivalent gating charges (z) derived from the voltage-gating of junctional conductance of paired oocytes expressing rCx46 showed no significant change by ELF-EMF exposure (Figure 65). But the conductance of exposed paired oocytes is smaller than the conductance of sham exposed cell pairs. This finding is not significant on the basis of the 3 paired oocytes analysed so far.



polarity of trans-junctional voltage	$G_{\pm 70}$ [µS]	$V_{1/2}$ [mV]	z
	Sham		
negative	13.53	-82.94	0.96
positive	28.97	69.32	-1.03
	Field		
negative	08.00	-82.54	1.19
positive	16.27	68.94	-0.99

Figure 65. Mean junctional conductance (G) of paired oocytes expressing cell-to-cell channels composed of rCx46 as function of transjunctional voltage. The table summarizes the maximal conductance $G_{\pm 70}$ = G(V=±70 mV) and the voltage-dependent gating parameters: the half-activation voltage ($V_{1/2}$) and number of apparent equivalent gating charges (z) of cell-to-cell channels after intermittent exposure (5 min on / 10 min off) for 8 hours at 1.0 mT, 50 Hz powerline (closed symbol: exposed cell pairs (n = 3); open symbol: sham exposed cell pairs (n = 3)). The parameter values were obtained by fitting the experimental data of G vs. V by a simple Boltzmann-distribution.

No significant influence of ELF-EMF on gap junctional coupling of rat granulosa cells was observed.

Gap junctional coupling by cell-to-cell channels of pairs of cultured granulosa cells was recorded after continuous exposure to ELF-EMF of 2.3 mT for 30 min. Figure 66a shows the maximal gap junctional conductance in the absence and presence of ELF-EMF exposure. The data were obtained as function of days in culture after passage, respectively. The corresponding mean gap junctional conductance of sham- and field-exposed cell all pairs is given in Figure 66b. No significant influence of ELF-EMF exposure on gap junctional coupling of rat granulosa cells was found.

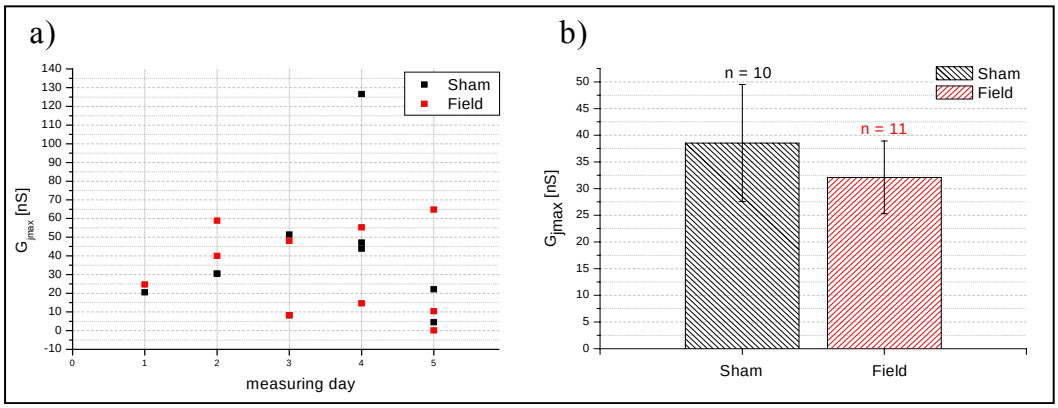


Figure 66. Gap junctional coupling of rat granulosa cell pairs as function of culture time in the presence and absence of ELF-EMF. **a.)** Maximal gap junctional conductance G_{jmax} of cultured pairs of granulosa cells in the absence (■, n = 10) and presence of ELF (■, n = 11) as function of time in culture. ELF-EMF was continuously applied with 2.3 mT for 30 min at room temperature, respectively. Measurements were performed by application of the double whole-cell patch clamp technique. **b**.) Mean ± s.e.m. of gap junctional conductance measured at 1 to 5 days (see a)), in the absence (n = 10) and presence of ELF-EMF (n = 11). n denotes the number of different cell-pairs.

An effect of ELF-EMF on cytoplasmic free calcium of cultured human fibroblasts and granulosa cells of rats was not observed.

After exposure of fibroblasts for 5, 6, 7, 9, 10 and 11 hours to ELF-EMF $[Ca^{2+}]_i$ was recorded. Measurement of $[Ca^{2+}]_i$ was started 10 min after end of exposure and recorded up to 40 min under exposure –free incubation conditions. In Figure 67 $[Ca^{2+}]_i$ was followed after exposure for 11 h (Figure 67a) and 15h (Figure 67b). During the presented recording time no significant change of $[Ca^{2+}]_i$ was observed. The described experiments were repeated for a cultured granulosa cell line (not shown). The observed variability in the time course of $[Ca^{2+}]_i$ of some sham- and field exposed cells seems not to be significant. As in the case of fibroblasts a long-lasting influence of ELF-EMF on the time course and amplitude of $[Ca^{2+}]_i$ was not observed for cultured rat granulosa cells. For clearer presentation the results of $[Ca^{2+}]_i$ recorded for fibroblasts and rat granulosa cells are summarised in Table 9.

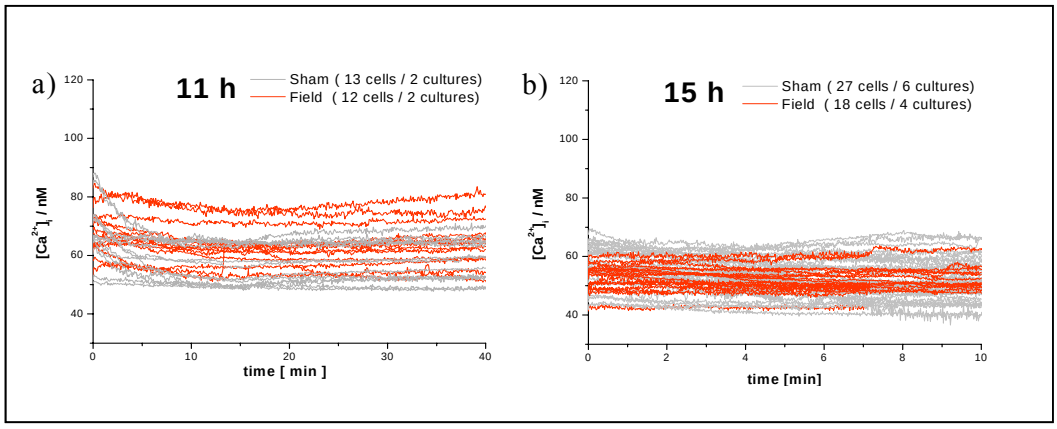


Figure 67. Time course of $[Ca^{2+}]_i$ in fibroblasts after ELF-EMF exposure at 50 Hz sinusoidal, 1.0 mT, intermittent (5 min on/10 min off) for **a)** 11 hours and **b)** 15 hours exposure time (grey curves denote sham- and red curves field-exposure)

Table 9. Summary of $[Ca^{2+}]_i$ data obtained on cultured fibroblasts and rat granulosa cells after ELF-EMF (5 min on/10 min off, sinusoidal 50 Hz, 1.0 mT) exposure

cell system	exposure time	7 h	9 h	11 h	15 h	17 h
Fibroblasts	sham	35 cells / 6 cultures	27 cells / 5 cultures	13 cells / 2 cultures	27 cells / 6 cultures	56 cells / 7 cultures
	field	37 cells / 6 cultures	15 cells / 3 cultures	12 cells / 2 cultures	18 cells / 4 cultures	14 cells / 3 cultures
		no ELF-EMF effect	**no ELF-EMF effect**	**no ELF-EMF effect**	**no ELF-EMF effect**	**no ELF-EMF effect**
Granulosa	**exposure time**	**4 h**	**5 h**	**6.5 h**	**7.75 h**	
	sham	11 cells / 1 culture	54 cells / 5 cultures	60 cells / 5 cultures	51 cells / 3 cultures	
	field	19 cells / 2 cultures	57 Cells / 5 cultures	47 cells / 3 cultures	7 cells / culture	
		no ELF-EMF effect	**no ELF-EMF effect**	**no ELF-EMF effect**	**no ELF-EMF effect**	
ELF-EMF stimulation: 50 Hz, sinusoidal, 1 mT (5 min on / 10 min off)						

In a further series of experiments the cells were exposed to an additional stressor added to the bath after the end of ELF-EMF exposure. Figure 65 shows the time course of $[Ca^{2+}]_i$ of fibroblasts during an additional exposure to 200 µM H_2O_2 in the bath. No significant effect on $[Ca^{2+}]_i$ could be found during a consecutive treatment by H_2O_2. Application of another stress condition like cell- depolarisation by high external KCl (30 mM) also did not affect the time course and amplitude of $[Ca^{2+}]_i$ for ELF-EMF exposed cells (data not shown).

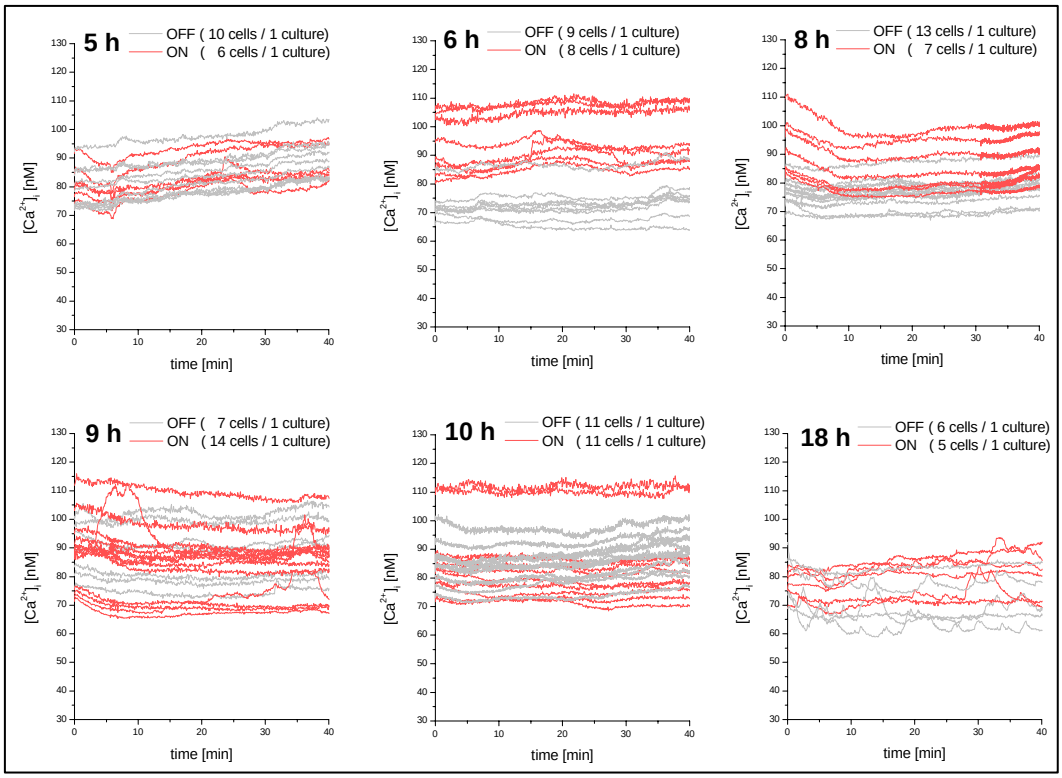


Figure 68. Time course of $[Ca^{2+}]_i$ in fibroblasts after end of ELF-EMF (5 min on/10 min off, 50 Hz sinusoidal, 1.0 mT) exposure for 5 , 6, 8, 9, 10 and 18 hours which was followed by an addition of 200 µM H_2O_2 to the bath for further 10 min (grey curves denote sham- and red curves field-exposure). For further details see text.

A summary of $[Ca^{2+}]_i$ measurements on fibroblasts and rat granulosa cells after application of ELF-EMF followed by addition of H_2O_2 to the bath is given in Table 10. $[Ca^{2+}]_i$ was recorded in the presence of 200 µM H_2O_2.

Table 10. Summary of $[Ca^{2+}]_i$ data obtained on cultured fibroblasts and rat granulosa cells after ELF-EMF (5 min on/10 min off, sinusoidal 50 Hz, 1.0 mT) exposure followed by addition of H_2O_2 to the bath.

cell system	stimulation	exposure time	5 h	6 h	8 h	9 h	10 h	18 h
Fibroblasts	ELF-EMF and 200 µM H_2O_2	sham	10 cells / 1 culture	9 cells / 1 culture	13 cells / 1 culture	7 cells / 1 culture	11 cells / 1 culture	6 cells / 1 culture
		field	6 cells / 1 culture	8 cells / 1 culture	7 cells / 1 culture	14 cells / 1 culture	11 cells / 1 culture	5 cells / 1 culture
			no ELF -EMF effect	no ELF -EMF effect	no ELF -EMF effect	no ELF -EMF effect	no ELF -EMF effect	no ELF -EMF effect
	ELF-EMF and 30 mM KCl	**exposure time**	**6.5 h**	**7.5 h**	**8.5 h**	**9.5 h**		
		sham	7 cells / 1 culture	7 cells / 1 culture	6 cells / 1 culture	7 cells / 1 culture		
		field	5 cells / 1 culture	5 cells / 1 culture	10 cells / 1 culture	8 cells / 1 culture		
			no ELF -EMF effect	no ELF -EMF effect	no ELF -EMF effect	no ELF -EMF effect		
Granulosa	ELF-EMF and 200 µM H_2O_2	**exposure time**	**5 h**	**6 h**	**7 h**	**8 h**	**18 h**	
		sham	14 cells / 1 culture	14 cells / 1 culture	20 cells/ 2 cultures	14 cells / 1 culture	14 cells / 1 culture	
		field	5 cells / 1 culture	5 cells / 1 culture	10 cells / 1 culture	8 cells / 1 culture	14 cells / 1 culture	
			no ELF -EMF effect	no ELF -EMF effect	no ELF -EMF effect	no ELF -EMF effect	no ELF -EMF effect	
ELF-EMF stimulation: 5o Hz, sinusoidal, 1 mT, 5 min on / 10 min off								

The volume regulatory response of granulosa cells appeared not to be influenced by ELF-EMF.

The volume regulatory response of cultured granulosa cells was studied after application of a hypotonic shock followed by a hypertonic shock. For clearer presentation for each experiment the volume change (v(t)–v(t=0)) of 10 cells was analysed as function of time and normalised to the maximal value v_{max}, respectively. As exposure period again 18 h were selected, since after this exposure period rat granulosa cells show the maximal response on the genomic level. The volume regulatory behaviour of rat granulosa cells appears not to be influenced by ELF-EMF. In addition, there was no significant difference between exposed and sham exposed cells for hypotonic (Figure 69) as well as hypertonic conditions (not shown). The volume analysis was started 15 min after end of ELF-EMF exposure.

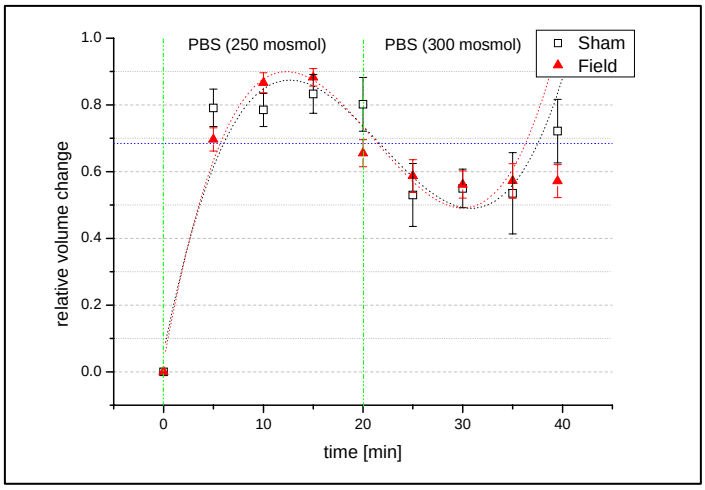


Figure 69. Relative volume change of granulosa cells (GFSHR-17) after addition of a hypotonic solution at t = 0. Prior to this treatment the cells were sham exposed (n = 7) or ELF-EMF exposed (n = 24) for 18 hours at 50 Hz, 1.0 mT, (5 min on/10 min off)). The mean ± s.e.m. is given, respectively.

3.1.4.5 Whole-genome analysis of various cell lines exposed to ELF-EMF (Participant 12)

Altogether, 58 whole-genome analyses of 10 different cell lines (sham-exposed cells and control cells) were performed (Table 1). After primary data analysis, we only worked on genes which were reproducibly regulated in several experiments (see materials and methods) and which belonged to certain gene families (Table 11). We defined gene families which are potentially relevant for the cellular answer on EMF exposure: signal transduction, ion/electron transport, metabolism of energy/proteins, cell proliferation/apoptosis, immune answer/inflammation and extracellular matrix/ cytoskeleton. Each gene family was sub-divided in subgroups again, e.g. GTP proteins in the signal transduction family (Tables 11, 12). In a first step, we did not go into single genes, but simply counted genes up- or down-regulated in the different gene families. The total number of regulated genes in a certain gene family is not very meaningful, because the sizes of the gene families are of course very different. Therefore, the total numbers of genes on the human array belonging to a gene family are shown in the first column of Tables 11 and 12. Although a single gene might appear in different categories (e.g. all small G proteins are GTP binding proteins), the Tables give a good overview on what might happen in the cells after EMF exposure on the molecular level.

In human fibroblasts (Participant 3), a number of G proteins and calcium associated proteins involved in signal transduction seem to be strongly regulated. Genes involved in adhesion of cells and cytoskeletal genes appear strongly regulated in several hybridizations, although the variances in numbers between the experiments are high (Table 11).

The fibroblast experiments (Participant 3) were also assessed by bio-statistics (Participant 8: Dr. Remondini, Table 12): Mitochondrial and ribosomal genes appeared strongly regulated, also Ca-related genes, cell cycle, apoptosis, extracellular matrix, and the cytoskeleton. The overall number of significantly regulated genes is higher in the ELF-EMF treated fibroblasts than e.g. in endothelial cells

exposed to RF-EMF (Participant 6). This was expected, since the number of regulated genes in fibroblasts after ELF-EMF exposure is pretty uniform in the non-statistically evaluated gene numbers (compare Table 12 with Table 11). From the experiments with SY5Y human neuroblastoma cells (Participant 11) and mouse embroyonic stem cells (Participant 4) it was not possible to extract bio-statistically significant data.

In detail, the following genes were extracted by bio-statistics so far:

Actin associated proteins (belong to cytoskeleton):
- Caldesmon (tropomysin binding, actin binding. Activation of ERK MAP kinases lead to phosphorylation of caldesmon. Regulatory protein of the contractile apparatus): down-regulated (fibroblasts, participant 3).
- Gamma-actin: down-regulated (fibroblasts, Participant 3).
- "coactosin-like": down-regulated (fibroblasts, Participant 3).
- "actin-binding": down-regulated (fibroblasts, Participant 3).
- "procollagen-proline 2": down-regulated (fibroblasts, Participant 3).
- "actin modulating activity": up-regulated (fibroblasts, Participant 3).
- "actin-binding, calcium ion binding": down-regulated (fibroblasts, Participant 3).
- CD2-associated protein, actin binding: down-regulated (fibroblasts, Participant 3).
- Tropomodulin 3: actin binding down-regulated (fibroblasts, Participant 3).

Calcium (Ca2+)-associated proteins:
- protein phosphatase 4: down-regulated (fibroblasts, Participant 3).
- Thrombospondin (cell adhesion): down-regulated (fibroblasts, Participant 3).
- "EGF-containing fibulin-like…" (cell adhesion): down-regulated (fibroblasts, Participant 3).
- matrix metalloproteinase 2 MMP 2 (extracellular matrix, collagen metabolism): down-regulated
- follistatin (extracellular matrix, heparin binding): down-regulated (fibroblasts, Participant 3).
- SPARC (extracellular matrix, collagen binding): down-regulated (fibroblasts, Participant 3).
- ("myosin light polypeptide": up-regulated (fibroblasts, Participant 3).
- ("hypothetical protein": up-regulated (fibroblasts, Participant 3).

Extracellular matrix (ECM):
- thrombospondin (see Ca): down-regulated (fibroblasts, Participant 3).
- "EGF-containing…." (see Ca): down-regulated (fibroblasts, Participant 3).
- MMP2 (see Ca): down-regulated (fibroblasts, Participant 3).
- Connective tissue growth factor CTGF (cell adhesion, obviously not influenced by Ca): up-regulated (fibroblasts, Participant 3).
- Collagen XV (obviously not influenced by Ca): up-regulated (fibroblasts, Participant 3).
- Lysyl oxidase (also processed by bone morphogenetic protein 1 BMP1, obviously not influenced by Ca): up-regulated (fibroblasts, Participant 3).

Cytoskeleton (see also actin and calcium-associated proteins):
- "hypothetical protein": down-regulated (fibroblasts, Participant 3).
- "protein phosphatase 4, caldesmon): down-regulated (fibroblasts, Participant 3).
- "SH3 protein interacting with Nck": down-regulated (fibroblasts, Participant 3).
- "in kinesin complex": down-regulated (fibroblasts, Participant 3).

Ion transport:
- "potassium channel activity": down-regulated (fibroblasts, Participant 3).
- SLC12A5 KCl (potassium chloride) transporter: down-regulated (fibroblasts, Participant 3).
- SLC26A3 sulfate porter: down-regulated (fibroblasts, Participant 3).
- "ferric ion binding": down-regulated (fibroblasts, Participant 3).
- (ATP synthase, H+ transport): down-regulated (fibroblasts, Participant 3).
- ("H+ transporter): down-regulated (fibroblasts, Participant 3).
- "iron ion transport": down-regulated (fibroblasts, Participant 3).

Ribosomal proteins:
- 7 ribosomal proteins down-regulated, 3 ribosomal proteins up-regulated, 1 ribosomal protein up-regulated in profile nr.1, down-regulated in profile nr.2 (fibroblasts, Participant 3).

Table 11. Numbers of genes regulated within different gene families

Gene Family	total number of clones in Human Unigene RZPD-2	partner 3 fibroblasts Exp1 ELF up-regulated genes	partner 3 fibroblasts Exp2 ELF up-regulated genes	partner 3 fibroblasts Exp1 ELF down-regulated genes	partner 3 fibroblasts Exp2 ELF down-regulated genes	Gene "Superfamily"
Signal	2528	251	232	296	190	signal transduction
GTP	560	66	73	66	52	signal transduction
Small G	235	31	32	28	27	signal transduction
Jak	23	0	0	5	3	signal transduction
Rab	80	0	9	11	13	signal transduction
Ras	66	10	7	10	5	signal transduction
wnt	5	0	0	0	0	signal transduction
phosphatase	334	39	36	35	26	signal transduction
protein kinase	304	28	29	35	24	signal transduction
phospholipase	72	9	8	10	7	signal transduction
calcium	715	67	72	80	50	signal transduction
calmodulin	131	6	13	17	11	signal transduction
channel	348	31	25	36	22	ion/electron transport
voltage-gated	164	16	13	16	6	ion/electron transport
electron transport	423	52	52	57	37	ion/electron transport
ion transport	501	49	48	45	29	ion/electron transport
metaboli	1241	122	128	135	96	metabolism of energy/proteins
ATP	1234	113	112	157	82	metabolism of energy/proteins
mitochon	574	84	82	70	65	metabolism of energy/proteins
ribosom	254	47	48	32	39	metabolism of energy/proteins
translation	168	30	28	20	13	metabolism of energy/proteins
transcript	1991	201	190	228	136	metabolism of energy/proteins
cell cycle	478	46	52	54	43	cell proliferation/apoptosis/differentiation
apoptos	373	31	37	37	23	cell proliferation/apoptosis/differentiation
differentiat	177	14	21	22	11	cell proliferation/apoptosis/differentiation
immun	390	31	37	38	32	immune answer/inflammation/stress answer
inflamma	184	10	20	24	11	immune answer/inflammation/stress answer
stress	118	12	11	14	13	immune answer/inflammation/stress answer
peroxidase	32	6	5	4	6	immune answer/inflammation/stress answer
heat shock	188	4	7	5	3	immune answer/inflammation/stress answer
DNA repair	154	14	19	19	10	immune answer/inflammation/stress answer
early	8	2	2	0	2	immune answer/inflammation/stress answer
adhesion	573	46	49	53	43	extracellular matrix/cytoskeleton/adhesion
extracellular matrix	226	19	34	31	16	extracellular matrix/cytoskeleton/adhesion
cytosk	529	45	50	47	42	extracellular matrix/cytoskeleton/adhesion
junction	129	11	10	13	10	extracellular matrix/cytoskeleton/adhesion
actin	494	32	32	38	32	extracellular matrix/cytoskeleton/adhesion

Table 12. Numbers regulated genes in different expression profiling experiments (bio-statistical analysis by Dr. Remondini/Participant 8)

Gene Family	total number of clones in Human Unigene RZPD-2	partner 3 fibroblasts ELF up-regulated genes	partner 3 fibroblasts ELF down-regulated genes	Gene "Superfamily"
Signal	2528	0	12	signal transduction
GTP	560	0	2	signal transduction
Small G	235	0	1	signal transduction
Rab	80	0	1	signal transduction
Ras	66	0	1	signal transduction
phosphatase	334	1	2	signal transduction
protein kinase	304	0	1	signal transduction
calcium	715	2	6	signal transduction
calmodulin	131	0	1	signal transduction
channel	348	0	2	ion/electron transport
voltage-gated	164	0	1	ion/electron transport
ion transport	501	0	7	ion/electron transport
electron transport	423	0	3	ion/electron transport
metaboli	1241	0	4	metabolism of energy/proteins
ATP	1234	2	7	metabolism of energy/proteins
mitochon	574	0	5	metabolism of energy/proteins
ribosom	254	3	7	metabolism of energy/proteins
translation	168	3	0	metabolism of energy/proteins
transcript	1991	2	9	metabolism of energy/proteins
cell cycle	478	1	4	cell proliferation/apoptosis/differentiation
apoptos	373	0	4	cell proliferation/apoptosis/differentiation
differentiat	177	0	2	cell proliferation/apoptosis/differentiation
immun	390	0	2	immune answer/inflammation/stress answer
DNA repair	154	0	0	immune answer/inflammation/stress answer
inflamma	184	0	1	immune answer/inflammation/stress answer
adhesion	573	2	3	extracellular matrix/cytoskeleton/adhesion
extracellular matrix	226	2	5	extracellular matrix/cytoskeleton/adhesion
cytosk	529	1	6	extracellular matrix/cytoskeleton/adhesion
actin	494	1	4	extracellular matrix/cytoskeleton/adhesion
junction	129	0	0	extracellular matrix/cytoskeleton/adhesion

3.1.4.6 Summary (Participant 1)

Our data indicate that ELF-EMF may affect the gene and protein expression in various cell systems. This conclusion is based on the following findings:

- ELF-EMF at a flux density of about 2 mT up-regulated the expression of early genes, such as p21, c-jun and erg-1, in p53-deficient mouse embryonic stem cells, but not in healthy wild-type cells suggesting that the genetic background affects the responsiveness of the cells (see 3.1.4.1).
- ELF-EMF at a flux density of 2 mT up-regulated in neural progenitor cells the transcript levels of the GADD45 gene and down-regulated the transcript levels of the bax gene by which the apoptotic process may be modulated (see 3.1.3.1 and 3.1.4.1).
- ELF-EMF at a flux density of 0.8 mT up-regulated the expression of cardiac specific genes in cardiomyocytes derived from embryonic stem cells thus promoting cardiogenesis (see 3.1.4.3).
- ELF-EMF did not affect the expression of neuronal genes in neuroblastoma cells (SY5Y) such as nAchRs, DβH, Phox2a and Phox2b, either at mRNA or protein level (see 3.1.4.2).
- ELF-EMF did not affect either the expression level of conducting hemi-channels composed of rCx46, nor their gating properties by voltage, pH, Ca^{2+} in *Xenopus laevis* oocytes (see 3.1.4.4).
- ELF-EMF appeared to regulate the expression of a series of genes and proteins in human fibroblasts such as mitochondral and ribosomal genes as well as Ca-, cell cycle-, apoptosis-, extracellular matrix-, and cytoskeleton-related genes, although it must be considered that the variances observed between the various experiments was high (see 3.1.4.5).

3.2 Results in RF-EMF research

3.2.1 Genotoxic effects

3.2.1.1 Human HL-60 cell line (Participant 2)

Genotoxic effects of EMF may occur directly either by damage to chromosomes and/or by damage to DNA repair mechanisms. Indirect genotoxic effects may arise by various processes such as generation of oxygen radicals or impairment of radical-scavenging mechanisms. Direct and indirect genotoxic effects of defined RF-EMF were investigated in the human cell line HL-60.

A. Direct genotoxicity

RF-EMF increased the micronucleus frequency and the number in DNA strand breaks in HL-60 cells dependent on the energy of radiation as determined by the cytokinesis-block in vitro micronucleus assay and the Comet assay.

The effect of RF-EMF on the formation of micronuclei (MN) and DNA strand breaks was examined by use of the cytokinesis-block in vitro micronucleus assay and the alkaline Comet assay. To validate the MN assay and to prove the susceptibility of HL-60 cells to physical noxes, cells were exposed to ionising-irradiation. As shown in Figure 70, a dose-dependent induction of micronuclei in HL-60 cells was found for doses of exposure increasing from 0.5 to 3.0 Gy. Cell division was effected by ionising-irradiation at doses ≥1.0 Gy as shown in Figure 71, inferred from the ratio of BNC against mono-, bi-, tri- and tetranuclear cells (in %).

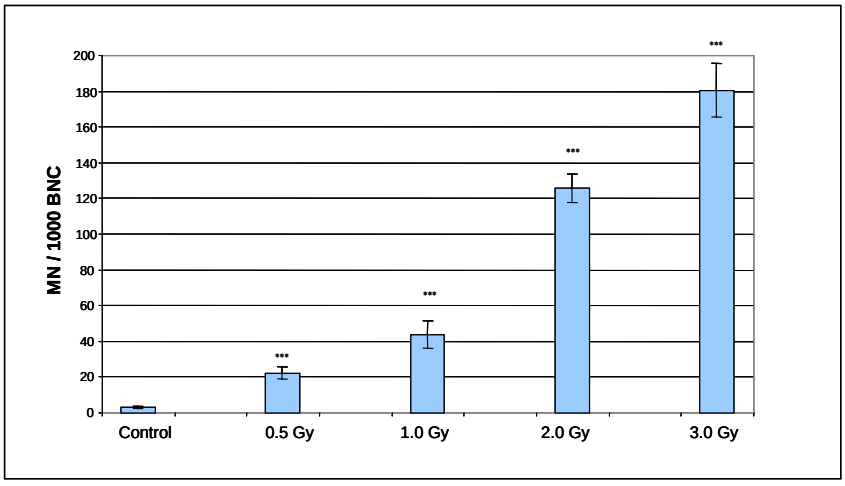


Figure 70. Effect of ionising-irradiation (6 MeV) on micronuclei formation in binucleated HL-60 cells. Each data point is based on at least three independent experiments. Each bar represents the mean ± SD of results obtained in three independent experiments. *** P<0.001 (Student's t-test, two-sided). All in all (mono-, bi-, tri-, and tetranucleated) 15000 cells were analysed.

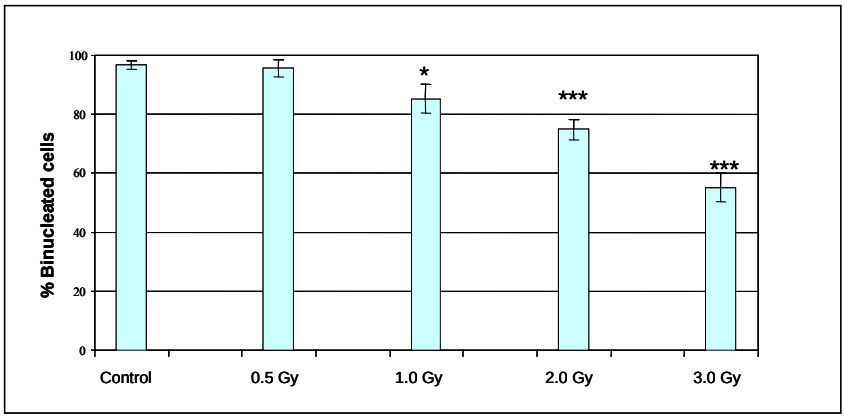


Figure 71. Effect of ionising-irradiation on HL-60 cell division. The number of binucleated cells relative to the number of mono-, bi-, tri-, and tetranuclear cells following ionising-irradiation (6 MeV) of HL-60 cells. Each bar represents the mean ± SD in % of results obtained in three independent experiments; *p<0.05, ***p<0.001 (Student's t-test, two-sided).

In a series of experiments SAR levels ranging from 0.2 W/kg to 3.0 W/kg were examined in order to clarify whether the effects of RF-EMF exposure (1800 MHz, continuous wave, 24 h) on MN frequencies in HL-60 cells are energy dependent (Figure 72). Whereas at SAR of 0.2 W/kg, 1.0 and 3.0 W/kg MN frequencies were not changed in RF-EMF-exposed cells as compared to sham controls and incubator controls, MN frequencies were significantly increased at SAR of 1.3 W/kg and above. The maximum increase was noted at a SAR of 1.3 and 1.6 W/kg. This effect was approximately 66 % of the effect observed after 0.5 Gy ionising-irradiation (6 MeV, exposure time: 5.2 s). At a SAR of 3.0 W/kg the MN frequency was similar to that found in sham-exposed cells. While MN frequencies of incubator controls were around 3.5, the MN frequency determined after RF-exposure at a SAR of 1.3 W/kg was 13.3 (approximately 3.8 fold higher). The MN frequency determined in cells after exposure to ionising-irradiation (0.5 Gy, exposure time: 5.2s), used as a positive control, was 22.3 ± 3.5 (n=3; 6.3 fold increase compared to control).

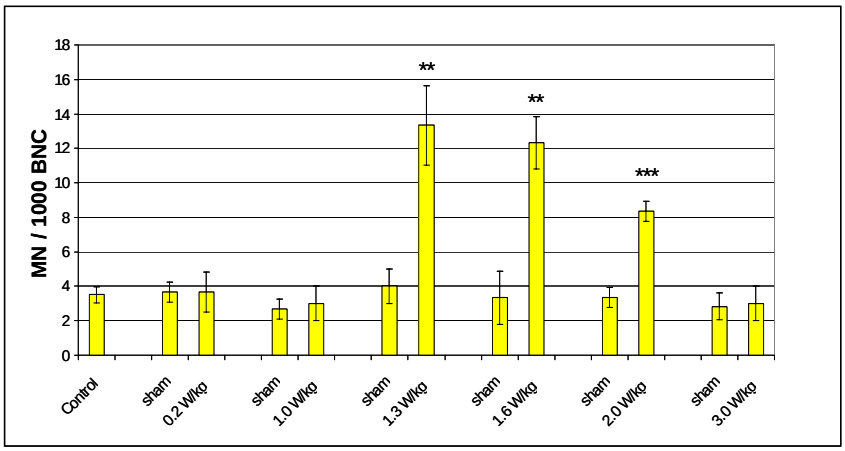


Figure 72. Micronucleus frequencies in binucleated HL-60 cells after exposure to RF-field (1800 MHz, continuous wave) for 24h ranging from SAR 0.2 to 3.0 W/kg, compared to control and sham-exposure. Each bar represents the mean ± SD of results obtained in three independent experiments (except of control: n = 11). Each data point is based on at least three independent experiments except of the control with 11 independent experiments and on a total number of 11000 (control), 18000 (sham-exposed) and 18000 (RF-exposed) bi-nucleated HL-60 cells. All in all (mono-, bi-, tri-, and tetra-nucleated) 47000 cells were analysed. The micronuclei frequency of BNC after exposure to ionising-irradiation (0.5 Gy, exposure time: 5.2s), which was used as a positive control, were on average 22.3 ± 3.5 (n=3). ** P<0.01; *** P<0.001 (Student's t-test, two-sided).

In order to compare micronuclei induction in cells exposed to RF-fields at different ranges of SAR, the average micronuclei frequencies (MN/1000 BNC) were calculated for the following groups: experiments performed at all SAR tested (range 0.2 W/kg to 3.0 W/kg, number of independent experiments n=18), experiments performed at SAR ranging from 1.0 W/kg to 2.0 W/kg (number of independent experiments n=12), and experiments at SAR of 0.2 W/kg and 1.0 W/kg (number of independent experiments n=6). While the calculated average of MN/1000 BNC in HL-60 cells at a SAR of 0.2 W/kg to 1.0 W/kg was not significantly different from that observed in sham-exposed controls, both groups ranging from 0.2 W/kg to 3.0 W/kg ($p<0.01$) or from 1.0 W/kg to 2.0 W/kg ($p<0.001$) exhibited a significant increase in micronuclei induction after RF-exposure as compared to sham-exposed controls (Figure 74A).

Previous experiments had clearly shown that RF-EMF exposure results in an increase of DNA strand breaks in HL-60 cells. In order to achieve a better understanding of whether these effects are energy dependent, additional experiments were performed applying RF-exposure (1800 MHz, continuous wave, 24h) at SAR of 0.2 W/kg to 3.0 W/kg. As shown in Figure 73 the effect of RF-EMF on DNA strand breaks at these exposure conditions exhibited a similar energy dependency as the effect of RF-EMF on micronucleus formation (Figure 72). RF-EMF exposure at a SAR of 1.0 W/kg and below had no effect on Comet formation in HL-60 cells (expressed as Olive Tail Moment OTM) as compared to control and sham-exposed cells. On the other hand RF-EMF at SAR of 1.3 W/kg and above caused a significant increase in DNA strand breaks. The maximum of this effect was observed at SAR 1.3 W/kg (OTM = 2.20 ± 0.16) and 1.6 W/kg (2.24 ± 0.10). At a SAR of 3.0 W/kg Comet formation in RF-EMF exposed cells (OTM 1.23 ± 0.12) was similar to that observed in sham-exposed cells (OTM 1.18 ± 0.03). While the Olive Tail Moment was around 1.0 in sham-exposed and incubator controls, the OTM determined after exposure at a SAR of 1.3 W/kg was approximately 2.2 fold higher (Figure 73). The OTM determined in cells after exposure to hydrogen peroxide (100 µmol/l, 1h), used as a positive control, was 8.3 ± 1.3 (n=3; 8 fold increase compared to control).

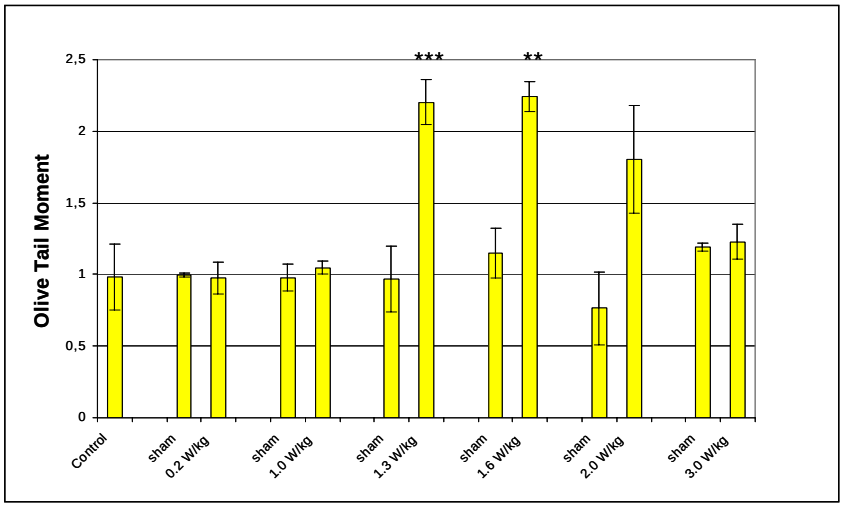


Figure 73. Comet formation in HL-60 cells after exposure to RF-field (1800 MHz, continuous wave) for 24h ranging from SAR 0.2 to 3.0 W/kg, expressed as Olive Tail Moment, compared to control and sham exposure. Each bar represents the mean ± SD of results obtained in at least three (except SAR 1.3 W/kg: n=4) independent experiments. The OTMs of the Comets after exposure to hydrogen peroxide (100 µmol/l, 1 h), which was used as a positive control, were on average 8.3 ± 1.3 (n=3). ** $P<0.01$; *** $P<0.001$ (Student's t-test, two-sided).

In order to compare Comet formation in cells exposed at different SAR ranges the average values of the Olive Tail Moments were calculated for the following groups: experiments performed at all SAR tested (range 0.2 W/kg to 3.0 W/kg, number of independent experiments n=18), experiments performed at SAR ranging from 1.0 W/kg to 2.0 W/kg (number of independent experiments n=12), and experiments at SAR of 0.2 W/kg and 1.0 W/kg (number of independent experiments n=6). While the calculated average of OTMs in HL-60 cells at SAR of 0.2 W/kg and 1.0 W/kg was not significantly different from that observed in sham-exposed controls, both groups ranging from 0.2 W/kg to 3.0 W/kg ($p<0.01$) or from 1.0

W/kg to 2.0 W/kg ($p<0.001$) exhibited a significant increase in Comet formation after RF-exposure as compared to sham-exposed controls (Figure 74B).

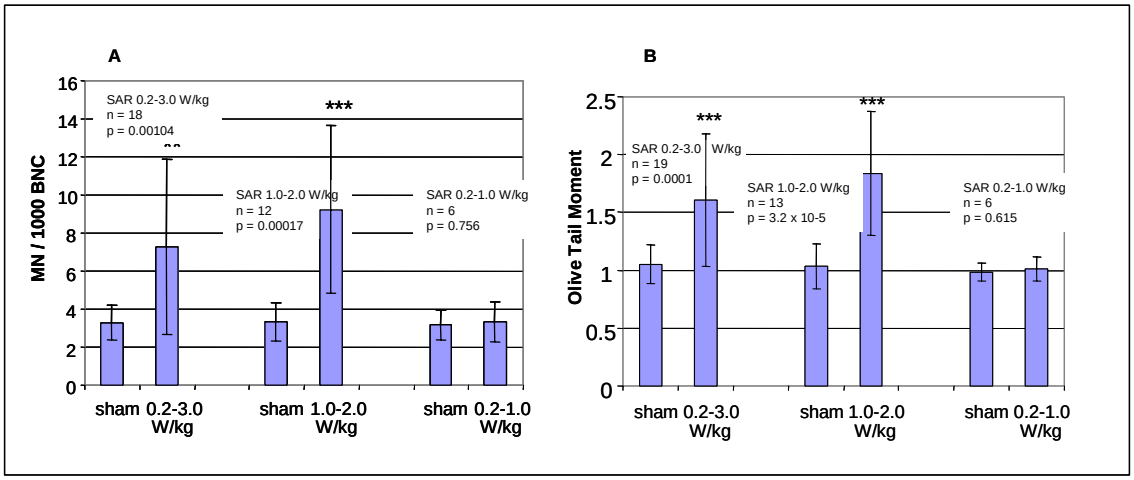


Figure 74. MN induction and Comet formation in HL-60 cells after RF-field exposure (1800 MHz, continuous wave, 24 h) over all SAR groups tested versus total sham, expressed as MN per 1000 BNC (A) and as Olive Tail Moment (B). Each bar represents the mean ± SD of results obtained in indicated number of experiments. ** $P<0.01$; *** $P<0.001$ (Student's t-test, two-sided).

RF-EMF increased the micronucleus frequency and the number of DNA strand breaks in HL-60 cells dependent on the exposure time as determined by the cytokinesis-block in vitro micronucleus assay and the Comet assay.

Using the cytokinesis-block MN assay it was also investigated, whether the duration of exposure of HL-60 cells to RF-fields has an influence on MN induction (Figure 75). Short exposure periods (6 h) caused no or less pronounced effects compared to longer exposure periods of 24 and 72h. The level of the effect on MN frequency noted after RF-EMF exposure for 72h (MN/1000 BNC: 20.22 ± 2.08) was comparable to that observed after 0.5 Gy ionising-irradiation (6 MeV, exposure time: 5.2 s) (MN/1000 BNC: 22.33 ± 2.48).

Furthermore, it was investigated, whether the duration of exposure of HL-60 cells to RF-EMF has an influence on Comet formation (Figure 76). Short exposure periods (2 and 6h) caused less pronounced effects compared to the longer exposure period of 24h. After 72h of exposure Comet formation was similar to that observed after short exposure times (2 and 6h).

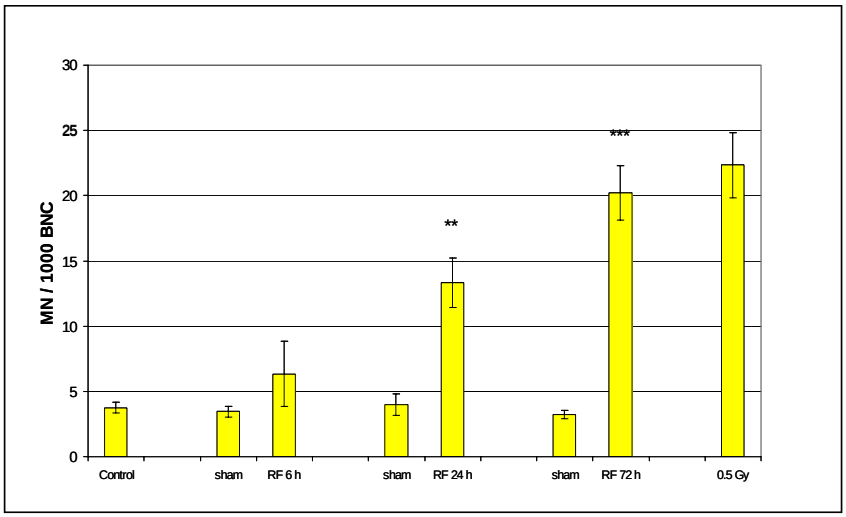


Figure 75. Micronucleus frequencies in binucleated HL-60 cells after exposure to RF-fields (1800 MHz, continuous wave, SAR = 1.3 W/kg) for 6, 24 and 72h, compared to control and sham-exposure. Positive control: 0.5 Gy ionising-irradiation (6 MeV). Each bar represents the mean ± SD of results obtained in three independent experiments (except control: n = 4). Data points are based on a total cell number of 4000 (control), 9000 (sham-exposed), 9000 (RF-exposed) and 3000 (0.5 Gy-exposed, exposure time: 5.2s) binucleated HL-60 cells. All in all (mono-, bi-, tri-, and tetranucleated) 25000 cells were analysed. ** P<0.01; *** P<0.001 (Student's t-test, two-sided).

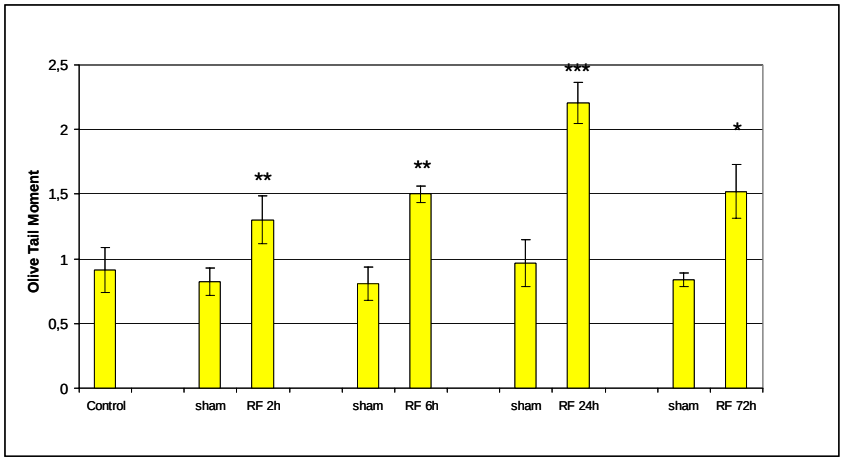


Figure 76. Comet formation in HL-60 cells after exposure to RF-fields (1800 MHz, continuous wave, SAR 1.3 W/kg) for 2, 6, 24 and 72h, expressed as Olive Tail Moment, compared to control and sham-exposure. Each bar represents the mean ± SD of results obtained in at least three (except RF-field exposure, 24h with n=4) independent experiments. * P<0.05; ** P<0.01; *** P<0.001 (Student's t-test, two-sided).

The effects of RF-EMF on genomic integrity of HL-60 cells were exposure-signal-dependent as determined by the cytokinesis-block in vitro micronucleus assay and the Comet assay.

In a further series of experiments it was studied, whether different RF-signals (1800 MHz, SAR 1.3 W/kg: continuous wave, C.W., 5 min on/10 min off; GSM-217Hz, GSM-Talk) for 24h are capable to cause MN induction in HL-60 cells (Figure 77) The number of independent experiments was extended to at least three independent experiments for each of the different types of RF-signals at that SAR with the most pronounced effect (SAR 1.3 W/kg). Using the cytokinesis-block MN assay the different RF-signals had similar effects on MN induction as observed following continuous wave exposure. While the MN frequency of continuous wave-exposed cells was 13.33 ± 1.89, the MN frequencies determined after different other RF-exposure signals were 16.11 ± 3.10 (C.W., 5 min on/10 min off), 13.22 ± 2.88 (GSM-

217Hz) and 17.66 ± 1.70 (GSM-Talk). The MN frequency determined in cells after exposure to ionizing-irradiation (0.5 Gy, 6 MeV, exposure time. 5.2 s), used as a positive control, was 22.3 ± 3.5 (n=3; 6.3 fold increase compared to control).

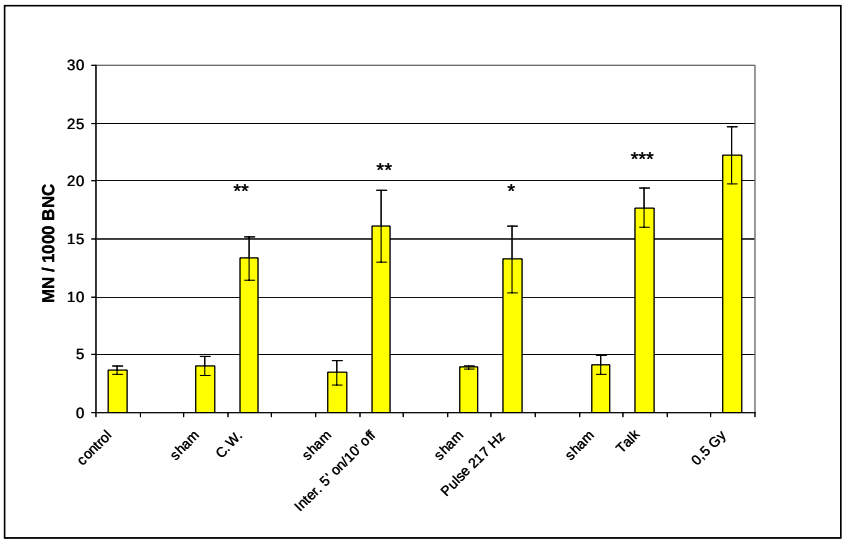


Figure 77. Micronucleus frequencies in binucleated HL-60 cells after exposure to RF-field (1800 MHz, SAR 1.3 W/kg, 24h) compared to control and sham-exposure for different signal modulations. Positive control: 0.5 Gy ionising-irradiation (6 MeV, exposure time: 5.2s). Bars represent means ± SD of three independent experiments (except control = 5). * P<0.01; ** P<0.01; *** P<0.001 (Student's t-test, two-sided). Each data point is based on at least three independent experiments except the control with five and the positive control with three independent experiments and on a total of 5000 (control), 12000 (sham-exposed), 12000 (RF-exposed) and 4000 (0.5 Gy-exposed) binucleated HL-60 cells. All in all (mono-, bi-, tri-, and tetranucleated) 32000 cells were analysed.

Calculation of the average numbers of micronuclei per 1000 BNC determined after exposure (SAR of 1.3 W/kg, 24h) to all RF-signals tested (continuous wave, C.W., 5 min on/10 min off; GSM-217Hz, GSM-Talk) showed an increase in micronuclei induction as compared to sham-exposure at a significant level (number of independent experiments n=12, P<0.001) (Figure 79A).

Using the Comet assay the different RF-signals had similar effects on Comet formation as observed after continuous wave exposure (Figure 78). While the OTM of continuous wave-exposed cells was 2.20 ± 0.16, the OTMs determined after different other RF-exposure signals were 2.11 ± 0.05 (C.W., 5 min on/10 min off), 1.77 ± 0.01 (GSM-217Hz) and 2.26 ± 0.24 (GSM-Talk).

Calculation of the average value of Olive Tail Moments determined after exposure to all RF-signals tested (continuous wave, C.W., 5 min on/10 min off; GSM-217Hz, GSM-Talk) showed a significant increase in Comet formation compared to sham-exposed controls (n=14, P<0.001 (Figure 79B).

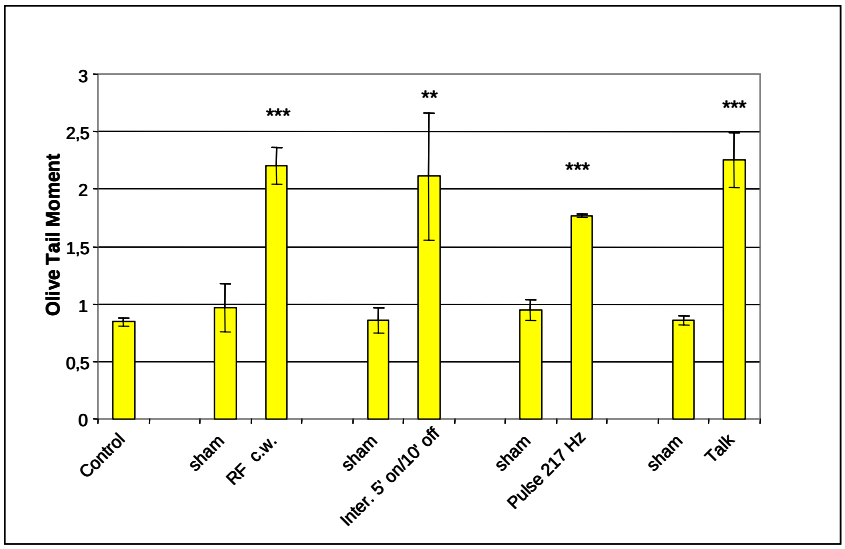


Figure 78. Comet formation in HL-60 cells after exposure to RF-fields (1800 MHz, SAR 1.3 W/kg) for different signal modulations, expressed as Olive Tail Moment, compared to control and sham exposure. Each bar represents the mean ± SD of results obtained in at least three (except continuous wave and C.W., 5 min on/10 min off: n=4) independent experiments. ** P<0.01; *** P<0.001 (Student's t-test, two-sided).

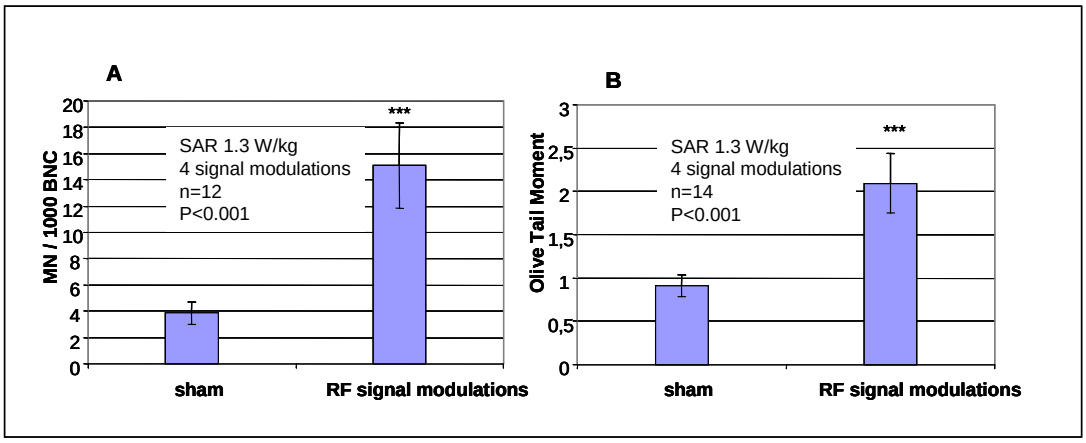


Figure 79. MN induction and Comet formation in HL-60 cells over all RF-field signal modulations at 1800 MHz, 24h, versus total sham, expressed as MN per 1000 BNC (A) and as Olive Tail Moment (B). Each bar represents the mean ± SD of results obtained in indicated number of experiments. *** P<0.001 (Student's t-test, two-sided).

As shown by flow cytometric analysis RF-EMF increased the micronuclei frequency, but did not affect cell cycle.

The results of the flow cytometric analysis of MN induction in HL-60 cells following RF-EMF exposure at the SAR level of 1.3 W/kg tested above using the continuous wave signal, according to the method of Nüsse et al. 1984, 1997, parallel the results obtained using the cytokinesis-block MN assay. Figure 80 shows a representative flow cytometric analysis of MN induction for sham-exposed (A) and RF-exposed (B) HL-60 cells after exposure to RF (1800 MHz, continuous wave, SAR 1.3 W/kg) for 24h. The DNA distribution of micronuclei (marker M1) and nuclei (marker M2) is obtained by projection of the particles defined by their side scatter intensities as micronuclei and nuclei. The percentage of MN is higher in the RF-exposed sample than in the sham-exposed sample (4.1% MN versus 2.7% MN).

The quantitative results of MN content analysis in four independent experiments are presented in Table 13. In all HL-60 cell experiments the MN content of the RF-exposed samples is higher than in sham-exposed samples. Normalisation of MN content in sham-exposed cells to 100% revealed a significant induction of MN after exposure to RF-field by 138.2 ± 18.4% ($P<0.01$; Student's t-test, two-sided). This result parallels those obtained using the microscopic analysis of MN frequencies.

Moreover, by means of flow cytometry the DNA-content of G1/G0, S and G2/M phase can be quantified by determining the fraction of each sub-population. The DNA-content distribution (ethidium bromide fluorescence) showed no differences between RF-field exposed and sham-exposed cells, indicating no influence of RF-EMF on cell cycle (Figure 80). Furthermore, cell cycle analysis demonstrated no accumulation of cells arrested in S and G2/M phase following exposure to RF fields. Additionally, for 24h RF-field exposure (1800 MHz, continuous wave, SAR 1.3 W/kg) no increase of the cell population in he sub G1 peak, which can be considered a marker of apoptotic cell death, was observed by flow cytometry.

Table 14 shows the data of DNA content distribution as percentage of gated cells for G1/G0, G2/M and S phase for RF-field exposed cells (continuous wave, SAR 1.3 W/kg, 24h), as compared to incubator control, sham-exposure and positive control hydrogen peroxide (100 µmol/l for 1 hour). The distribution of G1/G0, G2/M and S phase in the incubator control was 51.9 ± 4.2%, 18.3% ± 3.7 and 19.4 ± 1.7%; that of the positive control hydrogen peroxide: 28.4 ± 12.1%, 9.1 ± 5.2% and 9,0 ± 3.6%. Overall, the percentage of gated cells for the positive control was clearly lower than in all other conditions due to the fact, that here out of the gate analysed a high content of cellular debris was detected. This serves as a measure of cytotoxicity exerted by hydrogen peroxide. Sham-exposed and RF-exposed cells showed a similar DNA distribution as in the incubator control. No significant differences in DNA distribution of RF-exposed cells compared to sham-exposed cells were observed.

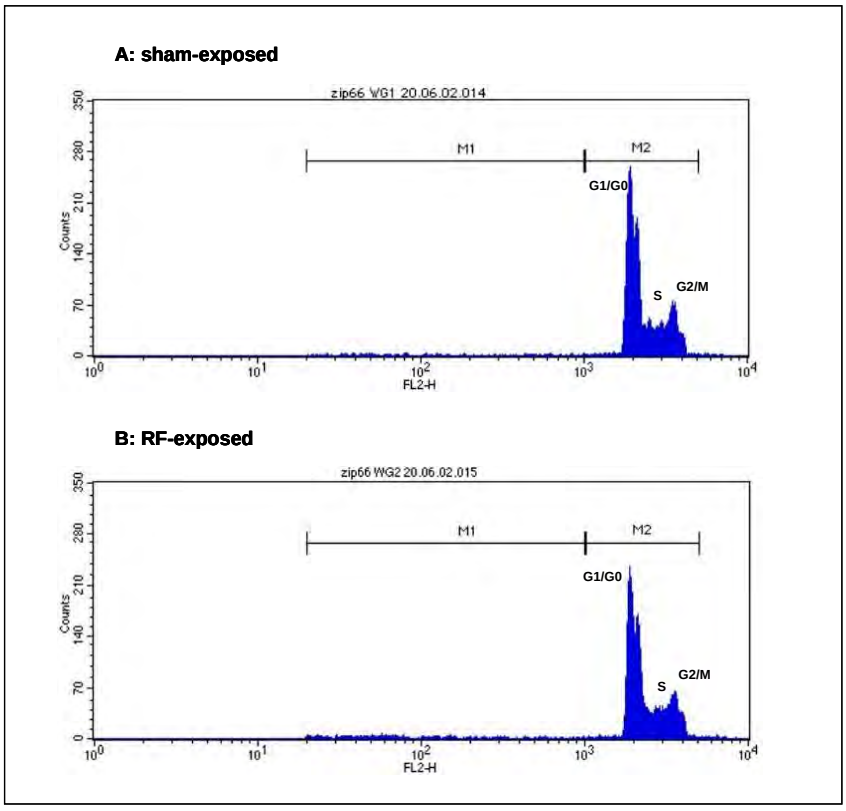


Figure 80. Flow cytometric analysis of micronuclei induction and determination of the proportions of cells in G1/G0, S and G2/M phases of the cell cycle after exposure of HL-60 cells to sham (A) or RF field (B, 1800 MHz, continuous wave, SAR 1.3 W/kg) for 24h.

The diagrams show representative ethidium bromide fluorescence histograms of micronucleated cells/nuclei suspension after treatment with FACS solution I and II. The method was performed according to Nüsse and Kramer (1984), Nüsse and Marx (1997) and Wessel and Nüsse (1995). G1/G0, S and G2/M peaks are indicated. For measurement of MN the G1/G0 peak was adjusted to approximately 2000 relative fluorescence units (FL2-H). The sort window for counting the MN comprised the relative DNA fluorescence units from 20 to 1000 (M1). For quantitative determination of MN the ratio of events in M1 (micronuclei) was compared to the events in M2 (nuclei) and expressed as % MN. For this representative experiment out of four the results for sham-exposure is 2.7% MN (A) and for RF-exposure is 4.1 % MN (B).

Table 13. Quantitative flow cytometric analysis of micronuclei frequencies after RF-field exposure (1800 MHz, continuous wave, SAR 1.3 W/kg, 24 h), compared to sham-exposure.

No. of experiment	Content of MN [%]		
	Sham-exposed	RF-field exposed	Content of MN in RF-exposed cells rel. to sham-exposed cells (100%)
1	2.69	4.08	151.67
2	5.49	6.37	116.03
3	4.24	6.57	154.95
4	1.85	2.41	130.70
mean	3.57 ± 1.62	4.86 ± 1.98	138.23 ± 18.41 **

Data of column 4 are values of RF-exposed cells in percentage relative to the corresponding sham-exposed value.
** Significant difference between the content of MN of RF-exposed cells to sham-exposed cells at P<0.01 (Student's t-test, two-sided).

Table 14. DNA distribution and cell cycle analysis of HL-60 cells after exposure to RF-field (1800 MHz, continuous wave, SAR 1.3 W/kg) for 24 h, compared to control, sham-exposure and positive control hydrogen peroxide (100 µmol/l for 1 h). Data represent DNA content distribution as percentage of gated cells in G1/G0, G2/M and S phase.

Group	n	G1/G0 [%]	S [%]	G2/M [%]
control	3	51.90 ± 4.17	18.25 ± 3.65	19.37± 1.71
sham	4	54.98 ± 6.69	19.45 ± 3.50	18.43 ± 4.07
RF-field	4	52.94 ± 6.19	19.01 ± 3.54	20.06 ± 2.73
positive control H_2O_2 (100 µmol/l for 1h)	3	28.37 ± 12.11	9.11 ± 5.17	8.96 ± 3.59

RF-EMF did not affect apoptosis as demonstrated by the Annexin V and TUNEL assay.

As the findings of structural alterations on the genomic level correlated with an external cellular stimulus do per se not prove a genotoxic effect, it has to be ruled out, that such changes are due to induction of apoptosis. Apoptotic cells typically undergo a series of structural changes: blebbing of the plasma membrane, condensation of the cytoplasm and intact organelles, and nuclear fragmentation. The most common biochemical property of apoptosis is the endonucleolytic cleavage of chromatin, initially to large fragments of 50-300 kilobase pairs and subsequently to monomers and multimers of 180-200 base pairs.

By establishing two flow cytometry methods for detection of apoptosis, Annexin V assay and TUNEL assay, a differentiation approach was included in the experimental strategy. By means of these two tests the detection of apoptotic changes at different stages in the apoptotic process became feasible. As a positive control for apoptosis induction by camptothecin, a topoisomerase I inhibitor, was used in the flow cytometry assays.

After initiation of apoptosis most cell types translocate phosphatidylserine (PS) from the inner plasma membrane leaflet to the cell surface. Once on the cell surface, PS can easily be detected by staining with a FITC conjugate of Annexin V, a protein that has strong natural affinity for phosphatidylserine. As

externalisation of phosphatidylserine occurs before nuclear changes, associated with apoptosis, take place, the Annexin V test detects apoptotic cells significantly earlier than do DNA-based assays.

Figure 81 shows a representative flow cytometric analysis of Annexin V staining following RF- (1800 MHz, 1.3 W/kg, continuous wave, 24h) and sham-exposure of HL-60 cells. As a positive control the apoptosis inducer camptothecin, a topoisomerase I inhibitor, was used to prove inducibility of apoptosis in the HL-60 cell system. The histograms show apoptosis associated Annexin V-FITC-signals (FL-1) versus DNA content propidium iodide (PI) signals. In order to prove that the gating of the corresponding cell populations for scoring the content of apoptotic cells did not select sub-populations, the histograms for "gated" and "not gated" cells are presented.

The TUNEL method (Terminal Deoxynucleotidyl Transferase Biotin-dUTP Nick End Labeling) identifies apoptotic cells *in situ* by using terminal deoxynucleotidyl transferase (TdT) to transfer FITC-dUTP to the free 3´-OH of cleaved DNA. These labelled cleavage sites can then be detected by flow cytometry.

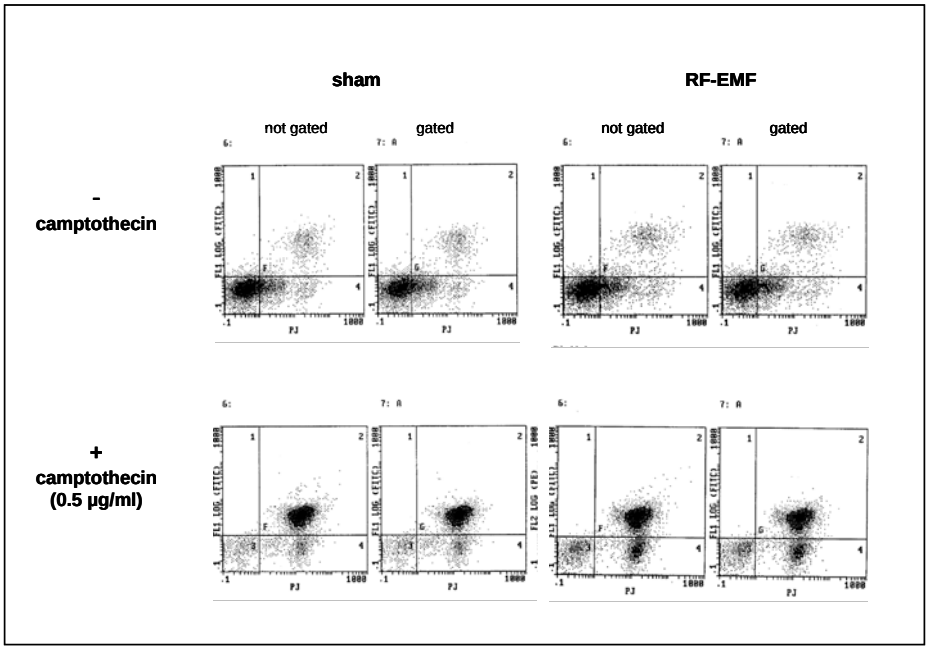


Figure 81. Flow cytometric analysis of RF- (1800 MHz, continuous wave, 1.3 W/kg, 24h) and sham-exposed HL-60 cells after staining with Annexin V-FITC (FL-1) and propidium iodide (PI, DNA content). The apoptosis inducer camptothecin is included as a positive control.

The histograms show apoptosis associated Annexin V-FITC (FL-1) versus DNA content propidium iodide (PI) signals. In order to prove that the gating of the corresponding cell populations for scoring the content of apoptotic cells did not select certain sub-populations, the histograms for "gated" and "not gated" cells are presented.

Figure 82 shows a representative flow cytometric analysis of TUNEL staining following RF-exposure (1800 MHz, , continuous wave, SAR 1.3 W/kg, 24h) and sham exposure of HL-60 cells. As a positive control the apoptosis inducer camptothecin, a topoisomerase I inhibitor, was used to prove inducibility of apoptosis in the HL-60 cell system. The histograms show apoptosis associated TUNEL-FITC-signals (FL-1) versus DNA content propidium iodide (PI) signals. In order to prove that within the quantification procedure no differences in cell population analysed occurs, histograms for "gated" and "not gated" cells are presented (Figure 82).

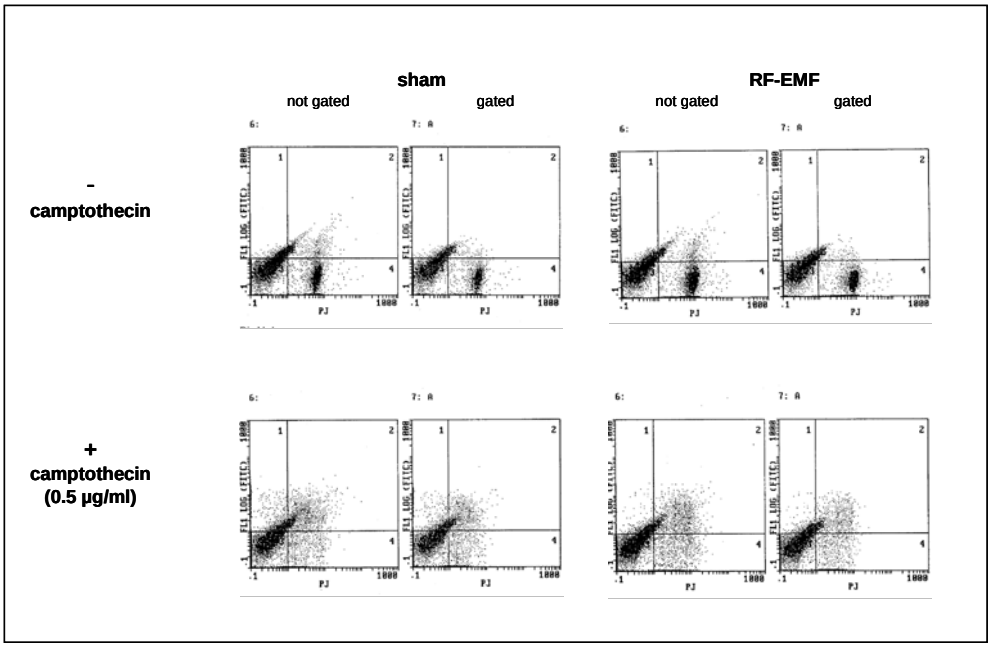


Figure 82. Flow cytometric analysis of RF- (1800 MHz, continuous wave, 1.3 W/kg, 24h) and sham-exposed HL-60 cells after labelling with TUNEL reaction mixture (Roche, Mannheim) for 1 hour at 37°C. The cells then underwent flow cytometric analysis in order to determine the number of green stains (representing apoptotic DNA fragmentation). DNA content analysis was performed on a Becton Dickinson FACScan by using the manufacturer's protocol. The apoptosis inducer camptothecin is included as a positive control.

The histograms show apoptosis associated TUNEL-FITC-signals (FL-1) versus DNA content propidium iodide (PI) signals. In order to prove that the gating of the corresponding cell populations for scoring the content of apoptotic cells did not select certain sub-populations, the histograms for "gated" and "not gated" cells are presented.

Neither by the Annexin V assay nor by the TUNEL assay, apoptosis induced by RF-electromagnetic fields (1800 MHz, continuous wave, SAR 1.3 W/kg, 24h) could be detected in HL-60 cells. Moreover, HL-60 cells exposed to RF-field at SAR 1.3 W/kg and continuous wave signal for 24h show no induction of the cell population in the sub G1 peak, which can be considered a marker of cell death by apoptosis (Figure 80).

RF-EMF did not exert a cytotoxic effect on HL-60 cells.

The trypan blue vitality test did not reveal any cytotoxic effects on the HL-60 cells from any RF-field applied. The vitality of the exposed cells was on the same order of magnitude (≈ 90%) as the cells of the sham-exposed and the incubator control. To exclude minor effects of RF-fields on viability of HL-60 cells, cell viability was examined spectrophotometrically by the MTT assay (Figure 83). Supplementary, also by this method no cytotoxic effect was detectable: absorbance A_{570nm} (RF-exposed HL-60 cells) = 0.91 ± 0.13 ; A_{570nm} (sham-exposed HL-60 cells) = 0.98 ± 0.15.

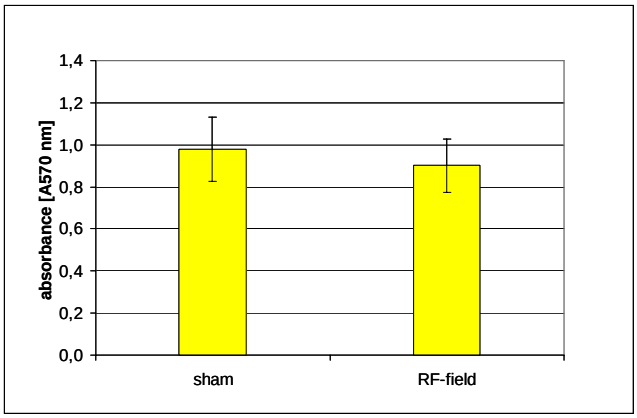


Figure 83. Viability of HL-60 cells after exposure to RF-field (1800 MHz, continuous wave, SAR 1.3 W/kg, 24h) compared to sham exposure. Cell viability was evaluated by the MTT assay and reported as absorbance at 570 nm. Bars represent means ± SD of 12 independent experiments.

In addition, the ratio of binucleated cells (BNC) against mono-, bi-, tri- and tetranucleated cells (% BNC) was determined as a measure of cell division and cell cycle progression. No effect of RF-EMF exposure on % BNC for different energies (Figure 84) or for different signal modulations (Figure 85) was found in comparison to sham-exposed or ionising-irradiated (0.5 Gy, 6 MeV, exposure time: 5.2s) HL-60 cells.

Additionally, no significant differences in DNA distribution of RF-exposed cells compared to sham-exposed cells were observed with respect to increased incidence of cellular debris as a measure of cytotoxicity (Table 14).

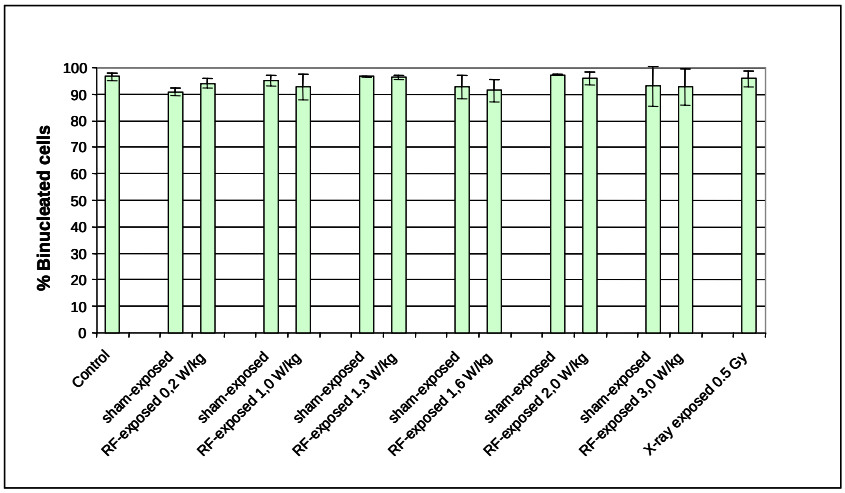


Figure 84. Effect of RF-field exposure on cell division. Shown is the number of binucleated HL-60 cells relative to the number of mono-, bi-, tri-, and tetra-nuclear cells (% BNC) following RF-field exposure (1800 MHz, continuous wave, different SAR levels, 24h). Positive control: 0.5 Gy ionising-irradiation (6 MeV, exposure time: 5.2s). Each bar represents the mean ± SD of results obtained in three independent experiments.

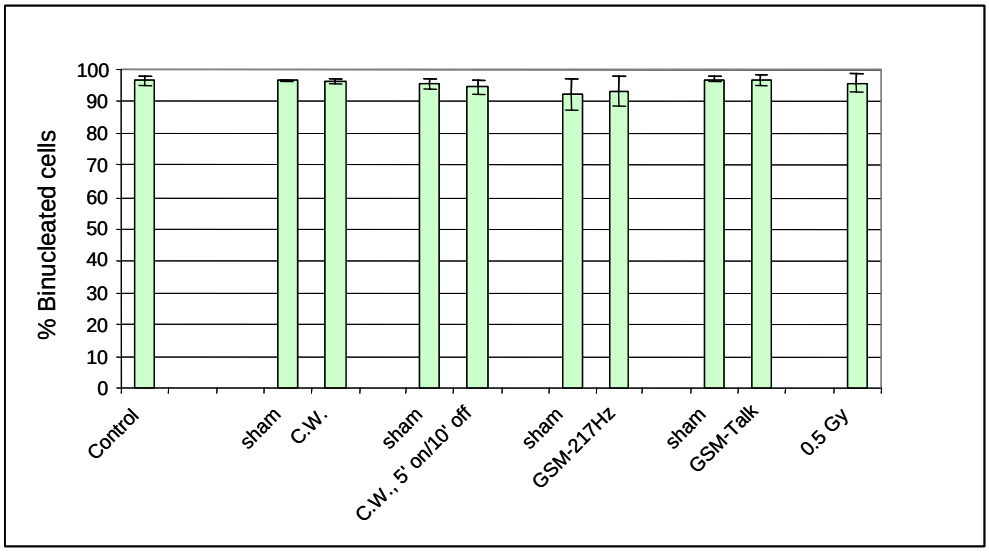


Figure 85. Effect of RF-field exposure on cell division. Shown is the number of binucleated HL-60 cells relative to the number of mono-, bi-, tri-, and tetranuclear cells (% BNC) following RF-field exposure (1800 MHz, SAR 1.3 W/kg, different signal modulations, 24h). Positive control: 0.5 Gy ionising-irradiation (6 MeV, exposure time: 5.2s). Each bar represents the mean ± SD of results obtained in three independent experiments.

Concludingly no in vitro cytotoxic effects of RF-EMF could be detected in RF-EMF-exposed and sham-exposed cells for the exposure conditions tested using either microscopic evaluation (trypan blue exclusion, % BNC), colorimetric MTT assay or flow cytometric analysis (nuclear ethidium bromide staining).

B. Indirect genotoxicity (by reactive oxygen species)

RF-EMF induced formation of reactive oxygen species as shown by flow cytometric detection of oxyDNA and rhodamine fluorescence.

It was the aim of these series of experiments to examine whether RF-EMF (1800 MHz at SAR 1.3 W/kg, 24h exposure) is capable to induce indirect genotoxic effects by affecting the generation and elimination of reactive oxygen species (ROS). For monitoring these ROS-formation and elimination steps, different assays, measuring nitric oxide, oxyDNA, oxidative DNA-damage via Dihydrorhodamine 123 (DHR123), lipid peroxidation, glutathione peroxidase activity, superoxide dismutase activity, have been established and were applied following RF-field exposure of HL-60 cells at that exposure condition with the most significant effect on DNA integrity (1800 MHz, continuous wave, 1.3 W/kg, 24h).

Nitric oxide (NOx)

Nitric oxide (NOx), was measured using the colorimetric Nitric Oxide Assay Kit, Calbiochem, Bad Soden, Germany. The data in Table 15 show the NOx production from HL-60 cells after exposure to RF-field (1800 MHz, continuous wave, SAR 1.3 W/kg, 24h), compared to control and sham-exposed cells. For an amount of 0.25 x 10^5 cells, in neither treatment group the detection limit of 1 µmol NOx/l was exceeded. The results presented are the means of three independent experiments. Concludingly, with this assay no in vitro effect of RF-field exposure on NOx formation was detected for the exposure conditions tested.

Table 15. NO_x formation in HL-60 cells after exposure to RF-field (1800 MHz, continuous wave, SAR 1.3 W/kg, 24h), compared to control and sham-exposure.

Group	NOx [µmol/l]
control	< 1
sham	< 1
RF-field	< 1

0.75×10^6 viable HL-60 cells/3 ml cell culture medium were cultivated for 24h at 37°C. After centrifugation, aliquots of culture media corresponding to 0.25×10^5 cells were collected and analysed for nitric oxide (NOx) by the colorimetric Nitric Oxide Assay Kit, Calbiochem, Bad Soden, Germany.
The results presented are representative for three independent experiments.

Flow cytometric detection of oxidative DNA damage (oxy-DNA)

The presence of oxidised DNA (by a fluorescent probe, directly binding to 8-oxoguanine as the major oxidative DNA product) was indicated by a green/yellow fluorescence that could be detected using a flow cytometry system. In Figure 86 a partial augmentation (occurring as a shoulder on the right side of the signal, see arrow Figure 86 of FL-1 fluorescence intensity), indicating the presence of oxidised DNA, was observed for the RF-exposed signal (green) in contrast to sham-exposed signal (blue). Additionally, RF-exposed cells showed a significant shift to the left as compared to sham-exposed cells. Table 16 shows the data for the quantification of ROS levels using oxyDNA-FITC conjugate to stain 8-oxoGuanosine residues on oxidatively damaged DNA of HL-60 cells. RF-field exposure of HL-60 cells induced a mean increase of oxidative DNA damage of 21.7 ± 2.0 %.

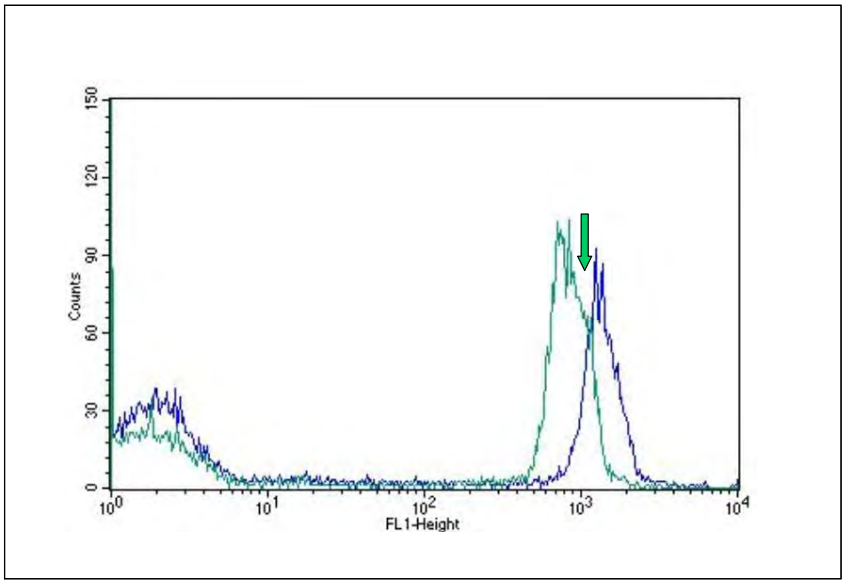


Figure 86. Flow cytometric detection of ROS levels using oxyDNA-FITC conjugate to stain 8-oxoGuo residues on oxidatively damaged DNA of HL-60 cells. The diagram shows the signal of oxidatively damaged DNA of RF-field exposed cells (green line) compared to sham-exposed cells (blue line). One representative histogram plot out of four independent experiments is shown. A partial augmentation (shoulder at the right side of the signal, indicated by arrow) of FL-1 fluorescence intensity was observed for the RF-exposed signal in contrast to sham-exposed signal.

Table 16. Quantification of ROS levels of HL-60 cells after exposure to RF-field (1800 MHz, continuous wave, SAR 1.3 W/kg) for 24h using oxyDNA-FITC conjugate to stain 8-oxoGuo residues on oxidatively damaged DNA.

No. of experiment	% augmentation of fluorescence signal of RF-field exposed cells (area under curve AUC of shoulder at the right side of the signal)
1	21.38
2	24.44
3	19.80
4	20.98
mean ± SD	21.65 ± 2.0

0.75 x 10^6 HL-60 cells/dish were sham- or RF-field exposed for 24 h. oxyDNA-FITC was used to stain 8-oxoGuo residues on oxidatively damaged DNA using the oxyDNA assay from Calbiochem-Novabiochem GmbH, Bad Soden, Germany. Oxidatively damaged DNA was quantified by determination of the area under the curve (AUC) of the shoulder at the right side of the signal fluorescence intensity (see arrow Figure 83) in RF-field exposed cells * Significant difference between the median of fluorescence intensity of RF-field exposed cells and sham-exposed cells at P<0.05 (n=4, Student's t-test, two-sided).

Oxidative DNA damage measured by DHR123 and flow cytometry

Cellular production of ROS was determined by measuring the rhodamine fluorescence of HL-60 cells, incubated in growth medium containing 5 µmol/l dihydrorhodamine 123 (DHR123) for 24h at 37°C. DHR123 is a non-fluorescent reduced Rhodamine 123 (Rh123) derivative that is freely permeable through cell membranes. Intracellular oxidation converts DHR123 to the fluorescent Rh123, which is retained intracellularly by the mitochondrial potential.

Figure 87 displays the overlay fluorescence histograms for RF-field exposed cells (1800 MHz, continuous wave, SAR 1.3 W/kg), compared to sham-exposed cells after simultaneous incubation with DHR123 for 24h. The figure shows that, in RF-field exposed cells, the fluorescence intensity signal shifts to the right in comparison to the signal of sham-exposed cells. In contrast, treating cells with 100 µmol/l H_2O_2 resulted in an even more pronounced shift of fluorescence signal. These shifts indicate enhanced fluorescence intensities and thereby increased production of intracellular ROS during RF-field exposure or H_2O_2 treatment of HL-60 cells.

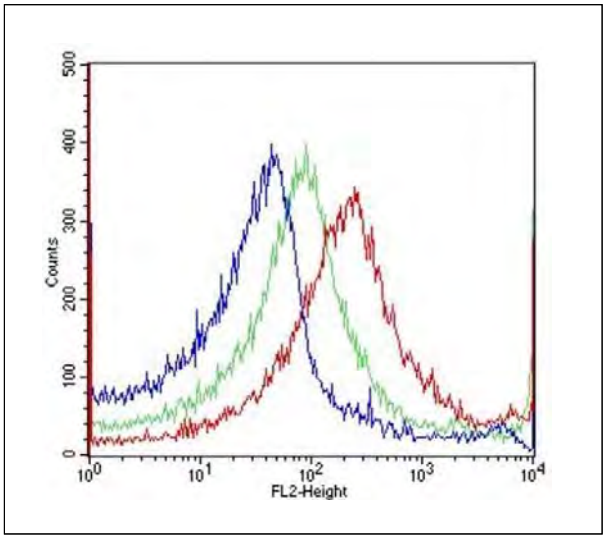


Figure 87. Fluorescence histograms for RF-field exposed (1800 MHz, continuous wave, SAR 1.3 W/kg, 24h) and sham-exposed HL-60 cells simultaneous treated with 5 µmol/l dihydrorhodamine 123 (DHR123). Blue line represents sham-exposed sample, green line represents RF-field exposed sample and red line represents H_2O_2-treated positive control (100 µmol/l for 1 hour). DHR123 reacts with intracellular ROS to form fluorescent Rh123, which is then retained by the mitochondria, enabling a flow cytometric assessment of cellular oxidant production.

By means of the DHR123 flow cytometry detection assay the shift of the signal can be quantified by determining the medians of the fluorescence intensities and the increase in rhodamine fluorescence for each population compared to sham, expressed in percent (Table 17). The data show that there is no difference in the level of oxidatively damaged DNA for control cells and sham-exposed HL-60 cells, expressed as the median of fluorescence intensity. The median values for control cells were 41.8 ± 5.8 and for sham-exposed cells 39.9 ± 8.5 (n=3). The values for the positive control (H_2O_2, 100 µmol/l, 1h) were 230.6 ± 100.3. In contrast, exposing cells to RF-fields resulted in a significant increase in median (75.5 ± 19.2; P<0.05, n=3). The percentage increase of rhodamine fluorescence for RF-field exposed cells compared to sham-exposed cells is 17.8 ± 9.7%, that of H_2O_2-treated cells 31.9 ± 12.4% (Table17).

Table 17. Detection and quantification of ROS levels with Dihydrorhodamine 123 after exposure to RF-field (1800 MHz, continuous wave, SAR 1.3 W/kg, 24 h), compared to control, sham-exposed and H_2O_2-treated HL-60 cells. For quantitative measurement the shift in median and the increase of fluorescence intensity was evaluated.

No. of exp.	Median of fluorescence intensity [units]				% increase of rhodamine 123 fluorescence relative to sham-exposure [a]	
	control	sham-exposed	RF-field exposed	H_2O_2-treated	RF-field exposed	H_2O_2-treated
1	39.95	30.51	95.60	149,89	28.98	43.12
2	37.18	42.17	57.26	199.02	12.35	18.53
3	48.26	46.98	73.65	342.89	11.97	33.91
mean	41.8 ± 5.8	39.9 ± 8.5	75.5 ± 19.2 *	230.6 ± 100.3	17.8 ± 9.7	31.9 ± 12.4

[a] differences of rhodamine 123 fluorescence (AUC, area under curve) of cells exposed to RF-field or those treated with H_2O_2 was determined and the values were plotted as percentage increase relative to sham-exposure.
* Significant difference between the median of RF-exposed cells to the median of sham-exposed cells and the median of RF-exposed cells to that of control cells at P<0.05 (Student's t-test, two-sided).

Lipid peroxidation

Lipid peroxidation was measured using the colorimetric Lipid Peroxidation Assay Kit, Calbiochem, Bad Soden, Germany. Malondialdehyde (MDA) and 4-hydroxy-2(E)-nonenal (4-HNE), products of lipid peroxidation, were estimated spectrophotometrically at 586 nm after reaction with a chromogenic reagent at 45°C. The absorbance values obtained for the samples were compared with a standard curve of known concentrations of MDA / 4-HNE (1 - 20 µmol/l). For MDA and 4-HNE the amounts of lipid peroxidation markers in all experiments and samples were below 1 µmol/l. Table 18 shows that there is no difference in the level of lipid peroxidaton for RF-field exposed HL-60 cells, compared to control and sham-exposed cells (n=3).

Table 18. Lipid peroxidation (LPO) in HL-60 cell homogenates after exposure to RF-field (1800 MHz, continuous wave, SAR 1.3 W/kg, 24h), compared to control and sham-exposed cells.

Group	amount of (MDA + HNE) [µmol/l]		
	exp. 1	exp. 2	exp. 3
control	< 1.0	< 1.0	< 1.0
sham	< 1.0	< 1.0	< 1.0
RF-field	< 1.0	< 1.0	< 1.0

Lipid peroxidation was measured using the colorimetric Lipid Peroxidation Assay Kit from Calbiochem, Bad Soden, Germany. Malondialdehyde (MDA) and 4-hydroxy-2(E)-nonenal (4-HNE), products of lipid peroxidation, were estimated spectrophotometrically at 586 nm in an aliquot corresponding to 6 x 10^5 cells after reaction with a chromogenic reagent at 45°C. The results presented are means of three independent experiments.

Antioxidant enzyme activities

RF-EMF did not affect antioxidant enzyme activities of HL-60 cells (SOD and GPx activity).

To screen the possible effect of RF-EMF on endogenous antioxidant enzyme activity, the activities of superoxide dismutase (SOD) and glutathione peroxidase (GPX) were determined in the HL-60 cells that were exposed to RF-fields (1800 MHz), continuous wave, SAR 1.3 W/kg for 24h. Positive controls as indicated in the assays by the manufacturer were included in the analysis.

Superoxide dismutase (SOD) activity

Superoxide dismutase (SOD) activity of cell homogenates was determined using the Superoxide Dismutase Assay Kit from Calbiochem, Bad Soden, Germany. The data in Table 19 show SOD activities in HL-60 cells after exposure to RF-field (1800 MHz, continuous wave, SAR 1.3 W/kg, 24h), compared to control and sham-exposed cells. For an amount of 4 x 10^5 cells, in neither treatment group the detection limit of 0.2 U/ml SOD activity was exceeded. The results presented are means of two independent experiments. Concludingly, no in vitro effect of RF-field exposure on SOD activity was detected for the exposure conditions tested.

Table 19. Superoxide dismutase (SOD) activity in HL-60 cell homogenates after exposure to RF-field (1800 MHz, continuous wave, SAR 1.3 W/kg, 24h), compared to control and sham-exposed cells.

Group	SOD_{525} activity [U/ml]	
	exp. 1	exp. 2
control	< 0.2	< 0.2
sham	< 0.2	< 0.2
RF-field	< 0.2	< 0.2

Superoxide dismutase (SOD) activity of cell homogenates was determined using the Superoxide Dismutase Assay Kit from Calbiochem, Bad Soden, Germany. The SOD-mediated increase in the rate of autooxidation of the reaction mixture was utilized to yield a chromophore with maximum absorbance at 525 nm. SOD activity was measured in an aliquot corresponding 4 x 10^5 cells (n=2). Detection limit for SOD activity is 0.2 U/ml.

Glutathione peroxidase (GPx) activity

Glutathione peroxidase (GPx) activity of cell homogenates was determined using a cellular Glutathione Peroxidase Assay Kit, Calbiochem, Bad Soden, Germany. The data in Table 20 show the GPx activity in HL-60 cells after exposure to RF-field (1800 MHz, continuous wave, SAR 1.3 W/kg, 24h), compared to control and sham-exposed cells. For an amount of 1 x 10^6 cells, in neither treatment group the detection limit of 5.6 mU/ml GPx activity was exceeded. The results presented represent two independent experiments. Concludingly, no in vitro effect of RF-field exposure on GPx activity was detected for the exposure conditions tested.

Table 20. Glutathione peroxidase (GPx) activity in HL-60 cell homogenates after exposure to RF-field (1800 MHz, continuous wave, SAR 1.3 W/kg, 24h), compared to control and sham-exposed cells.

Group	GPx activity [mU / ml]	
	exp. 1	exp. 2
control	< 5.6	< 5.6
sham	< 5.6	< 5.6
RF-field	< 5.6	< 5.6

Glutathione peroxidase (GPx) activity of cell homogenates was determined in two independent experiments using the cellular Glutathione Peroxidase Assay Kit from Calbiochem, Bad Soden, Germany.
Cell homogenisate of 1 x 10^6 cells is added to a 1050 µl of a solution containing glutathione (GSH, 1mmol/l), GSH reductase (0.4 U/ml) and NADPH. The reaction is initiated by the addition of 350 µl of the diluted organic peroxide t-butyl hydroperoxide and the absorbance at 340 nm was recorded over a period of 5 minutes. The rate of decrease in the absorbance is directly proportional to the GPx activity in the cell homogenisate. Detection limit for GPx activity is 5.6 mU/ml.

Summarising, the endogenous antioxidant enzyme activities of HL-60 cells (SOD and GPx activity) were not altered by RF-field exposure compared to sham-exposure using the conditions of the assays described above. This screening approach revealed, that the analysis of antioxidant enzyme activities does not show enough methodological sensitivity for the amounts of ROS to be generated by RF-field exposure of HL-60 cells.

Indirect genotoxicity by modulation of cellular toxifying and detoxifying capacities

The generation of genotoxic effects through RF-EMF was inhibited by ascorbic acid.

In a further series of experiments it was examined, whether ascorbic acid as a free radical scavenger and inhibitor of reactive oxygen species is capable to inhibit MN induction and DNA damage by co-administration to RF-field-exposure (continuous wave, SAR 1.3 W/kg, 24h). The inhibition of micronuclei induction and Comet formation was measured by use of the cytokinesis-block in vitro Micronucleus assay and the Comet assay. In both tests systems, ascorbic acid effectively reduced the RF-field induction of micronuclei and DNA damage (Figures 85 and 86).

Figure 88 displays the inhibition of MN induction induced by RF-fields (continuous wave, SAR 1.3 W/kg, 24h) and simultaneous treatment of cells with ascorbic acid (AA, 10 µmol/l) for 24h. MN frequencies for sham-exposed and RF-field exposed cells were 4.1 ± 0.2 and 11.6 ± 1.9 expressed as MN / 1000 BNC. After co-incubation of sham-exposed and RF-exposed HL-60 cells with ascorbic acid (AA, 10 µmol/l) for 24h the frequencies were 4.3 ± 0.4 and 4.8 ± 1.9. The MN frequency for the incubator control was 3.4 ± 0.4. Data show, that ascorbic acid inhibits RF-field associated MN induction significantly (n=3, P<0.05). The inhibition resulted in an induction by factor 1.08 compared to sham.

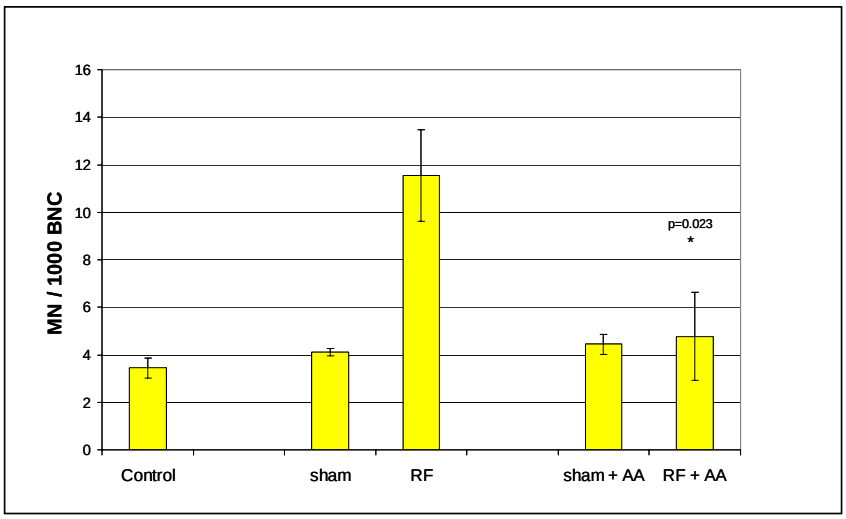


Figure 88. Effect of ascorbic acid (AA, 10 µmol/l) on RF-field (1800 MHz, continuous wave, SAR 1.3 W/kg, 24h) induced MN frequencies in HL-60 cells, compared to control and sham-exposed cells. Each bar represents the mean ± SD of results obtained in at least three independent experiments. Significant differences between RF-field exposure and sham-exposure with co-administration of AA is given by * P<0.05 (Student's t-test, two-sided).

Figure 89 displays the inhibition of DNA damage induced by RF-fields (continuous wave, SAR 1.3 W/kg, 24h) and simultaneous treatment of cells with ascorbic acid (AA, 10 µmol/l) for 24h. The values of Olive Tail Moment for sham-exposed and RF-field exposed cells were 0.9 ± 0.1 and 2.0 ± 0.2. After co-incubation of sham-exposed and RF-field exposed HL-60 cells with ascorbic acid (AA, 10 µmol/l) for 24h the values were 1.0 ± 0.1 and 1.2 ± 0.03. The OTM for the incubator control was 0.8 ± 0.05. Data show, that ascorbic acid inhibits the RF-field induced DNA damage significantly (n=3, P<0.01). The inhibition resulted in an induction by factor 1.2 compared to sham. Additionally, no induction of cytotoxicity (trypan blue test), no alteration of cell medium pH value and no influence on cell growth or cell cycle progression was observed for ascorbic acid alone and for co-administration of ascorbic acid together with RF-field exposure over 24h.

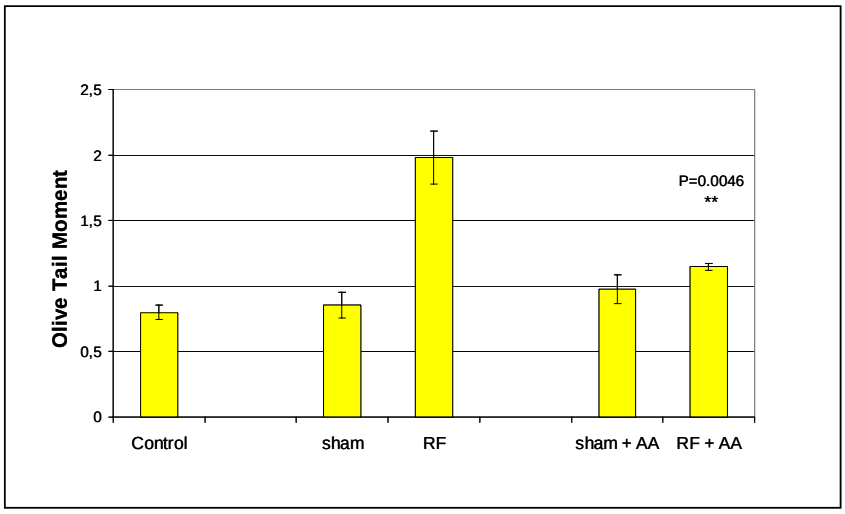


Figure 89. Effect of ascorbic acid (AA, 10 µmol/l) on RF-field induced Comet formation in HL-60 cells (1800 MHz, continuous wave, SAR 1.3 W/kg, 24 h), compared to control and sham-exposed cells. Each bar represents the mean ± SD of results obtained in at least three independent experiments. Significant differences between RF-field exposure and sham-exposure with co-administration of AA is given by ** P<0.01 (Student's t-test, two-sided).

Concludingly, this observed inhibition of genotoxicity by ascorbic acid supports the hypothesis that the effect of RF-field on genomic integrity may be explained by the generation of free oxygen radicals.

3.2.1.2 Human fibroblasts and granulosa cells of rats (Participant 3)

RF-EMF generated DNA strand breaks in human fibroblasts and in granulosa cells of rats.

The influence of RF-EMF exposure on the generation of DNA strand breaks in cells of two different tissues (human fibroblasts, rat granulosa cells) was evaluated using alkaline and neutral Comet assay. Four different sets of exposure conditions were tested: continuous (1800 MHz, 2 W/kg), intermittent (5 min on/10 min off, 1800 MHz, 2 W/kg), pulse modulation (1800 MHz, 2 W/kg, amplitude 217 Hz, 5 min on/10 min off) and talk modulation (1800 MHz, 1.2 W/kg, DTX 66%, GSM basic 34%, continuous). Different exposure duration was applied (4, 16 and 24 hours).

An elevation of Comet assay levels in exposed cells compared to sham-exposed controls could be detected in each of these experiments, even at continuous exposure (Figure 90). This elevation became significant at 16 hours of exposure, but no significant differences between 16 and 24 hours could be detected. At intermittent, pulse modulation and talk modulation Comet assay levels were significantly higher than at continuous exposure. Human fibroblasts and granulosa cells responded equally to RF-EMF, albeit the latter exhibited higher basal and higher end levels (Figure 91). The Comet factors with neutral Comet assay were similar, albeit lower (Figures 92, 93). Dose response investigations with human fibroblasts, which were exposed intermittently (5 min on/10 min off) for 24 hours, revealed a dose dependent increase of the Comet tailfactor beginning already at a SAR of 0.3 W/kg with a peak level at 1.0 W/kg (Figure 94).

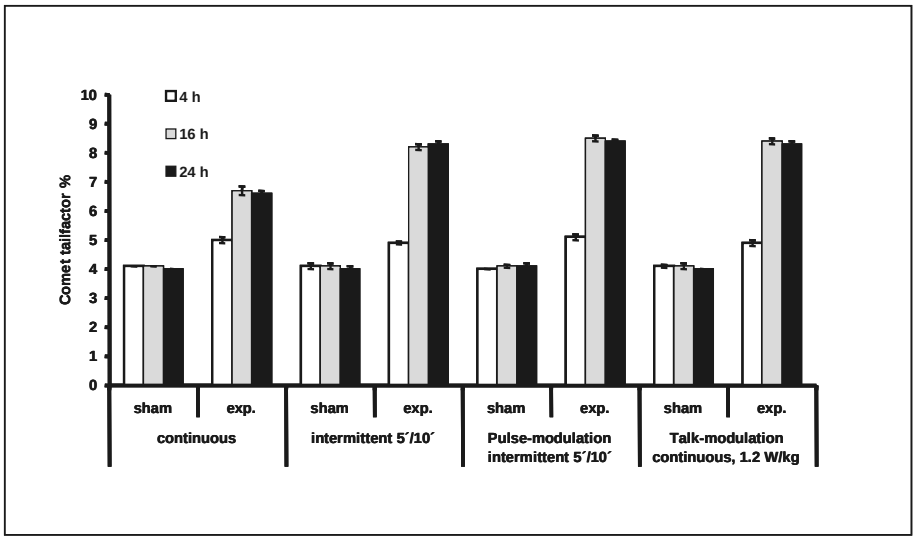


Figure 90. Influence of exposure time and of different exposure conditions on formation of DNA single and double strand breaks in human fibroblasts determined with Comet assay under alkaline conditions (cell strain ES 1, 1800 MHz, SAR 2 W/kg).

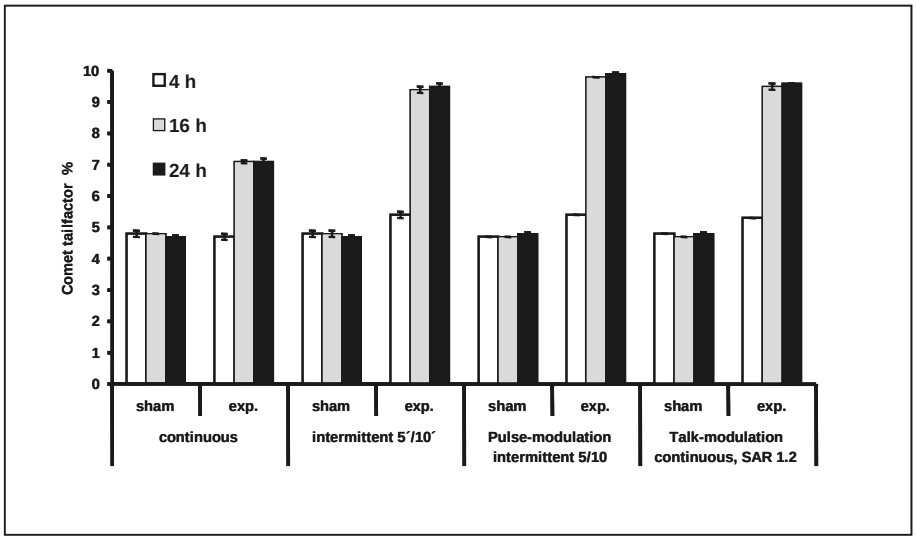


Figure 91. Influence of exposure time and of different exposure conditions on formation of DNA single and double strand breaks in granulosa cells determined with Comet assay under alkaline conditions.

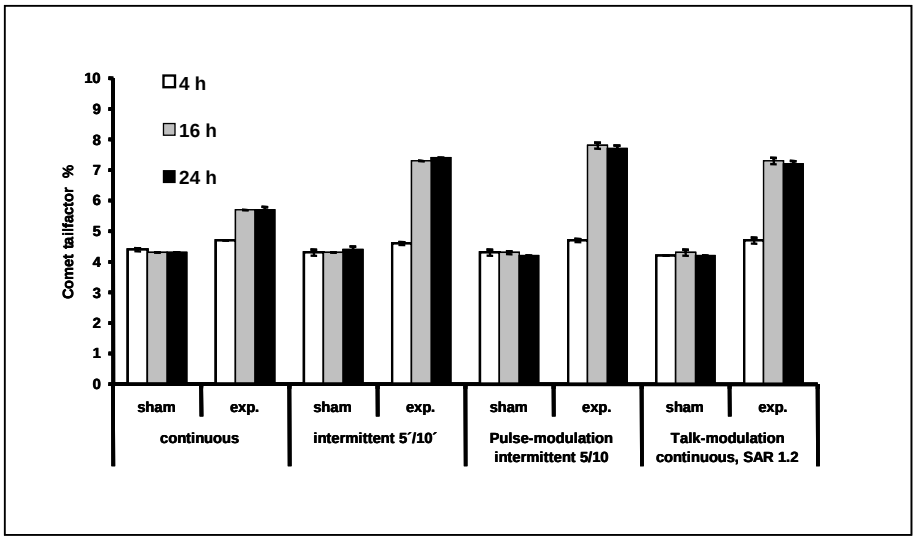


Figure 92. Influence of exposure time and of different exposure conditions on formation of DNA double strand breaks in human fibroblasts determined with Comet assay under neutral conditions (cell strain ES 1, 1800 MHz, SAR 2 W/kg).

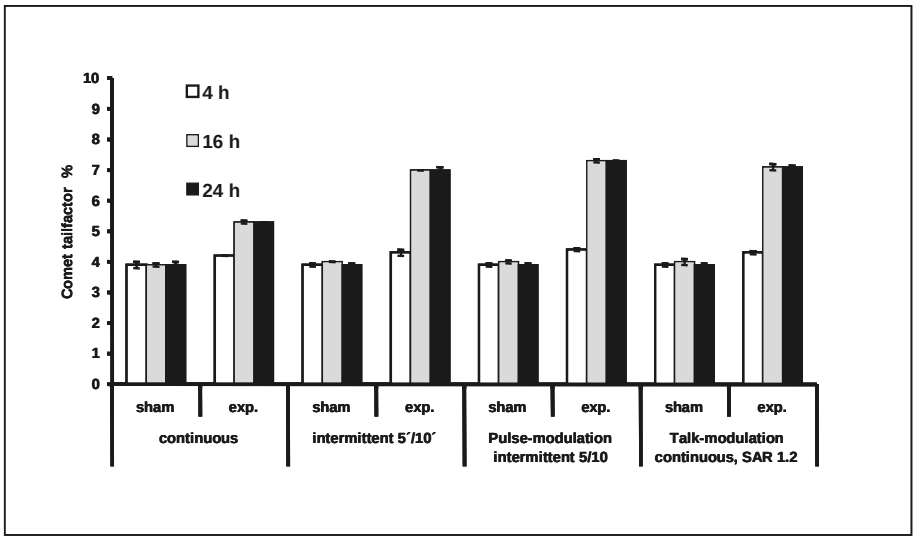


Figure 93. Influence of exposure time and of different exposure conditions on formation of DNA double strand breaks in granulosa cells determined with Comet assay under neutral conditions (1800 MHz, SAR 2 W/kg).

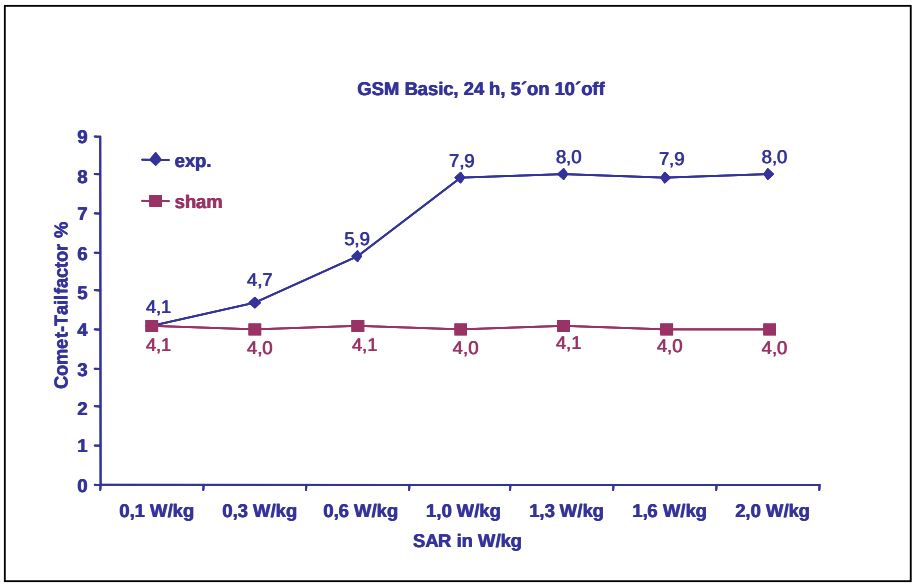


Figure 94. Dose dependent increase of DNA single and double strand breaks in human fibroblasts determined with the Comet assay under alkaline conditions

RF-EMF generated chromosomal aberrations in human fibroblasts.

Since we could demonstrate an induction of DNA strand breaks in human fibroblasts after RF-EMF exposure and due to the results of chromosome aberrations upon ELF-EMF exposure, we also evaluated the chromosome aberrations after RF-EMF exposure (GSM basic 1950 MHz, 1 W/kg, 5 min on/10 min off, 15 hours). These preliminary experiments (500 metaphases scored) revealed in RF-EMF exposed fibroblasts a 10-fold increase in chromosome gaps, a 4-fold increase in chromosome breaks and very high incidences of dicentrics and acentric fragments (Table 21).

Table 21. Percentage of chromosomal aberrations induced by RF-EMF exposure (GSM basic 1950 MHz, 1 W/kg, 5 min on/10 min off, 15h) in cultured human fibroblasts[a]

types of aberration	RF-exposed (% ± SD)	sham-exposed (% ± SD)	p-value[b]
chromosome gaps	57.5 ± 2.1 %	4.8 ± 1.6 %	< 0.001
chromosome breaks	8.1 ± 0.7 %	1.7 ± 0.1 %	< 0.001
dicentric chromosomes	4.5 ± 0.7 %	---	
acentric fragments	1.5 ± 0.7 %	---	

[a] a number of 1,000 metaphases were scored in each of five independent experiments. Results are expressed as percentage chromosomal aberrations per cell.
[b] Significant differences (p<0.05) as compared to sham-exposed controls using Student's t-test for independent samples

RF-EMF induced micronuclei in human fibroblasts.

Cultured human fibroblasts were exposed to RF-EMF (GSM basic 1950 MHz, 15h, 2 W/kg) and micronuclei frequencies were evaluated. These results showed an induction of micronuclei in RF-EMF exposed fibroblasts (Figure 95a). The observed increase in micronucleus frequencies was about 20-fold compared to sham exposed cells or non-exposed controls, but not as high as in bleomycin (10 µg/ml, 17h) treated cells, which were used as positive controls.

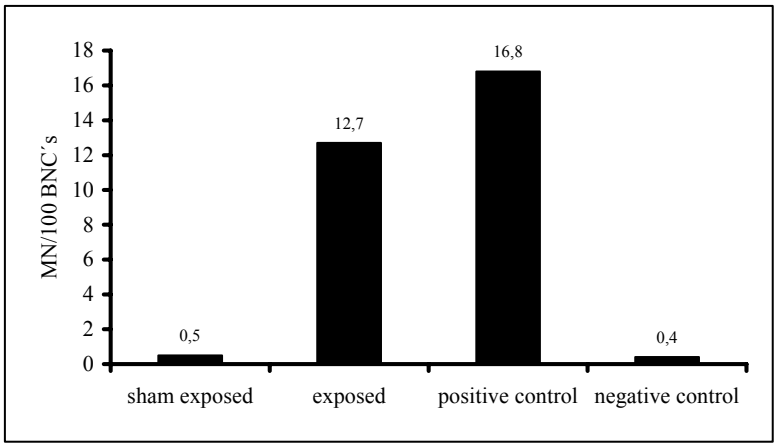


Figure 95. Micronucleus frequencies of RF-EMF exposed (GSM basic 1950 MHz, 15h, 2 W/kg) cultured human fibroblasts and control cells. Bleomycin-treated cell were used as a positive control.

Results on the influence of RF-EMF on the mitochondrial membrane potential were inconsistent.

Evaluating changes in the mitochondrial membrane potential after RF-EMF exposure (GSM basic 1950 MHz, 1 W/kg, 5 min on/10 min off, 15h) using JC-1, revealed a significant decrease in the mitochondrial membrane potential in one experiment, which could not be reproduced..

3.2.1.3 Mouse embryonic stem cells (Participant 4)

RF-EMF affected double-strand DNA break induction in ES cell derived neural progenitors immediately after exposure.

We studied the possible effects of RF-EMF on the integrity of DNA strands in differentiating ES cell from EB outgrowths. Two schemes were applied: (1) For RF-EMF exposure (GSM signal 217-Hz, 1.71 GHz, 1.5 W/kg, intermittency 5 min on/30 min off, 6h), the percentage of primary DNA damage was measured in the alkaline and neutral Comet assay immediately after the RF-EMF exposure at the stage of neural differentiation (4+4d - 4+6d) and 18 hours after the RF-EMF exposure. No differences in the induction of single-strand breaks as measured by the alkaline Comet assay were observed 0 and 18 hours after exposure. The tailfactor was slightly, but significantly increased in the neutral Comet assay immediately after exposure ($p<0.05$) (Table 26). In the second set of experiments, the same RF-EMF exposure conditions were applied for 48 hours instead of 6 hours, and the alkaline Comet assay was done immediately after exposure, while the neutral Comet assay was done 24 or 48 hours post exposure. However, no significant differences were observed in the induction of single- or-double DNA strand breaks between sham-exposed or EMF exposed neural progenitors after prolonged exposure (48h).

3.2.1.4 Summary (Participant 1)

Our data indicate a genotoxic action of RF-EMF in various cell systems. This conclusion is based on the following findings:

- RF-EMF exposure was able to induce DNA single and double strand breaks as well as an increase in micronuclei in HL-60 cells (3.2.1.1).
- The DNA damage generated by RF-EMF in HL-60 cells was dependent on the time of exposure, the field strength and the type of RF-EMF signals (3.2.1.1).
- The DNA damage in HL-60 cells probably resulted from an increase in free oxygen radicals induced during RF-EMF exposure (3.2.1.1).
- RF-EMF exposure at a SAR value between 0,3 and 2,0 W/kg produced DNA single and double strand breaks in human fibroblasts and in granulosa cells of rats dependent on the exposure time and the type of signals (3.2.1.2).

- RF-EMF exposure at a SAR value of 2 W/kg caused an increase in chromosomal aberrations in human fibroblasts demonstrating that the DNA repair was not error-free (3.2.1.2).
- RF-EMF exposure at a SAR value of 1,5 W/kg caused a slight, but significant increase in DNA double strand breaks in neural progenitor cells stemming from mouse embryonic stem cells (3.2.1.3).

3.2.2 Cell proliferation and cell differentiation

3.2.2.1 Human neuroblastoma cell line NB69 and neural stem cells (NSC) (Participant 5)

RF-EMF did not affect growth or viability of NB69 neuroblastoma cells and neural stem cells (NSC).

NB69 cells were exposed at day 3 postplating to GSM-Basic over a 24-hour period. After exposure, the cells were left to grow in the absence of field for an additional 24 hour lapse (5 days postplating). The field exposure was applied alone or in combination with bFGF. As it is shown in Figure 96, the GSM-Basic signal alone did not affect significantly cell growth or cell viability. The morphological analysis did not show significant differences between exposed and control groups, either (data not shown). The treatment with bFGF alone induces differentiation in NB69 cells, which exhibit significant increases in the number and the extension of processes per cell and in the cell size (see 3.2.4.2). An equivalent response was obtained after exposure to the combined treatment with bFGF plus GSM-Basic.

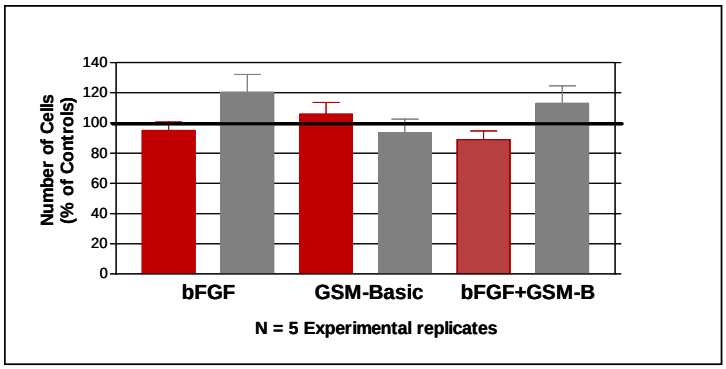


Figure 96. Number of living (red) and dead (grey) cells after a 24-h exposure to GSM-Basic signal followed by 24 additional hours of incubation in the absence of the RF-EMF. No significant changes were observed in the exposed samples when compared to the respective controls

NS cells were exposed at day 2 postplating to GSM-Basic over a 24-hour period. After exposure, the cells were left to grow in the absence of field for an additional 48-hour lapse (5 days postplating). At the end of this period, the samples were studied for cell growth and/or cell viability. The treatment did not affect cell growth (Figure 97A) and did not induce significant changes in the cells' morphology (data not shown). However, the cell size in the exposed samples was observed to be slightly, but not significantly augmented when compared to the respective controls (Figure 97B; image analysis of 60 microscope fields per condition of a total of 4 experimental replicates). The observed, slight reductions in the percent of dead cells, and in the increase of the cells' size after exposure to the GSM-Basic signal (Figure 97) could be due to an enhancement of the cell attachment to the substrate in the exposed samples. Such an attachment was microscope observed, though not image-analysis quantified (data not shown).

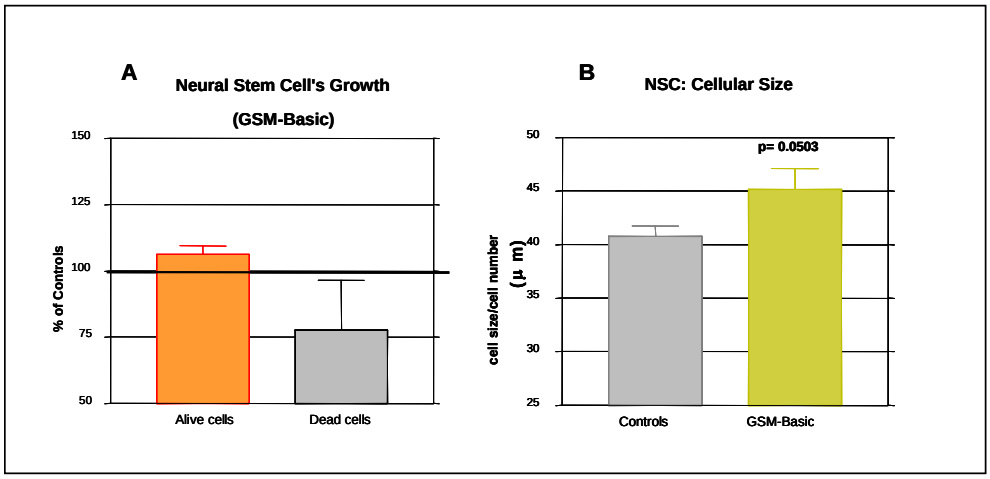


Figure 97. (A) Number of living and dead cells after a 21-h exposure to GSM-Basic signal followed by 48 additional hours of incubation in the absence of RF-EMF (5 days postplating). No significant changes were observed, even though a small reduction of dead cells occurs in the exposed samples. (B) The cell size in the exposed samples was observed to be slightly, but not significantly augmented when compared to the respective controls.

RF-EMF may affect the expression of FGF receptors in NB69 human neuroblastoma cells and in neural stem, potentially influencing cellular differentiation.

See 3.2.4.2

RF-EMF affected the differentiation of neural stem cells (NSC), but not of neuroblastoma cells (NB69).

The aim of the study was to determine whether the exposure of neural stem cells (NSC) to GSM-1800 signals (GSM-Basic signal, 21-hours, 5 min on/10 min off) can influence the evolution of the phenotypic differentiation at the middle term (6 additional days after exposure). As described in the methodology, the cells were exposed to RF-EMF at day 2 postplating. After the 21-hour exposure, the cells were grown for 6 additional days in the absence of the GSM stimulus. At the end of this period, the cells were immunostained with O1 for mature oligodendrocyte identification, GFAP for astrocytes and ß-tubulin III for neurons. All experiments and analysis were conducted following blind protocols. The data (N= 5 experimental replicates) indicate that the GSM-Basic signal promotes marked morphological changes in differentiating oligodendrocytes and astrocytes derived from NSC (Figure 98).

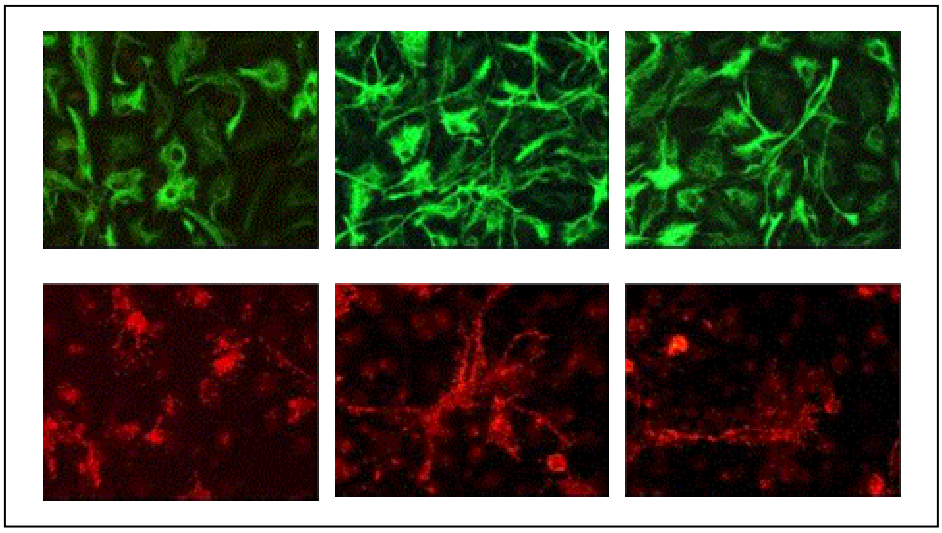


Figure 98. Photomicrographs of neural stem cells progeny. Astrocytes labelled with GFAP (green) and oligodendrocytes labelled with O1 (red) immunostaining: A, Control; B, and C, exposed to the GSM-Basic signal. The exposure increases cell extension in astrocytes and oligodendrocytes.

Similar experiments have been carried out to evaluate the phenotypic differentiation of NB69 cells treated with the above GSM-signal and exposure conditions, in the presence or absence of RA. As described in the methodology, the cells were exposed to the field at day 3 postplating, during a 24-hour period, and then grown for 2 additional days in the absence of GSM exposure. After that, the cells were analysed for expression of mature neuronal cell marker ß-tubulin III, and for tyrosine hydroxylase (TH) marker. Exogenous, basic fibroblast growth factor was used as a positive control. The data show that the neuronal outgrowth of NB69 cells (Figure 99) and the percent of TH+ cells seem not to be altered by the exposure to the GSM-Basic signal. Only the treatment with bFGF promoted the neuronal microtubules network in these cells.

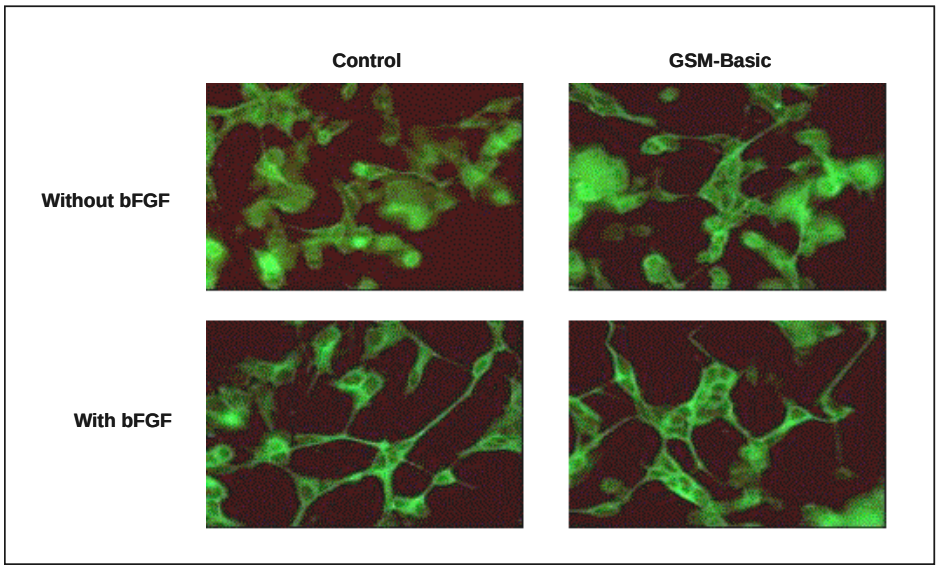


Figure 99. Photomicrographs of NB69 cells analysed 3 days after the exposure and/or incubation in the presence or absence of bFGF. Cells stained with anti-beta-tubulin antibody. The neuronal outgrowth of NB69 cells seems not to be altered by the exposure to the GSM-Basic signal. However, the treatment with bFGF promotes the neuronal microtubules network in these cells.

3.2.2.2 Human lymphocytes and thymocytes (Participant 8)

RF-EMF did not affect proliferation, cell cycle and activation of human lymphocytes.

The experiments with RF-EMF were performed at SAR 1,4-2,0 W/kg using different RF modulations or time exposure. Studies on cell proliferation were performed discriminating CD4+CD28+/- and CD8+CD28+/-lymphocytes subpopulation. Cells were exposed to Talk modulated RF-EMF (2 W/kg) and two intermittent types of exposure were applied: 1) 10 min on/20 min off for 44 hours; 2) 2 hours on/22 hours off for 72 hours. We performed experiments with cells from 6 donors using the former approach, from 11 donors using the latter approach. All cells were acquired and analysed after 72 hours and 120 hours of culture. A small increase (3%) of proliferating CD8+CD28+ T lymphocytes was observed in exposed cells both at 72 hours and 120 hours of culture. Since the differences observed are similar to the calculated standard error, we considered this effects not relevant. In Tables 22 and 23 data related to proliferating and not proliferating cells subsets after 72 hours and 120 hours of culture, respectively, are reported.

Table 22. T lymphocytes subsets exposed to talk modulated RF-EMF 2 hours on/22hours off for 72 hours. Data are reported as mean (% ± s.e.) of all experiments performed

Lymphocyte subsets	Sham % ± se	RF % ± se	p
P CD4+CD28+	30.7 ± 2	28.2 ± 2	ns
P CD4+CD28-	0.9 ± 0.4	0.7 ± 0.3	ns
NP CD4+CD28+	13.0 ± 2	12.4 ± 2	ns
NP CD4+CD28-	1.8 ± 0.9	0.8 ± 0.3	ns
P CD8+CD28+	19 ± 2	21 ± 2	0.042
P CD8+CD28-	7.9 ± 3	7.7 ± 3	ns
NP CD8+CD28+	8.0 ± 1	9.3 ±1	ns
NP CD8+CD28-	1.1 ± 0.2	1.0 ± 0.4	ns

P = proliferating; NP = non proliferating; ns = not significant

Table 23. T lymphocytes subsets exposed to Talk modulated RF-EMF 2 hours on/22 hours off for 72 hours and analysed at 120 hours of culture. Data are reported as mean (% ± s.e.) of all experiments performed

Lymphocyte subsets	Sham % ± se	RF % ± se	p
P CD4+CD28+	61.0 ± 2	62.1 ± 2	ns
P CD4+CD28-	0.60 ± 0.07	0.80 ± 0.11	ns
NP CD4+CD28+	9.3 ± 0.2	8.7 ± 0.2	ns
NP CD4+CD28-	1.4 ± 0.1	1.72 ± 0.11	ns
P CD8+CD28+	32 ± 4	35 ± 4	0.048
P CD8+CD28-	5.4 ± 3	4.3 ± 2	ns
NP CD8+CD28+	5.4 ± 0.7	4.7 ± 0.9	ns
NP CD8+CD28-	0.9 ± 0.3	0.8 ± 0.3	ns

P = proliferating; NP = non proliferating; ns = not significant

Cell cycle analysis was performed in PBMCs exposed at three RF-EMF modulations and in the case of Talk signal also PBMCs from old donors were analysed. In all the cases observed no differences were found between exposure and control cells. Some slight differences (1-2%, sometimes increase and sometimes decrease) were observed when analysis of activation markers on CD4+ and CD8+ T lymphocytes were performed on both young and old donors. Since the effects were really small, we

performed from 5 up to 8 replications of T lymphocyte phenotypical analysis, using cells from the same donor.

Results obtained from replications did not confirm the data previously obtained, thus suggesting that such small significant effects must be considered at the noise level of the statistical analysis. Moreover, we performed a more sophisticated analysis on fluorescence intensity in order to verify if the number of molecular markers could be changed in RF exposed cells in comparison with sham exposed cells. We found that in T helper lymphocytes from elderly, but not from young donors, and exposed to Talk modulated RF-EMF, CD95 molecules shifted significantly their fluorescence from bright to dim, as reported in Figure 96. This effect means that in the exposed cells the number of molecular markers on membrane surface was slightly decreased (around 9%).

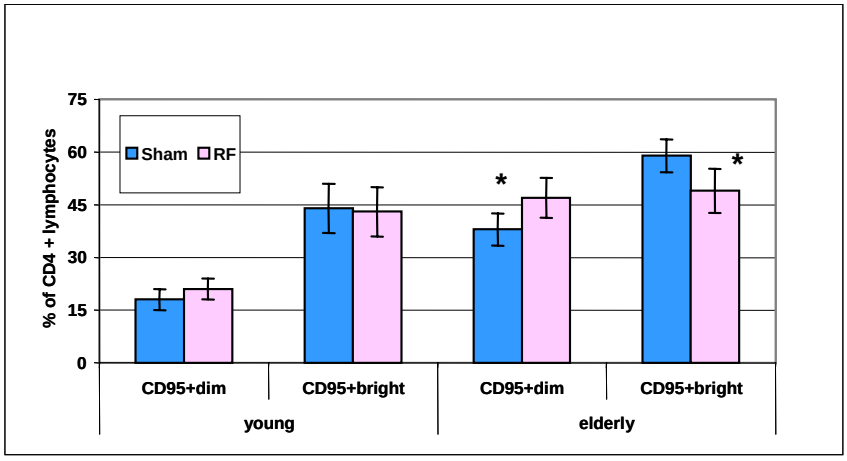


Figure 100. Analysis of fluorescence brightness of proliferating CD4+CD95+ from 10 young and 8 elderly donors, after sham and talk modulated RF-EMF exposure (2W/kg). * = $p < 0.05$. Data are represented as mean ± s.e.

RF-EMF (DTX) may inhibit the production of IL-1beta in human lymphocytes, but did not affect the production of IL 6.

The results obtained showed no significant differences between cells sham-exposed or differently modulated RF-EMF exposed, except in the case of IL-1beta. Indeed, we found a decrease of IL-1beta production (around 13%) in CD3-stimulated PBMCs exposed to DTX modulated RF-EMF in comparison with sham-exposed cells. As demonstrated in Figure 101, the decrease observed was statistically significant on 6 experiments performed. This result was not found when PBMCs were exposed to Talk modulated RF-EMF or using the other stimulus.

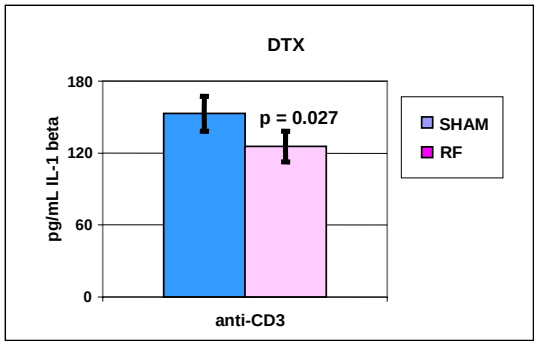


Figure 101. Effect of DTX modulated RF (SAR 1.4 W/kg) on IL-1 beta production in CD3-stimulated PBMCs.

RF-EMF did not affect thymocyte differentiation.

HTOC were performed in order to assess *in vitro* phenotypical differentiation and apoptotic phenomena due to negative selection, which usually occurs in vivo inside the thymus. Thus, different subsets of thymocytes were analysed such as CD71+CD4-CD8-, CD3-/+CD4+CD8+, abTCR-/+CD4+CD8+, gdTCR-/+CD4-CD8-, CD3+CD4+CD8-, CD3+CD4CD8+, abTCR+CD4+CD8-, abTCR+ CD4-CD8+ cells. Each population represents a different phase of development, which was monitored before the exposure and at the end of culture, in presence or absence of RF-EMF. Thymocyte apoptosis was assessed with two different methods, in the same conditions, but the data obtained from 6 human thymus on thymocyte differentiation and apoptosis did not suggest positive results on both the endpoints. Actually, a small increase (4%) of double positive thymocytes (CD4+CD8+) was found in RF-EMF-exposed cultures in comparison with sham-exposed tissue fragments. Also in this case the effect is of the order of the standard error thus we consider these results irrelevant.

3.2.2.3 Human promyelocytic cell line HL-60 (Participant 2)

RF-EMF did not affect the cell cycle of HL-60 cells as shown by flow cytometric analysis.

See 3.2.1.1

RF-EMF did not affect the growth behaviour of HL-60 cells with respect to growth velocity and DNA synthesis.

See 3.2.1.1

Indicators for HL-60 cell growth were the proliferation rate, reflected by the cellular doubling time, and the synthesis of the enzyme thymidine kinase (TK). The enzyme thymidine kinase plays an important role in DNA synthesis. It has been well established that the cellular activity of thymidine kinase is correlated with the growth rate of cells (Johnson et al. 1982). Its relation to the cell cycle has been shown in previous studies (Chang 1990; Kit 1976; Pelka-Fleischer et al. 1987; Piper et al. 1980).

Cellular doubling time

Cellular growth behaviour with respect to growth velocity was assessed by determination of the cellular doubling time. Cellular doubling time of HL-60 cells following RF-field exposure (1800 MHz, 24h) for different SARs (continuous wave, 0.2, 1.0, 1.3, 1.6, 2.0 and 3.0 W/kg) and different signal modulations (continuous wave, C.W., 5 min on/10 min off, GSM-217Hz, GSM-Talk) was compared to controls and sham-exposure. No alteration of the cellular doubling time was observed for any of the different SARs or signal modulations tested (Figures 101, 102). The value of the doubling time for the control was 20.8 ± 2.8 h. Calculation of the average HL-60 doubling time after exposure to all SAR levels tested revealed a value of 22.0 ± 3.8 h (n=21) versus that for all sham-exposed cells: 21.6 ± 4.5 hours (n=21). On the other hand the calculation of the average HL-60 doubling time after exposure to all signal modulations tested at SAR = 1.3 W/kg revealed a value of 21.3 ± 4.2 h (n=14) versus that for all sham-exposed cells: 21.7 ± 4.5 h (n=14).

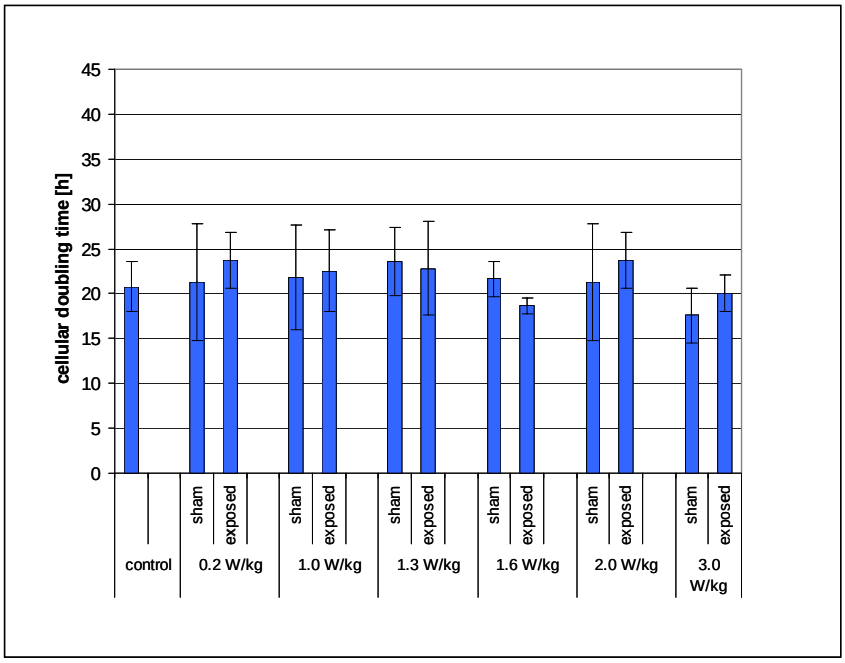


Figure 102. Effect of RF-field exposure (1800 MHz, continuous wave, SAR 0.2, 1.0, 1.3, 1.6, 2.0 and 3.0 W/kg, 24h) on HL-60 cell growth with respect to growth velocity compared to control and sham-exposure, determined by the cellular doubling time. Each bar represents the mean ± SD of results obtained in at least three independent experiments, except for control (n=6) and SAR 1.3 W/kg (n=6).

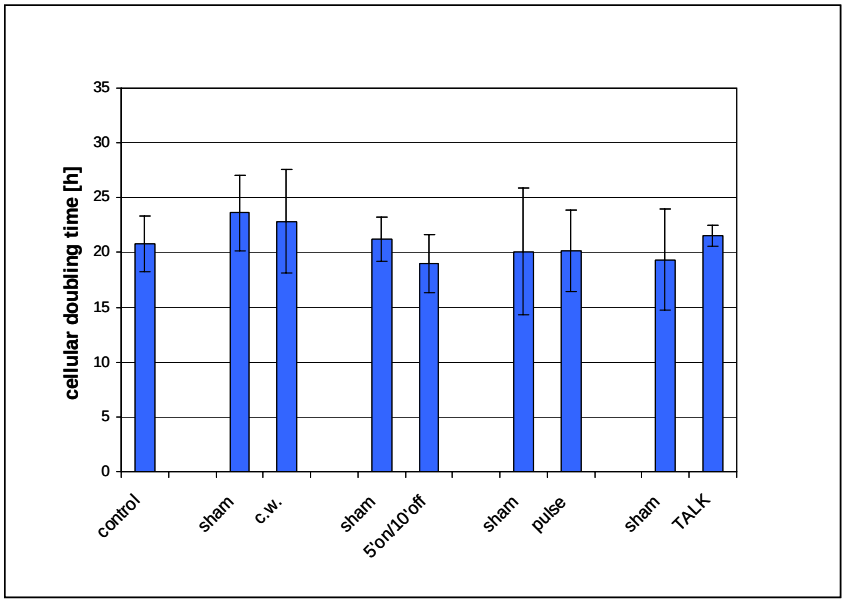


Figure 103. Effect of RF-field exposure (1800 MHz, different signal modulations, SAR 1.3 W/kg, 24h) on HL-60 cell growth with respect to growth velocity compared to control and sham-exposure, determined by the cellular doubling time. Each bar represents the mean ± SD of results obtained in at least three independent experiments, except for control (n=6), continuous wave (n=6) and GSM-Talk (n=2).

Thymidine kinase (TK) activity

Intracellular thymidine kinase (TK) activities were determined by radioenzyme assay with ^{125}I-deoxyuridine monophosphate as substrate (Prolifigen® TK-REA, AB Sangtec Medical, Bromma, Sweden). The level of radioactivity is directly proportional to the enzyme activity, the TK value is calculated from the standard curve and expressed as U/l.

TK activities of HL-60 cells following RF-field exposure (1800 MHz, continuous wave, 1.3 W/kg, 24 h) was compared to control and sham-exposure. Table 24 represent levels of thymidine kinase activities for two independent experiments. No changes in intracellular TK activities were found in HL-60 cells following RF-field exposure compared to control and sham-exposure. In summary, the growth behaviour of HL-60 cells with respect to growth velocity and DNA synthesis are not altered by RF-EMF exposure compared to control and sham-exposure using the assays described above.

Table 24. Thymidine kinase (TK) activity in HL-60 cells after exposure to RF-field (1800 MHz, continuous wave, 1.3 W/kg, 24h), compared to control and sham-exposed cells

Group	thymidine kinase activity [U/l]	
	exp. 1	exp. 2
control	121.4	126.8
sham	151.1	116.3
RF-field	121.9	118.4

3.2.2.4 Mouse embryonic stem cells (Participant 4)

RF-EMF did not induce cardiac differentiation of R1 ES cells and cardiac differentiation and proliferation of P19 EC cells, but may affect the bcl-2 mediated apoptotic pathway in ES-cell derived neural progenitors and neuronal differentiation by inhibiting nurr-1 and TH transcription.

See 3.2.4.1

3.2.2.5 Summary (Participant 1)

Our data did not reveal a significant effect of RF-EMF on proliferation and differentiation of various cell systems such as neuroblastoma cells (NB69) (see 3.2.2.1), R1-embryonic stem cells and embryonic cancer cells (P19) (3.2.2.4 and 3.2.4.1), human lymphocytes and human thymocytes (3.2.2.2) and HL-60 cells (3.2.2.3), though some effects on the differentiation process in neural stem cells were observed (see 3.2.2.1). These effects may be of indirect nature possibly through modulation of the expression of various genes and proteins. With respect to neural stem cells, RF-EMF may affect proliferation and differentiation via up-regulation of bcl-2 which mediates the apoptotic pathway and via inhibiting nurr-1 and TH transcription (see 3.2.2.4 and 3.2.4.1)

3.2.3 Apoptosis

3.2.3.1 Brain cells of different origin and human monocytes (Participant 9)

RF-EMF did not affect apoptosis in neuronal cells.

Spontaneous apoptosis was found higher in sensitive primary nerve cells than in the human neuroblastoma cell line SH-SY5Y) (around 20% versus 10% using the $DiOC_6$ dye). A high percentage of spontaneous apoptosis in granule cells was found using Annexin V staining compared to $DiOC_6$ staining. This observed difference seems to be cell type-dependent and it appeared difficult, in this case, to

correlate information given by these two dyes. Besides this technical consideration, exposure of primary granule cells to GSM-900 at 2.0 W/kg for one hour did not induce apoptosis as shown by the time-kinetics up to 24 hours after exposure (Figure 104). The same observation was made with the SH-SY5Y cell line. To test longer time exposure, we decided to expose granule cells and SH-SY5Y to GSM-900 during 24 hours and to quantify apoptosis at the end of exposure. In these exposure conditions, no significant difference was observed between sham and exposed cells in both cases (Figure 105).

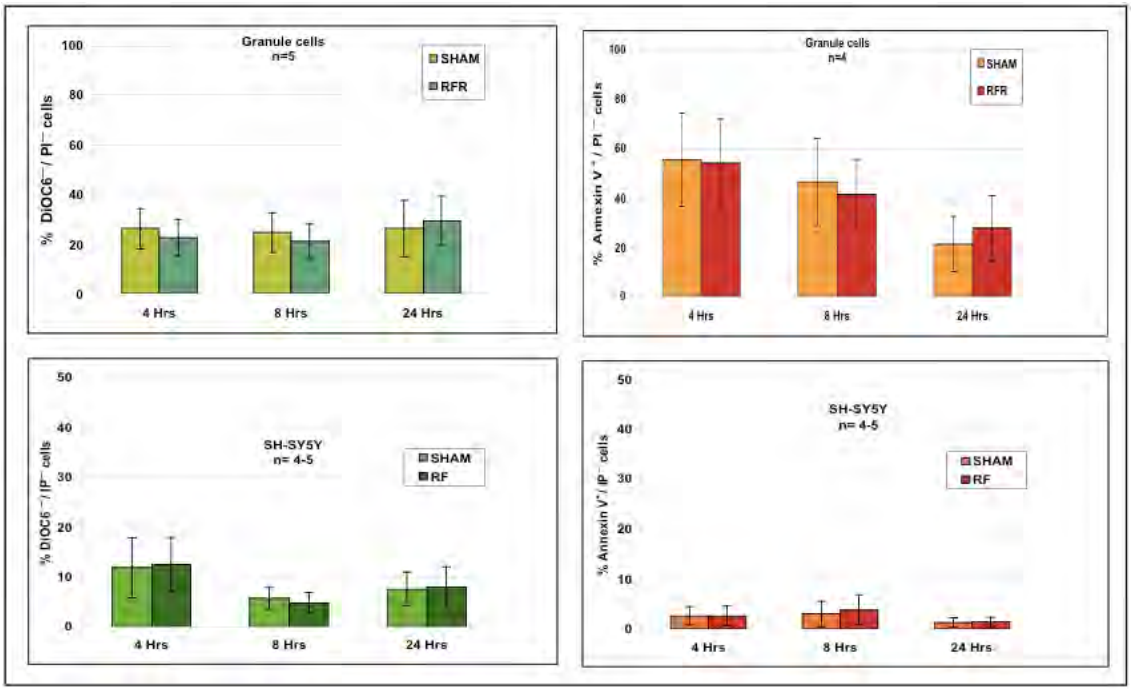


Figure 104. Effect of a one-hour exposure to GSM-900 on apoptosis in nerve cells. Results are expressed as the percentage of cells with depolarised mitochondrial transmembrane potential ($DIOC6^-/PI^-$, left panel) and apoptotic cells (ANX^+/PI^-, right panel) after exposure of primary granule cells (upper panels) and human neuronal cells (lower panels) to frame GSM-900 signal at 2.0 W/kg for 1 hour. Apoptosis was measured 4, 8 and 24 hours after the exposure began. Data are presented as the mean ± SEM of 4 to 5 independent and blind experiments.

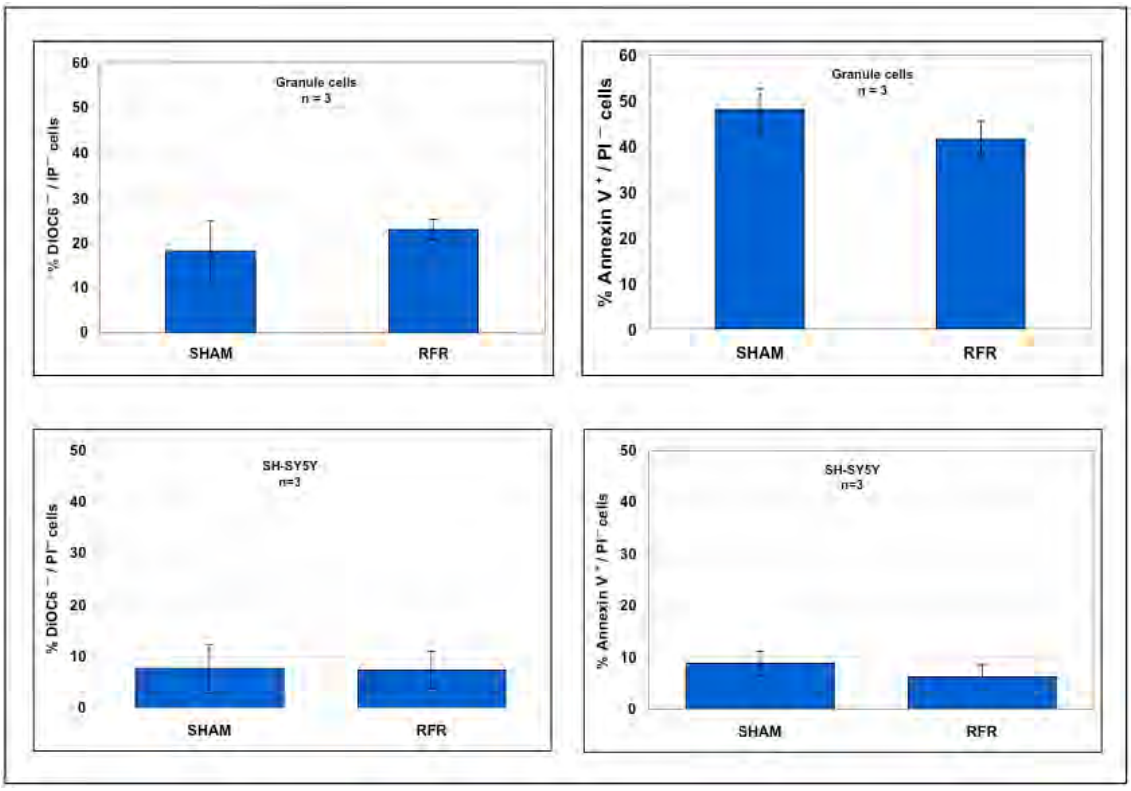


Figure 105. Effect of a 24-hour exposure to GSM-900 on apoptosis in nerve cells. Results are expressed as the percentage of cells with depolarised mitochondrial transmembrane potential ($DIOC6^-/PI^-$, left panel) and apoptotic cells (ANX^+/PI^{--}, right panel) after exposure of rat primary neurons (upper panels) and human SH-SY5Y neuroblastoma cells (lower panels) to frame GSM-900 signal at 2.0 W/kg for 24 hours. Apoptosis was measured immediately after exposure. Mean ± SEM of 3 independent, blinded experiments are presented.

We conclude from our results that granule cells and SH-SY5Y cells are not sensitive to GSM-900 exposure for up to 24 hours.

RF-EMF did not affect apoptosis in astrocytic cells.

Primary cultures of astrocytes and human U87 glioblastoma cells were sham-exposed or exposed to GSM-900 for one hour at 2.0 W/kg and apoptosis was followed up during 24 hours in the conditions described previously. Figure 105 shows the data obtained using the primary culture and the U87 cell line. In the two cell types, no difference in the number of cells with depolarised mitochondrial potential or in AnnexinV-positive cells could be evidenced after exposure to GSM-900. An increase in apoptotic astrocytic cell population measured with $DiOC_6$ was noticed, correlated to the time spent in culture. Nevertheless, no significant difference was observed between sham- and GSM-900 exposed cells. In the exposure conditions tested, no demonstration of a significant effect of GSM-900 signal on primary astrocytes or glioblastoma U87 cells could be made.

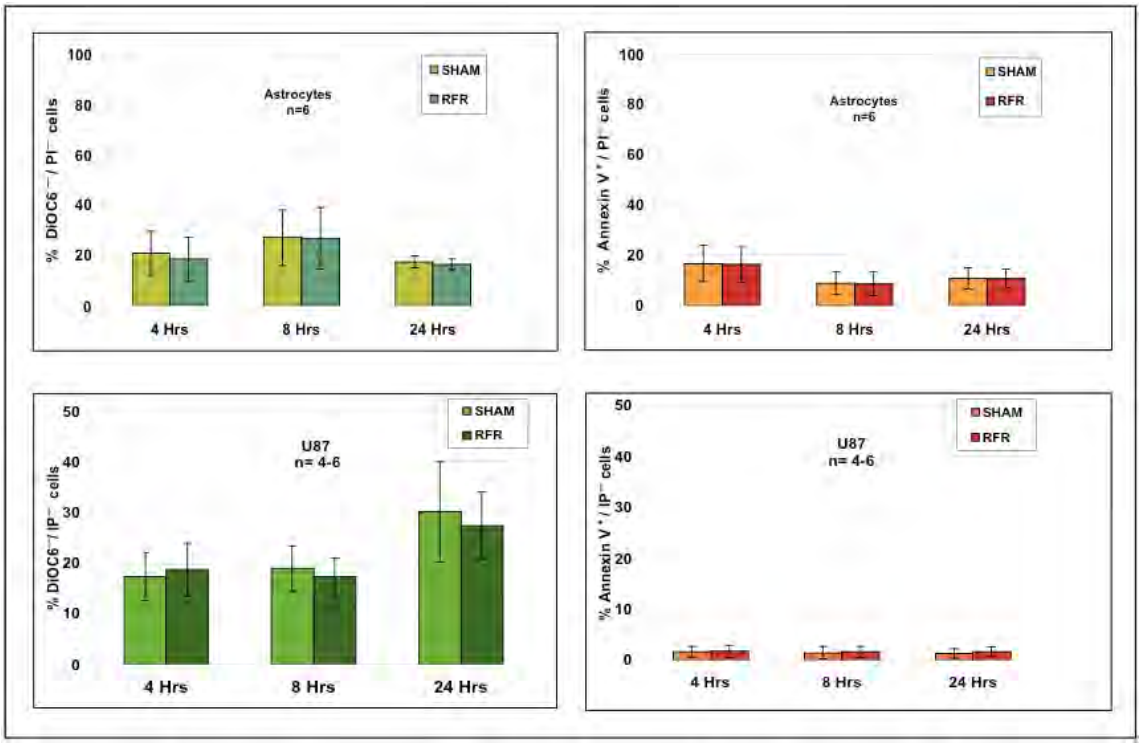


Figure 106. Effect of a 1-hour exposure to GSM-900 on apoptosis in astrocytic cells. Results are expressed as the percentage of cells with depolarised mitochondrial transmembrane potential ($DIOC6^-/PI^-$, left panel) and apoptotic cells (ANX^+/PI^-, right panel) after exposure of primary astrocytes (upper panels) and human astrocytic cells (lower panels) to GSM-900 signal at 2.0 W/kg for 1 hour. Apoptosis was measured 4, 8 and 24 hours after the exposure began. Data from 4 to 6 independent experiments are presented as the Mean ± SEM.

In summary, an immediate or delayed effect of RF-EMF on apoptosis in rat primary cells and human cell lines could not be demonstrated.

RF-EMF did not influence apoptosis in immune cells.

The ability of the human U937 promyeloma cells to undergo apoptosis was tested by using camptothecin as a positive control (Figure 107). We show a significant 4- to 8-fold increase ($p<0.01$), depending of the marker of apoptosis used, in U937 cells treated for 4 hours with CPT (4 µg/ml).

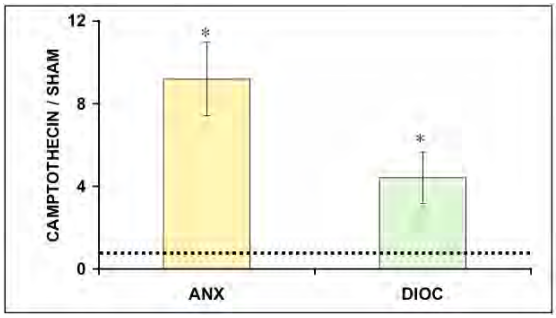


Figure 107. Effect of camptothecin treatment on apoptosis in human U937 cells. The results are expressed as the ratio of apoptotic cells (ANX^+/PI^-, left panel) and cells with depolarised mitochondrial transmembrane potential ($DIOC^-/PI^-$, right panel) in camptothecin-treated versus sham-exposed U937 promyeloma cells. U937 cells were sham-exposed for 1 hour and then treated for 4-hours with camptothecin (4 µg/ml). Data are presented as the mean ± SEM of 5 independent experiments. *=$p<0.01$

Sham-sham experiments (n=3-4) showed that inter-incubator (incubator used for RFR exposure versus incubator used for sham-exposure) variation of U937 apoptotic cells was around 1.1 ± 0.5 after one hour and 1.2 ± 0.6 when the cells were placed back in a control incubator for 4 hours after the one hour spent in the dedicated incubators (Figure 108). The effect of CPT treatment was very similar when the samples were put for one hour in either dedicated incubator. We therefore concluded that both dedicated incubators were equivalent within a range of 50% of ratio variation and that an at least two-fold increase in apoptosis in RFR-exposed cells could be considered as significant.

No statistically significant influence of GSM-900 could be evidenced on spontaneous apoptosis of U937 cells (Figure 109), when they were exposed for one hour at a SAR of 0.7 W/kg and even at the highest SAR tested (2.0 W/kg). No difference could be detected either immediately after exposure or after a 4-hour resting period in a control incubator. Hence, no delayed effect of GSM-900 on apoptosis could be evidenced. A longer exposure duration, i.e. 48 hours, to GSM-900 at the lowest SAR tested (0.7 W/kg) was also not able to alter spontaneous apoptosis in the human cell line.

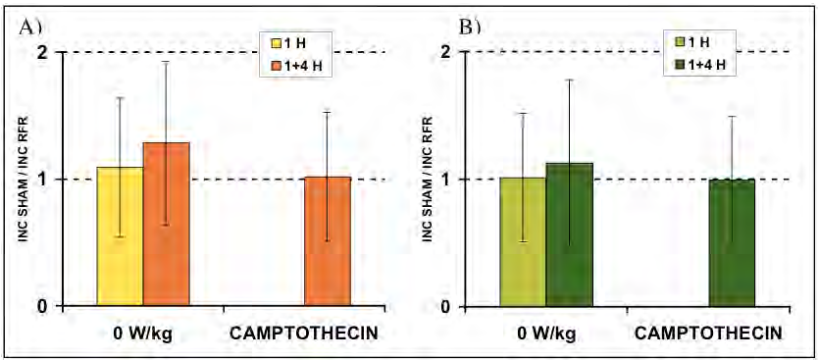


Figure 108. Comparison of occurrence of apoptosis in U937 cells in both dedicated incubators. Results are expressed as the ratio between apoptotic U937 cells in the incubator used for RFR exposure and apoptotic U937 cells in incubator used for sham-exposure. Sham-sham exposure lasted 1 hour and cells were harvested either immediately (1 H) or after an additional 4-hour resting period or campthotecin treatment (1+4 H). A) apoptotic U937 cells (ANX^+/PI^-) and B) U937 cells with depolarised mitochondrial transmembrane potential ($DIOC6^-/PI^-$). Data are presented as the mean ± SEM of 3 to 4 independent experiments.

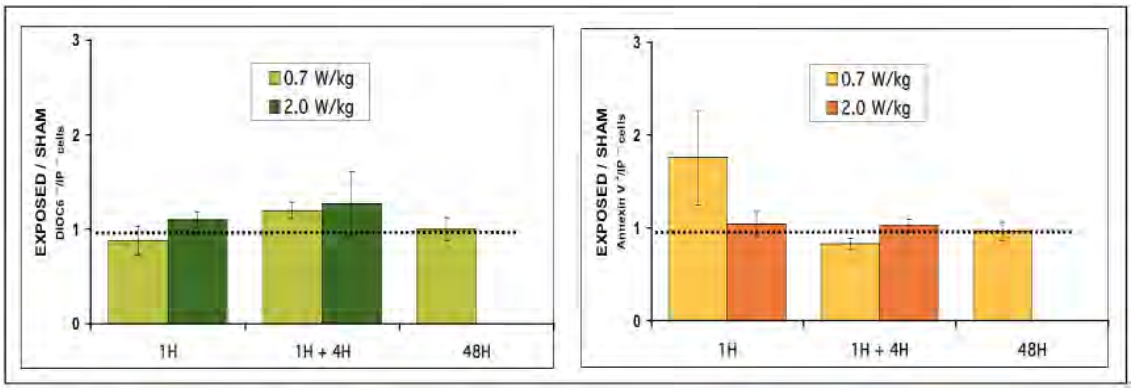


Figure 109. Effect of GSM-900 exposure on apoptosis in human U937 cells. Results are expressed as the ratio of cells with depolarised mitochondrial transmembrane potential ($DIOC6^-/PI^-$, left panel) and apoptotic cells (ANX^+/PI^-, right panel) in GSM-900- versus Sham-exposed U937 cells. U937 cells were exposed to GSM-900 at 0.7 W/kg (n=7) and 2.0 W/kg (n=6) for 1 hour, for 1 hour followed by a 4-hour resting period or for 48 hours. Data are presented as the mean ± SEM.

RF-EMF did not influence chemically-induced apoptosis in immune cells.

When possible interaction between RFR and camptothecin was tested, we show that a one-hour treatment with GSM-900 at either 0 .7 or 2.0 W/kg was not able to influence camptothecin-induced apoptosis (Figure 110).

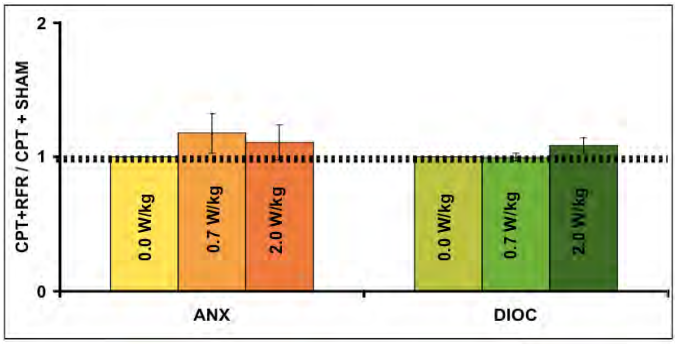


Figure 110. Effect of GSM-900 exposure on camptothecin (CPT)-induced apoptosis. The results are expressed as the ratio of apoptotic cells (ANX $^{+}$/PI $^{-}$, left panel) and cells with depolarised mitochondrial transmembrane potential (DIOC6 $^{-}$/PI $^{-}$, right panel) in GSM-900 plus camptothecin-treated versus GSM-exposed U937 promyeloma cells. U937 cells were sham-exposed or exposure to GSM-900 for 1 hour and then treated for 4-hour with camptothecin (4 µg/ml). The SAR level was 0.7 W/kg (n=7) or 2.0 W/kg (n=6). Data are presented as the mean ± SEM.

In summary, we showed no evidence for an immediate, cumulative or delayed effect of RF-EMF on apoptosis in a human monocytic cell line. We conclude from our results that U937 cells are not sensitive to GSM-900 exposure for up to 48 hours. Taken together, our results strongly suggest that the apoptotic process is not a major biological target for GSM mobile telephony-related signals.

3.2.3.2 Human lymphocytes (Participant 8)

RF-EMF did not affect apoptosis in human lymphocytes.

Negative results were obtained studying spontaneous or dRib-induced apoptosis, when PBMCs were exposed at all the three signal modulations. Moreover, PBMCs from old donors were exposed to Talk modulated RF, but also in this case no effects on spontaneous or oxidative stress-induced apoptosis were found. These results were also confirmed by mitochondrial membrane polarisation, since no differences were noticed in dependence of the exposure and age of donor.

RF-EMF did not increase the Hsp70 level in human lymphocytes after induction of apoptosis.

dRib induces an increase of hsp70 in treated cells in comparison with untreated cells already detectable after 3h of treatment (unpublished data) up to 44 hours of treatment. When we studied RF-EMF effects (1800 MHz, GSM talk signal, 2 W/kg), we did not found any alteration of hsp70 gene product, after 44 hours of intermittent exposure, in PBMCs from 7 young donors, as showed in Figure 111. In this figure it is possible to notice the significant difference between the level of hsp70 in untreated cells versus the dRib-treated cells. Additional analysis were performed in separated lymphocytes and monocytes, but no differences were found between RF-exposed and sham-exposed cell populations.

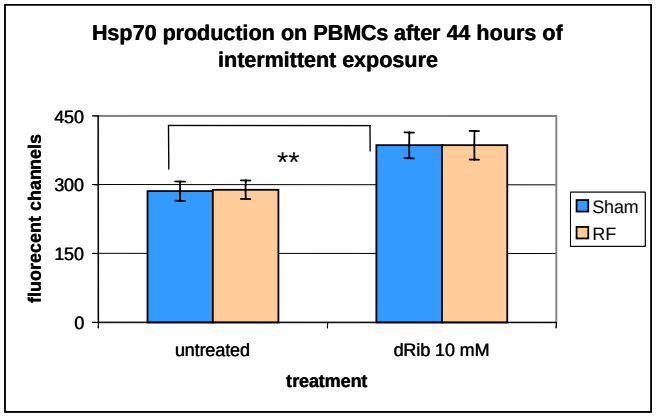


Figure 111. Hsp70 production in untreated and dRib treated PBMCs after 44hours of intermittent exposure. PBMCs were obtained from 7 donors and data are represented as mean of fluorescent channels ± s.e. ** = p< 0.01, untreated PBMCs versus dRib treated PBMCs.

RF-EMF did not affect apoptosis in thymocytes.

Thymocyte apoptosis was assessed with two different methods, in the same conditions, but the data obtained from 6 human thymus on thymocyte apoptosis did not suggest positive results on both the endpoints. Actually, a small increase (4%) of double positive thymocytes (CD4+CD8+) was found in RF-EMF-exposed cultures in comparison with sham-exposed tissue fragments. Also in this case the effect is of the order of the standard error thus we consider these results irrelevant.

3.2.3.3 Human promyelocytic cell line HL-60 (Participant 2)

RF-EMF did not affect apoptosis in HL-60 cells as shown by flow cytometric analysis and the Annexin V and TUNEL assay.

See 3.2.1.1

3.2.3.4 Embryonic stem cells of mice (Participant 4)

RF-EMF exposure may influence the bcl-2 mediated apoptotic pathway in ES-cell derived neural progenitors.

See 3.2.4.1

3.2.3.5 Human endothelian cell lines (Participant 6)

The RF-EMF-induced enhancement of hsp27 phosphorylation as well as the concomitantly RF-EMF-induced down-regulation of proteins of Fas/TNFα suggest that the anti-apoptotic pathway in RF-EMF exposed cell systems may be modified.

See 3.2.4.6

3.2.3.6 Summary (Participant 1)

Our data did not reveal a significant effect of RF-EMF on apoptosis in various cell systems such as brain cells and human monocytes (see 3.2.3.1), human lymphocytes and thymocytes (see 3.2.3.2), human endothelial cells (3.2.4.6) and HL-60 cells (see 3.2.3.3). On the other hand, an indirect effect on apoptosis through modulating the expression of various genes and proteins cannot be excluded at present. Up-

regulation of bcl-2 in differentiating embryonic stem cells (see 3.2.3.4 and 3.2.4.1) and of hsp27 in endothelial cells (see 3.2.4.6) both of which may affect the apoptotic process support such an assumption.

3.2.4 Gene and protein expression

3.2.4.1 Mouse embryonic stem cells (Participant 4)

Loss of p53 function rendered pluripotent ES cells sensitive to RF-EMF after prolonged exposure.

Exposure of undifferentiated cells to GSM-217 signals for 6 hours (Table 25) did not evoke any short-term modification of gene expression patterns neither in wt nor in p53-/- ES cells (Figure 112A). On the other hand, long-term (48 hours) GSM-217 exposure of p53-/- cells during EB development resulted in the up-regulation of transcript levels of 4 out of 6 analysed genes. Whereas c-jun, p21 and c-myc mRNA levels were only transiently up-regulated at early stages (days 2, 5 and 5+2 of EB differentiation, Figure 112B, a prominent induction of hsp70 levels in p53-/- cells was observed throughout the differentiation period. The same experimental protocol was applied to analyse the influence of GSM signals simulating talking and listening phases during a typical conversation (GSM-Talk). However, no changes of gene expression patterns in both wt and p53-/- cells were observed upon the exposure to GSM-Talk signals regardless of the protocol used. These observations indicate that the genetic constitution of cells determined by the p53 function affected cellular responsiveness to GSM-modulated EMF, whereas low frequency components characteristic for GSM-Talk modulation were not responsible for these effects.

Table 25. Conditions of the exposure of p53-proficient and deficient pluripotent embryonic stem cells to RF-EMF and summary of the effects on transcript levels of regulatory genes.

GSM-Basic	
1.5 W/kg (5min on/30 min OFF)	
$p53^{+/+}$ ES cells	**$p53^{-/-}$ ES cells**
6h, (5min ON/30 min OFF) (n=3) no EMF effect	6h, (5min on/30 min OFF) (n=3) no EMF effect
48h, (5min on/30 min OFF) (n=3) no EMF effect	48h, (5min on/30 min OFF) upregulation of hsp70, (n=3) c-jun,c-myc and p21

GSM-Talk (33% GSM-Basic, 66% DTX)	
0.4 W/kg (5min on/30 min OFF)	
$p53^{+/+}$	**$p53^{-/-}$**
6h, (5min on/30 min OFF) (n=3) no EMF effect	6h – (5min on/30 min OFF) (n=3) no EMF effect
48h,(5min on/30 min OFF) (n=3) no EMF effect	48h (5min on/30 min OFF) (n=3) no EMF effect

GSM – DTX (100% DTX)	
0.11W/kg	
$p53^{+/+}$	**$p53^{-/-}$**
6h, (5min on/30 min OFF) (n=3) no EMF effect	6h, (5min on/30 min OFF) (n=3) no EMF effect

n- number of experiments

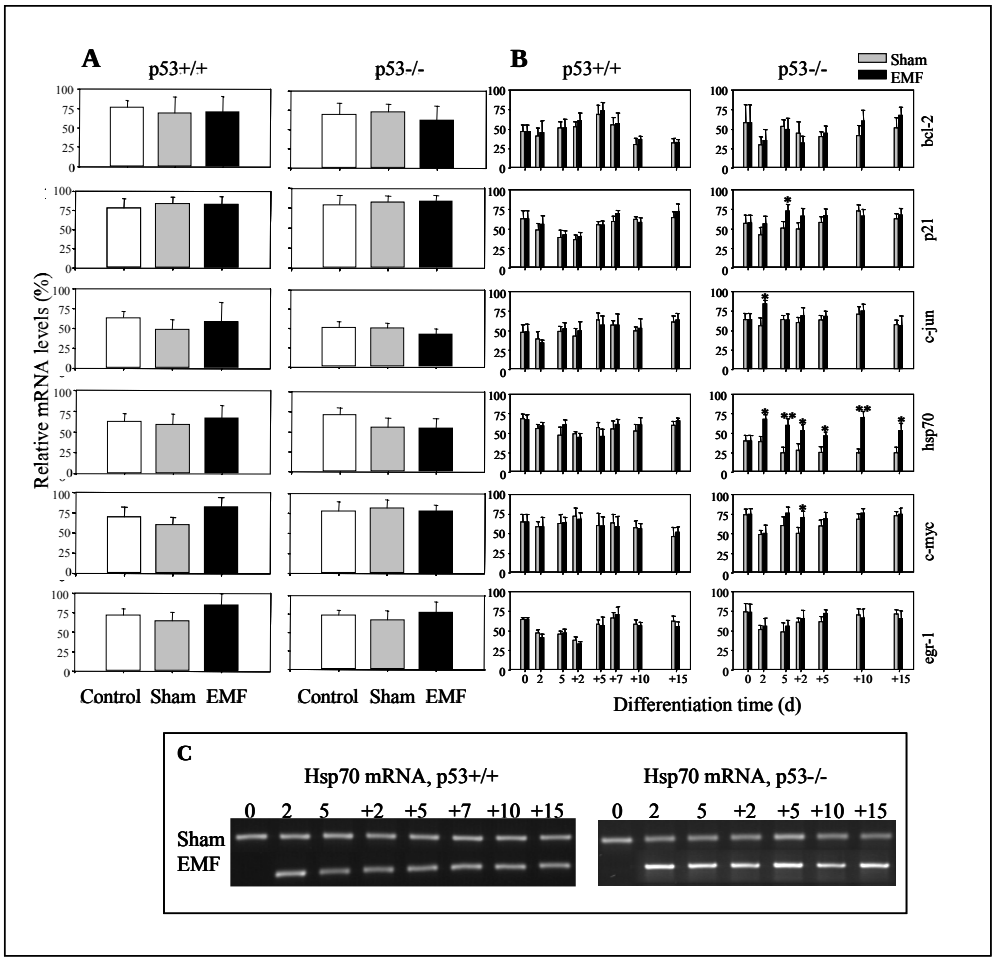


Figure 112. Relative mRNA levels of genes encoding bcl-2, p21, c-jun, hsp70, c-myc and egr-1 in p53-deficient ($p53^{-/-}$) and wild-type ($p53^{+/+}$) D3 ES cell-derived EBs after 6 (A) and 48 (B) hours GSM-217 Hz exposure. GSM-217 exposure resulted in a significant and long-lasting upregulation of hp70 transcripts (C) paralleled by a temporary upregulation of c-myc, c-jun and p21 levels in differentiating $p^{-/-}$ ES cells after 48h exposure (B), but not after 6h (A) exposure. Error bars represent standard deviations. Statistical significance was tested by the Student's t-test for significance levels of 1 and 5% (**, $p<0.01$, *,<0.05)

RF-EMF did not influence cardiac differentiation and gene expression levels in R1 ES cells.

For the evaluation of embryotoxic effects of chemical compounds in vitro, the mouse embryonic stem cell test, EST (Spielmann 1997), studying cardiac differentiation of ES cells as endpoint has been established. Therefore, we further analysed effects of EMF on cardiac differentiation in R1 ES and P19 EC cells. R1-derived EBs were exposed to EMF for 5 days and the cells were analysed after further differentiation. Similarly to D3 wt cells, no effect of GSM-217 signals on the expression of the regulatory genes including p53 was observed in R1 wt cells. Moreover, no significant differences in cardiac differentiation were found between sham- and EMF-exposed variants during EB differentiation. The results of morphological investigations were confirmed by RT-PCR analyses, where no differences in cardiac α-MHC mRNA levels between sham- and EMF-exposed variants were observed.

RF-EMF did not induce cardiac differentiation and gene expression and the proliferation of P19 EC cells.

Contrary to ES cells, which spontaneously differentiate into the cardiac lineage, differentiation of P19 cells has to be stimulated by external differentiation factors, i.e. DMSO or retinoic acid. To elucidate, whether EMF signals interfere with DMSO-induced cardiac differentiation, undifferentiated P19 EC cells were exposed to GSM-217 signals for 22 and 40h, respectively, and differentiated in the absence or

presence of 1% DMSO. The mRNA levels of regulatory genes were not affected by EMF exposition, and no significant differences were found between EMF-, sham-exposed and control variants of undifferentiated P19 cells, respectively.

Without DMSO induction, only low levels (5-10%) of spontaneous cardiac differentiation were found. By differentiation induction with DMSO during the first 48 hours of EB development, the differentiation of spontaneously beating cardiac cells was increased to a maximum level of 90% in both variants. Exposure of P19 cells to GSM-217 signals for 22 and 40 hours did not result in significant changes of cardiac differentiation suggesting that EMF had no effects on spontaneous or DMSO-induced cardiogenesis. Furthermore, undifferentiated EC cells were EMF- (GSM-217) and sham-exposed at a SAR value of 2.0 W/kg for 22 or 40 hours, and the lengths of cell cycle phases were analysed by flow cytometry. In both cases, no differences in the distribution of cells in G1, S or G2/M phases were observed between sham- and EMF-exposed variants.

RF-EMF exposure may affect the bcl-2 mediated apoptotic pathway in ES-cell derived neural progenitors and neuronal differentiation by inhibiting nurr-1 and TH transcription.

We used an experimental protocol that has been shown to be efficient for differentiation induction of ES cells into the neural lineage (Figure 6) for our experiments aimed at defining the influence of RF-EMF (1.71 GHz, 1.5 W/kg, 5 min on/30 min off) as shown in Table 26. Neural progenitors were analysed at stage 4+4d to 4+6d, where nestin-positive cells were detected in ca. 60-80% of the cells. The exposure conditions applied for 6 or 48 hours are shown in Table 23. The analysis of hsp70, bax and p21 mRNA levels after RF-EMF exposure of neural progenitor cells did not provide evidence of gene expression changes. Bcl-2 was significantly up-regulated after the RF-EMF exposure at the terminal stage 4+23d (Figure 109, $p<0.01$). In addition, we investigated the effect of RF-EMF exposure on GADD45 transcript levels and found that GADD45 mRNA levels were significantly up-regulated at the same stage of differentiation at 4+23d (Figure 113, $p<0.05$). The data on the influence of RF signals on gene expression pattern in differentiating neuronal cells revealed statistically significant down-regulation of nurr-1 at stage 4d+11 and TH at terminal stage 4d+23d, but no clear shifts in transcript levels of the tissue specific genes GFAP, Nestin and En-1 (Figure 113). Quantitative RT-PCR with specific primers and TaqMan probe confirmed the up-regulation of GADD45 at 4+23d stage, but not for bcl-2. According to the Q-RT-PCR data nurr-1 was down-regulated both at stage 4+7d and 4+11d, but the decrease in mRNA levels was statistically significant for stage 4+7d (Figure 114).

Table 26. Conditions of the exposure of neuronal progenitor cells to PL-MF or RF-EMF.

Intermittent exposure (5min on/30 min off)			
48h, GSM 217 Hz (1.71 GHz)		**6h, GSM 217 Hz (1.71 GHz**	
1.5 W/kg	Alterations of GADD45 transcript levels Down-regulation of nurr-1. No effect on DNA break induction (n=7)	1.5 W/kg	Low, but statistically significant induction of double-strand DNA breaks (n=7)

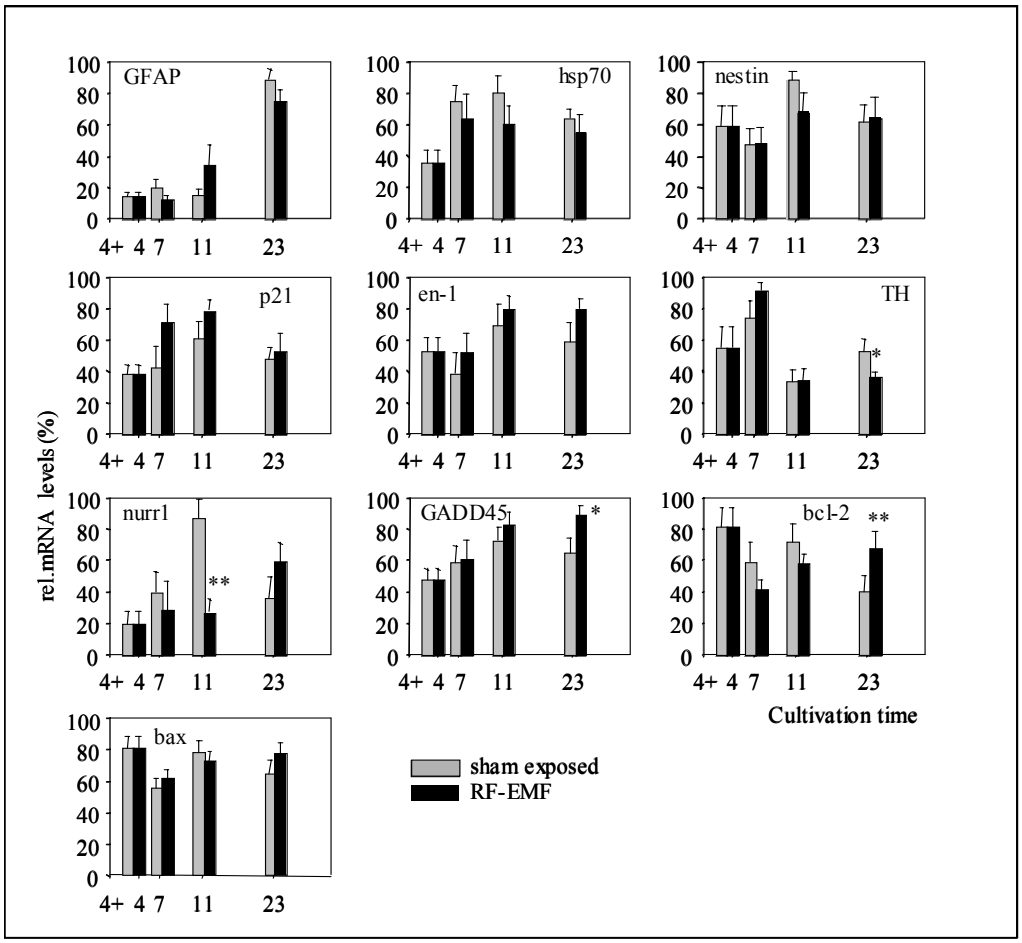


Figure 113. Relative mRNA levels analysed by semi-quantitative RT-PCR of genes encoding bcl-2, p21, c-jun, hsp70, c-myc and egr-1 in p53-deficient (p53-/-) and wild-type (p53+/+) D3 ES cell-derived EBs and EB outgrowths after 6 (A) and 48 hours (B) exposure to GSM-Talk. No effects of GSM-Talk on gene expression levels in ES cells were observed.

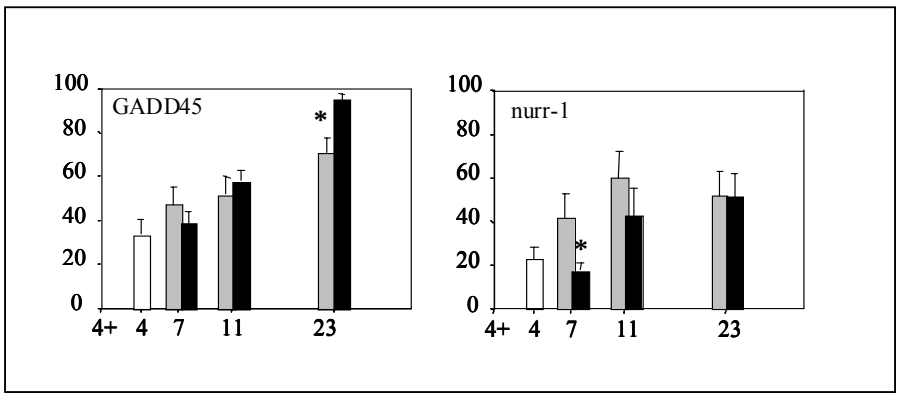


Figure 114. Quantitative RT-PCR with specific primers and Taqman probe for estimation of relative mRNA levels of the growth arrest and DNA damage inducible gene GADD45 and the nurr-1 gene involved in neuronal differentiation in ES-derived neural progenitors after 48 hours RF - EMF (GSM signal 217-Hz) exposure (1.5 W/kg, 1.71 GHz, intermittency 5 min ON/30 min OFF), at stage 4+4d - 4+6d. EMF exposure resulted in a significant transcript down-regulation, followed by up-regulation of GADD45 and down-regulation of nurr-1 at stage 4+7d. Error bars represent standard deviations. Statistical significance was tested by the Student's two-tailed paired t-test for a significance level of 5% (*, $p < 0.05$).

3.2.4.2 Human neuroblastoma cell line NB69 and neural stem cells (NSC) (Participant 5)

RF-EMF (GSM-CW and GSM-Basic) interfered with the expression of FGF receptors in NB69 human neuroblastoma cells.

In a first experiment the cellular response to a chemical promoter of differentiation was characterised. Immunocytochemical staining using antibodies against phenotype-specific antigens revealed that NB69 cells contain the neuroblast-specific protein ßIII-tubulin, but not the neuroepithelial marker nestin, which is present in immature progenitors and in some neuroblastoma cells (Kashima et al. 1995). Untreated NB69 cells remain in an undifferentiated state. Immunocytochemistry for FGFR1-3 revealed the three types of receptors in the human neuroblastoma cell line. On day 3 after plating approximately 70% of cells expressed R1, whereas FGFR3 and FGFR2 were present in a smaller proportion of cells, 30% and 20%, respectively (Figure 115, grey colour). Basic fibroblast growth factor (bFGF), which induces morphological changes including neurite extension at a 20-ng/ml concentration, was used as a positive control for the subsequent EM treatments. This growth factor reduced the number of NB69 cells expressing FGF receptors R1, R2 and R3. Such an effect was accompanied with changes in the cellular morphology associated to differentiate phenotypes. These changes included increased neural outgrowth, neural microtubule network and cell surface. As shown in this Figure 115, the 24-hour treatment with bFGF induced a consistent reduction in the percent of FGFR positive cells for the 3 receptors tested ($p<0.0001$; ANOVA).

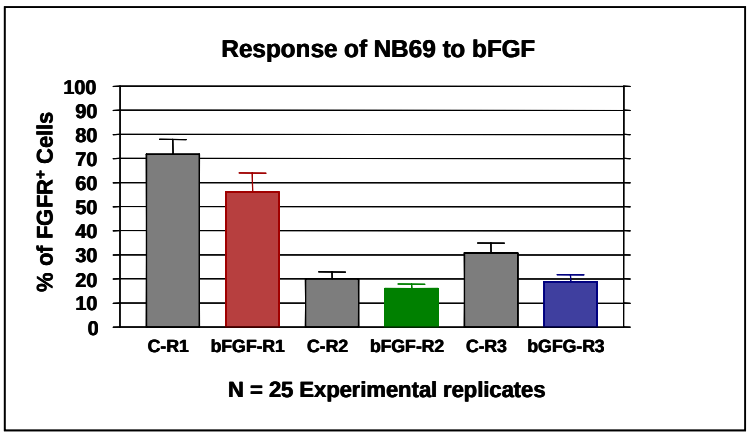


Figure 115. Expression of FGF Receptors 1, 2 and 3 in NB69 cells after a 24-hours treatment with 20-ng/ml bFGF. See text for details.

In the following experimental series, the cellular responsiveness to a differentiation-promoter was confirmed through treatment with bFGF, which induced a reduction of the percent of cells expressing FGF receptors R1, R2 and R3. Such an effect was associated with cell enlargement and neurite arborisation. In contrast, the treatment with GSM-Talk (SAR of 2 W/kg) signal alone (N= 4 experimental replicates), does not modify significantly the normal expression of the FGF protein receptors R1, R2 and R3 in NB69 cells (Figure 116). However, the results of the combined treatment bFGF + GSM-Talk signal show that the EMF seems to antagonize the significant reduction of FGFR-2 expression induced by bFGF. This indicates that the GSM-Talk signal might interfere with some of the cellular responses to bFGF.

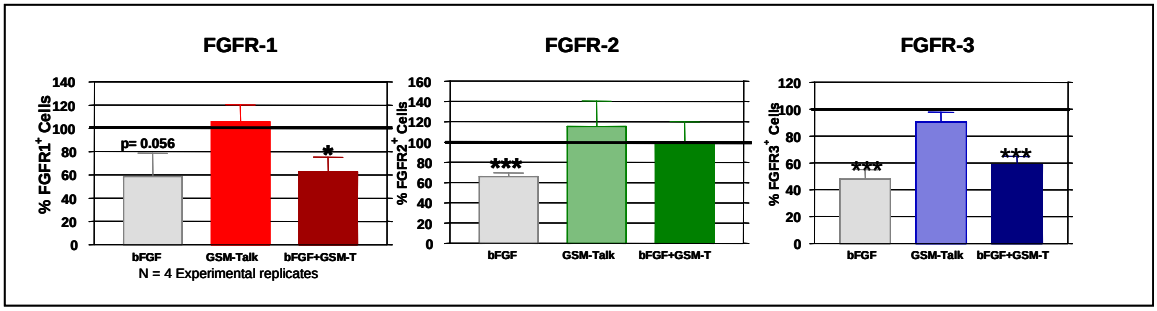


Figure 116. Response of NB69 cells to GSM-Talk signal. Percent of cells expressing FGF Receptors R1, R2 and R3. The treatment with bFGF induced significant reductions in the percent of cells expressing the receptors. Data normalised over the respective controls. (*, p<0.05; **, p<0.01; ANOVA followed by Student's T test).

When administered alone, the exposure to GSM 1800-Basic signal at a 2 W/kg SAR was found to induce a decrease in the number of cells expressing the FGFR-1 (15% reduction vs. controls, Figure 117) and photomicrographs of NB69 cell cultures) without affecting significantly the number of cells expressing receptors R2 and R3. The magnitude of the effect on R1 was equivalent to that induced by bFGF at a 20 ng/ml concentration. However, unlike bFGF, the exposure to GSM-Basic alone did not provoke changes in the cellular morphology. Provided that, as described previously, the GSM-Basic treatment does not induces significant changes in the total cell number or the cell viability, the present results indicate that the GSM-Basic-induced effect on FGFR-1 is not due to a reduction in the number of cells, but to a loss of expression of this receptor.

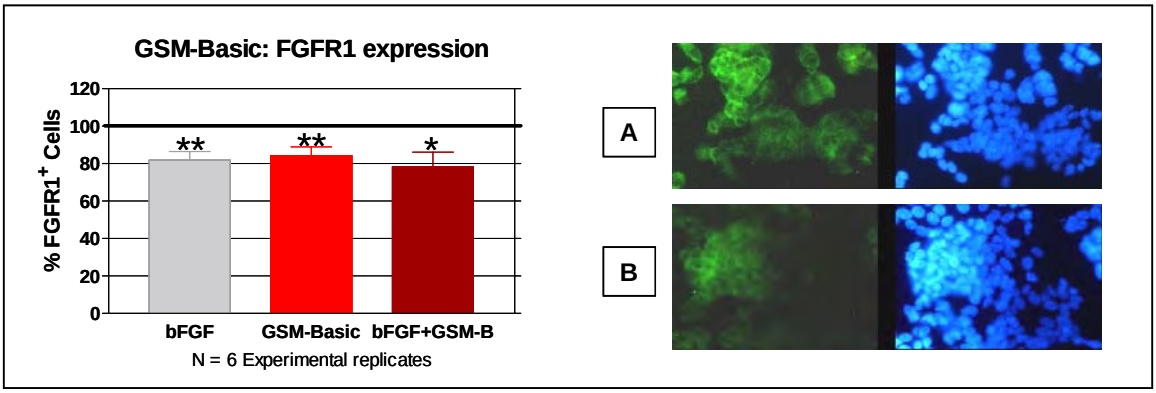


Figure 117. Photomicrographs of NB69 cell cultures: A, control; B, exposed to GSM-Basic signal. Immunocytochemistry for FGFR1. The image analysis showed a statistically significant reduction in the protein expression in the exposed cells (left). The matched fields (right) show the total cells stained with Hoechst.

The treatment with RF-CW signal (SAR 2 W/kg) induced effects on the expression of FGFR-1 equivalent to those induced by the GSM-Basic signal (Figure 118A), whereas the exposure to GSM-DTX signal at a lower SAR (1 W/kg) did not modify significantly the normal expression of the FGF protein receptors R1 in NB69 cells (Figure 118B).

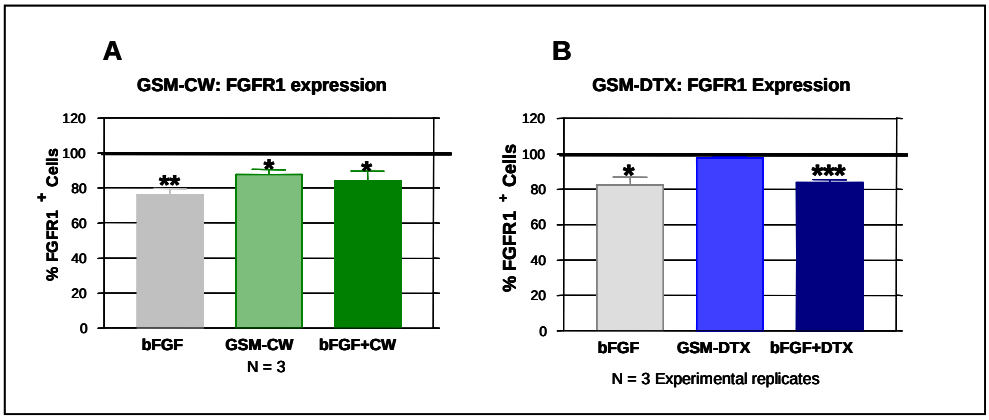


Figure 118. FGFR1 expression in NB69 cells after exposure to: (A) RF-CW signal, 2 W/kg and (B) GSM-DTX signal, 1 W/kg. Only RF-CW induced a significant response. *, $p<0.05$; **, $p<0.01$, ***, $p<0.001$ (ANOVA followed by Student's T test for unpaired data).

Taken together, the results on the expression of FGFRs in NB69 cells exposed to GSM-1800 MHz signals and RF-CW signals suggest that (1) these cells are sensitive to low-SAR signals, (2) the cellular response does not seem to be dependent on the tested low-frequency modulation and (3) the observed response on FGFR1 could be indicative of a EMF-induced promotion of cell differentiation. However, additional studies on the expression of differentiation markers have to be done to confirm this hypothesis. (4) The GSM signal does not seem to interfere significantly with the cellular response to bFGF.

RF-EMF affected the expression of FGF receptors in neural stem cells (NSC).

In order to enhance expansion of NS precursor cells, neurospheres were seeded onto adherent substrate and treated with the mitogen epidermal growth factor (EGF) during the first 3 days in culture. After this period the EGF was withdrawn, and cells grew in a defined medium, which promoted differentiation processes to neurons, astrocytes and oligodendrocytes. Between 2 h and 3 days cultures comprised mainly nestin-positive, undifferentiated precursors. At later stages, the total number of cells dropped, paralleling to a gradual loss of nestin content, and an enhancement in the differentiation processes of neurons, oligodendrocytes and astrocytes.

Immunocytochemistry for FGFR1-3 identified the three types of receptors in the progeny of EGF-expanded NSCs. During the first day after plating, approximately 70% and 50% of the precursors expressed FGFR1 and FGFR2, respectively, whereas FGFR3 was restricted to a less abundant population. At 3 days and thereafter the number of cells exhibiting FGFR1 and FGFR2 decreased gradually, so that at 3 days the percent of FGFR-1 was 35% and at 9 days postplating only approximately 15% of the cell population was immmunopositive for this receptor. FGFR1 immunostaining was preferentially localised in the cytoplasmic compartment, FGFR-3 was found in the cytoplasmic and/or nuclear compartments, and FGFR2 was frequently confined to the nucleus. In situ hybridisation studies on the third day postplating showed high levels of FGFR1 mRNAs in NSC.

On the basis of our previous data showing that the GSM-Basic signal induces a reduction in the percent of cells expressing FGF-R1 in human neuroblastoma cells, we tested firstly this signal (2 W/kg) on NSC. Like in NB69 cells, a significant decrease in FGFR1-positive NS cells was also observed after exposure to the GSM-Basic signal (50% reduction with respect to controls, Figure 119). Western blot analysis for FGFR1 confirmed this effect (data not shown). The study also indicates that the response of the neural stem cells seems to be dependent on the age of the culture (Figure 120).

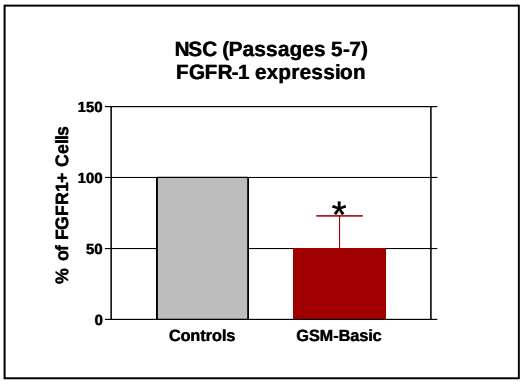
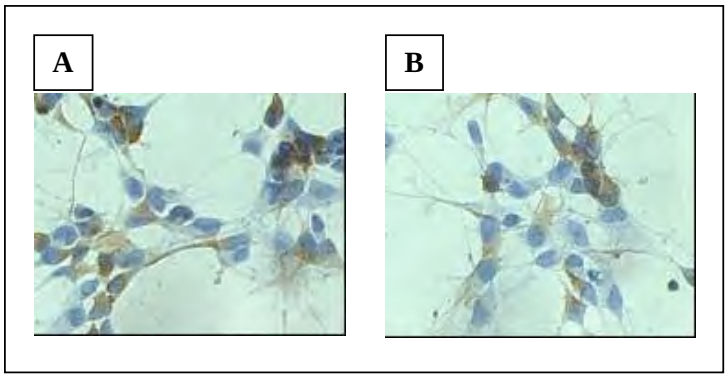


Figure 119. Percent of FGFR-1 positive labelling vs. total cell number normalised over their controls, quantification by the program for image analysis (IPWIN-3). Data represent the mean ± SEM of 3 independent experiments, done in duplicate (two coverslips), for the different treatment conditions. Student T-test *p< 0.05.



Photomicrographs of Neural Stem Cells, A, control and B exposed to GSM-Basic signal. Inmunocytochemistry for FGFR1-positive cells (brown).

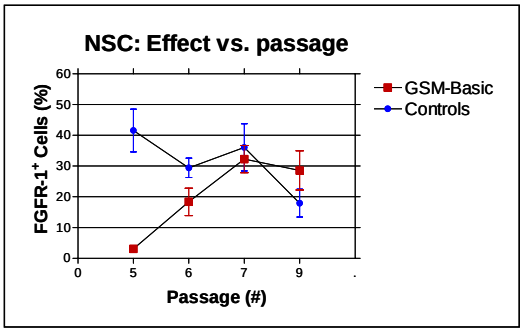


Figure 120. NSC exposed to GSM-Basic signal. The percent of FGFR-1 positive cells seems to be dependent on the passage number.

RF-EMF did not affect gene expression of FGF Receptor-1 in NB69 neuroblastoma cells and in neural stem cells (NSC)

In situ hybridisation studies were conducted on neural stem cells and in NB69 cells exposed for 21 hours (5 min on/10 min off) to the GSM-Basic signal. The objective is to evaluate potential EMF effects on gene expression of FGFR1. All experiments were conducted following blind protocols. The results on both cell types showed no differences in FGFR1 mRNA-expression between controls and exposed samples. An image-analysis study confirmed this result in NSC (Figure 121). Taken together, the

described effects on NB69 and NSC (FGFR1 protein-expression and FGFR1 mRNA-expression), suggest that the GSM-Basic signal can modulate FGFR1 protein translation without affecting protein transcription.

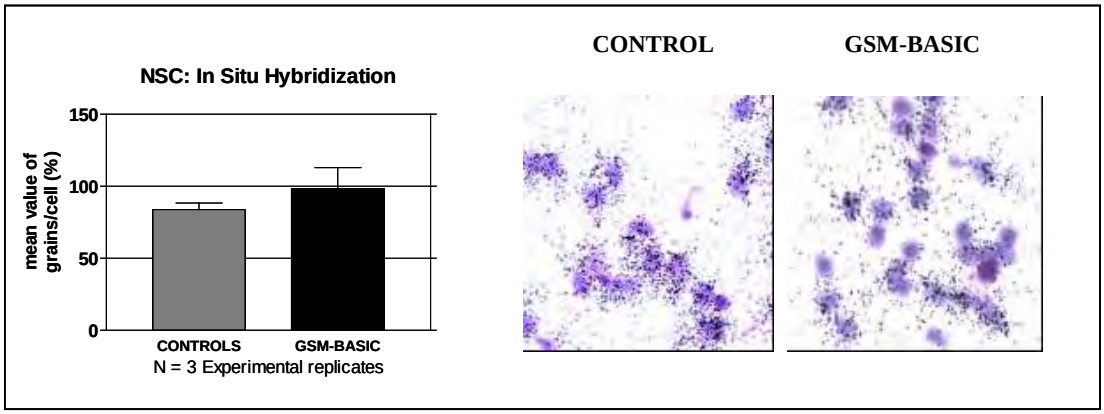


Figure 121. Quantification of in situ hybridisation staining by image-analysis technique (IPWIN 3.0) of the number of grains/cell. The photomicrographs show the developmental pattern of FGFR1 in NSC processed with Radiolabelled Probe Specific for Transcript of FGFR1.

3.2.4.3 Human promyelocytic cell line HL-60 (Participant 2)

RF-EMF exposure reproducibly up- and down-regulated protein expression in HL-60 cells (41 proteins showed to be up-, 1 protein to be down-regulated and 14 proteins appeared to be de-novo expressed).

The proteome screening approach included analysis of the entire HL-60 protein expression pattern by means of 2-D polyacrylamide gel electrophoresis (2D-PAGE). After having established the technique for HL-60 cells, cells were exposed to RF-fields at selected conditions in repeated independent experiments in order to obtain reproducible information on changes in the cellular protein pattern, correlated with RF-EMF-exposure.

HL-60 cells were exposed at 1800 MHz, continuous wave, SAR 1.3 W/kg, 24h, or were sham exposed in repeated independent experiments. Additionally, incubator controls were run and analysed for their protein expression pattern by 2D-PAGE. Cell samples were partly analysed as described above, partly stored at -80 °C for further analyses. Comparison of protein pattern after 2D-PAGE showed that optimal reproducibility is achieved when the 2-D separation step is performed in one series with identical reagent batches. In order to be able to perform statistics, appropriate numbers of comparable 2D-gels are required, also to have enough material for protein identification.

Figures 122, 123, and 124 show representative high-resolution 2-dimensional polyacrylamide gels (23 x 30 cm) for each of the conditions described above (incubator control, sham-exposure and RF-field exposure).

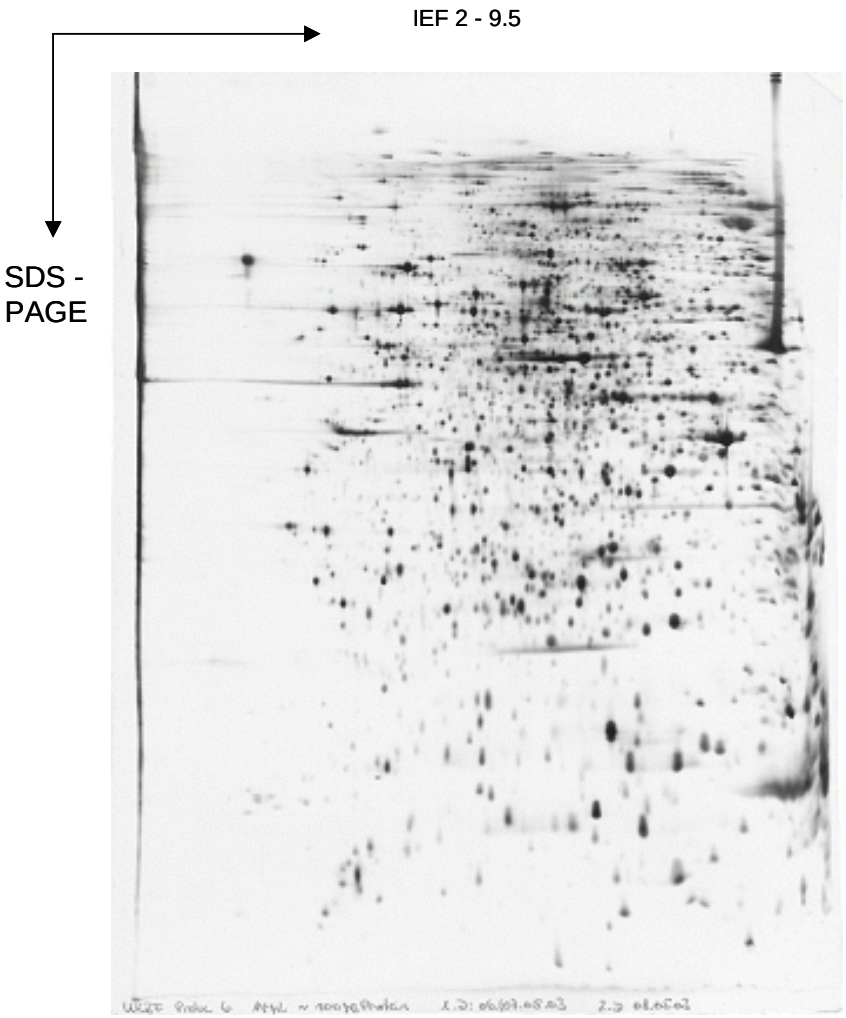


Figure 122. Two-dimensional polyacrylamide gel electrophoresis (2-D PAGE) profile of incubator control HL-60 cells (whole cell lysate). Incubation time: 24h. First dimension (isoelectric focussing): pH-gradient 2-9.5. Second dimension: 12.5% polyacrylamide gel, silver stain.

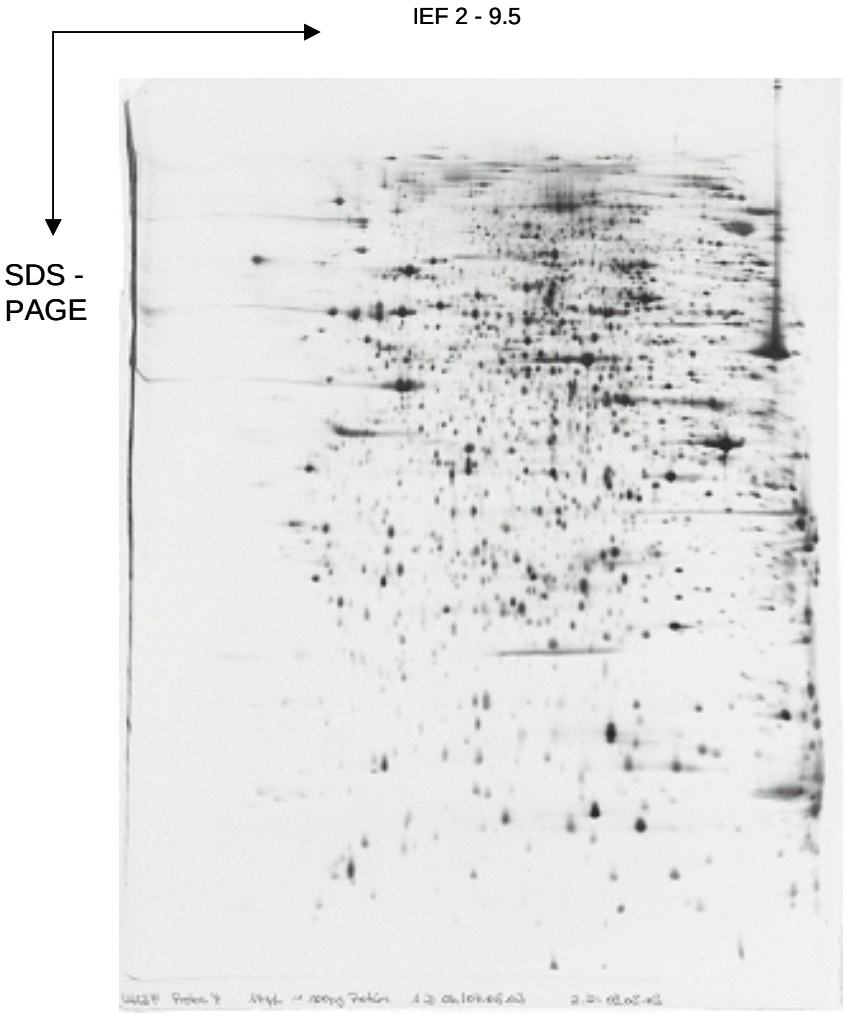


Figure 123. Two-dimensional polyacrylamide gel electrophoresis (2-D PAGE) profile of sham-exposed HL-60 cells (whole cell lysate). Exposure time: 24h. First dimension (isoelectric focussing): pH-gradient 2-9.5. Second dimension: 12.5% polyacrylamide gel, silver stain.

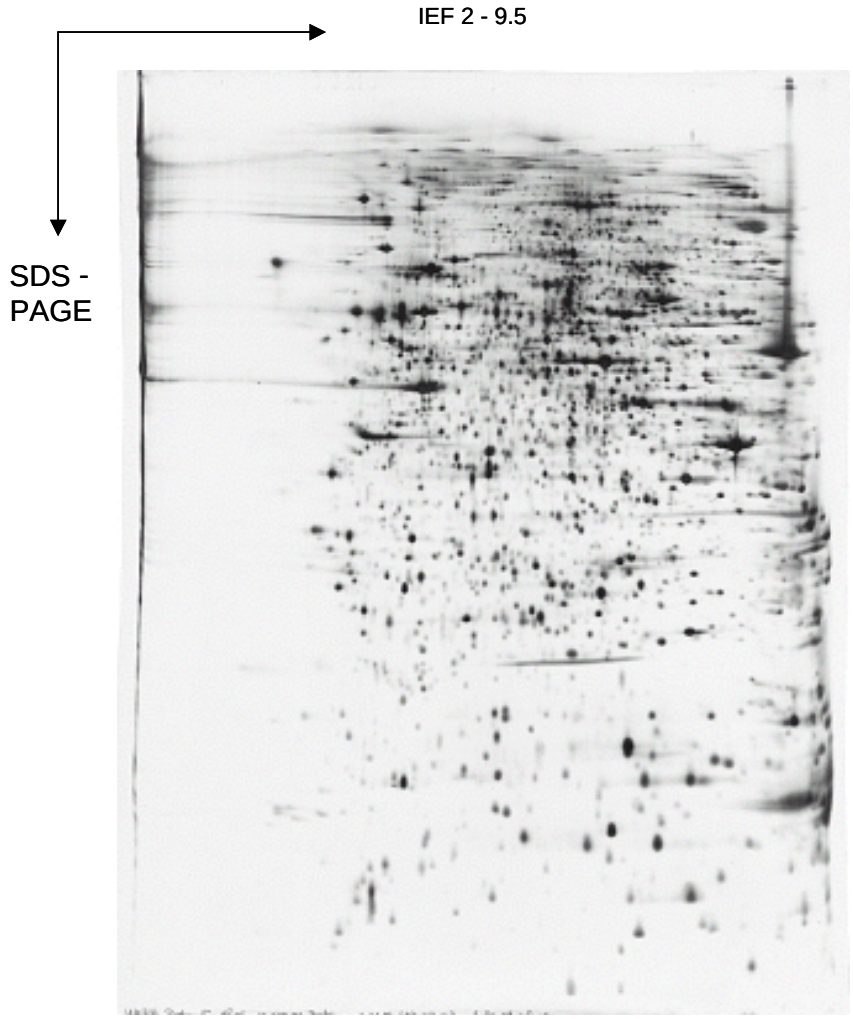


Figure 124. Two-dimensional polyacrylamide gel electrophoresis (2-D PAGE) profile of RF-exposed HL-60 cells (whole cell lysate). Exposure characteristics: 1800 MHz, continuous wave, 1.3 W/kg, 24h. First dimension (isoelectric focussing): pH-gradient 2-9.5. Second dimension: 12.5% polyacrylamide gel, silver stain.

Following digitalisation, in a second analysis step the qualitative and quantitative comparison of protein expression was performed by use of Proteom Weaver image analysis program. Figure 125 shows a representative comparative 2D-gel, in which expression differences between RF-field exposed and sham-exposed HL-60 cells are marked. Expression differences were quantified. In Table 27 (a-c) proteins up-or down-regulated and those that have disappeared after RF-field exposure are listed.

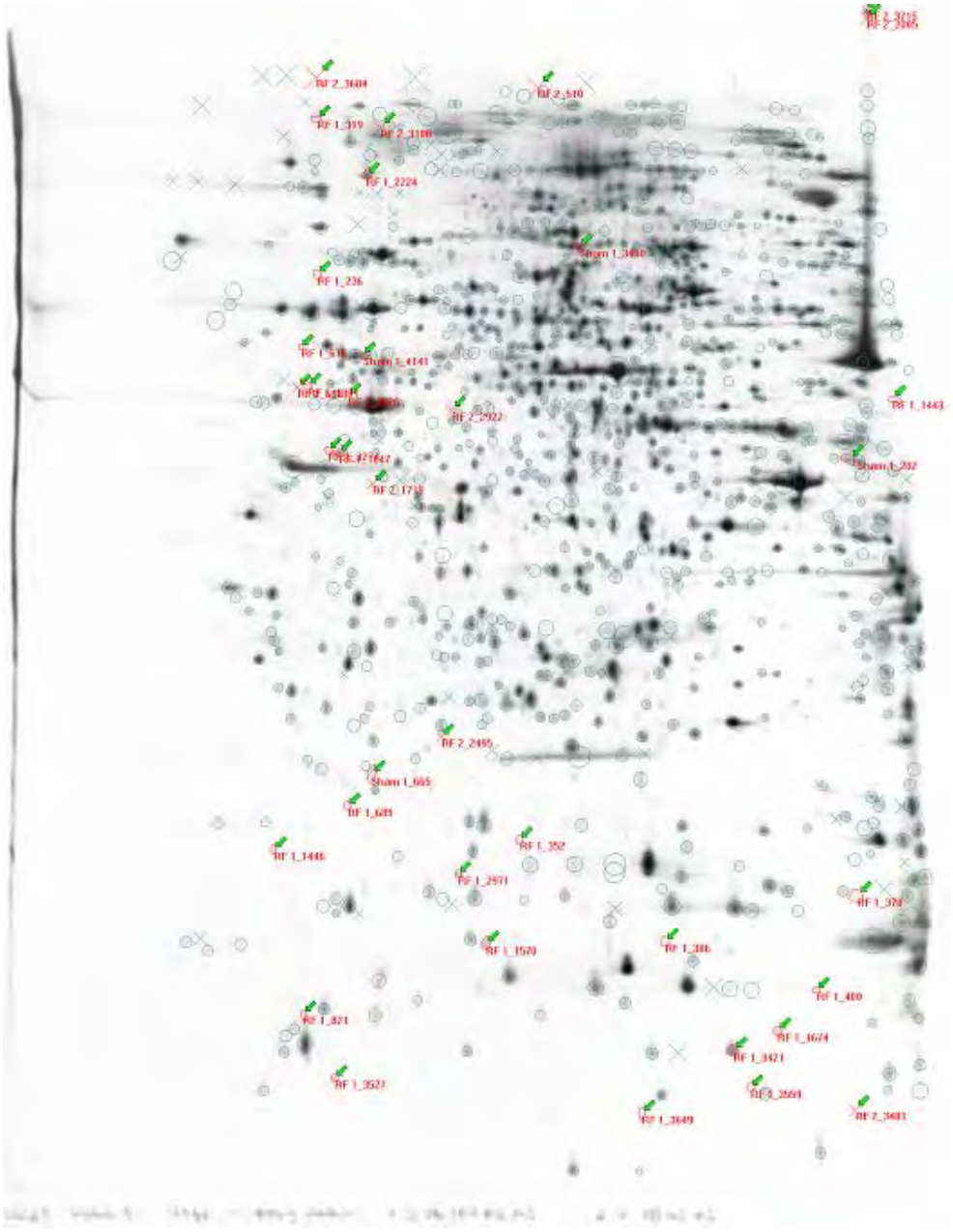
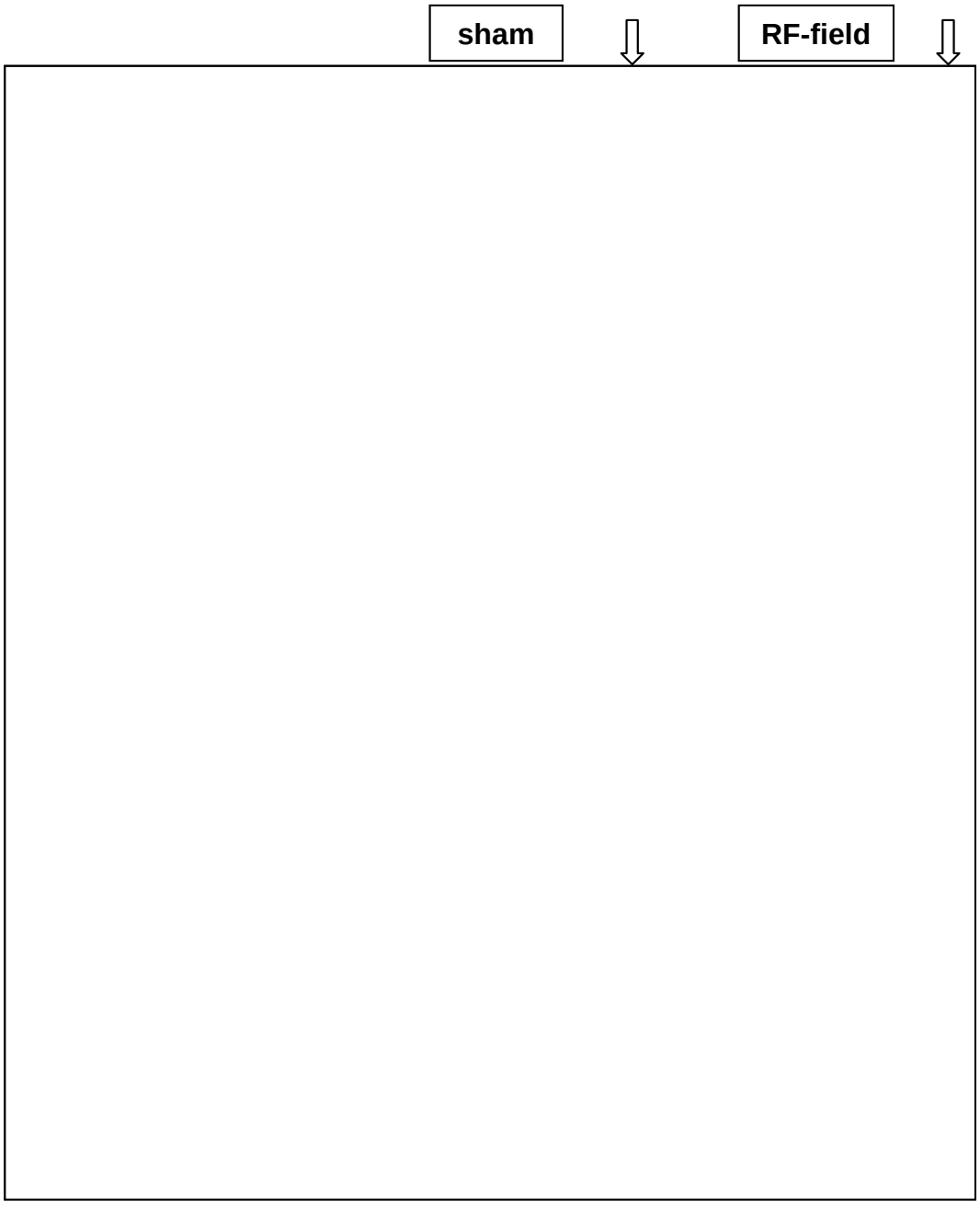


Figure 125. Representative qualitative and quantitative comparison of two-dimensional polyacrylamide gel electrophoresis (2-D PAGE) profiles of sham-exposed and RF-exposed HL-60 cells (whole cell lysate).

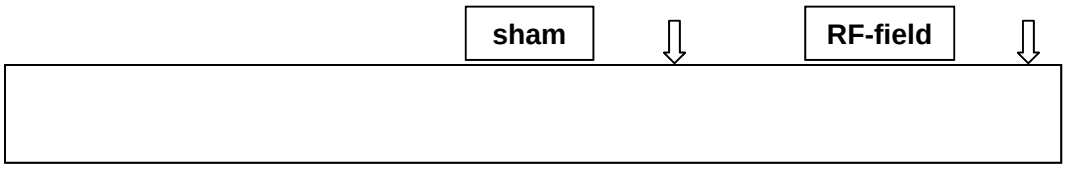
Exposure conditions: see above. Green arrows mark different proteins of sham-exposed cells compared to RF-field exposed cells (green arrows and red circles: up-regulated proteins; green arrows and red crosses: newly expressed or disappeared proteins).

Table 27. List of proteins in 2-DE patterns of HL-60 cells, differing between RF-field exposed and sham exposed cells. Exposure conditions: see above

a: List of proteins up-regulated in RF-field exposed HL-60 cells (average intensity see arrow) as compared to sham-exposed cells (average intensity see arrow).



b: List of proteins down-regulated in RF-field exposed HL-60 cells (average intensity see arrow) as compared to sham-exposed cells (average intensity see arrow).



c: List of newly expressed or disappeared proteins in RF-field exposed HL-60 cells (average intensity see arrow) as compared to sham-exposed cells (average intensity see arrow) .

Overall, 56 polypeptides of HL-60 cells are influenced in their expression under RF-EMF. Reproducibly, 41 proteins showed to be up- and 1 protein to be down-regulated following RF-field exposure. 14 proteins appeared to be de-novo expressed after RF-field exposure of HL-60 cells.

By use of these lists identification strategies are further performed. They will include in gel-cleavage, identification of selected proteins by mass spectrometry (MALDI-TOF) and mass spectrometric sequencing (ESI-MS/MS). Further identification of selected proteins will include immunoblotting and functional protein assays.

3.2.4.4 Human lymphocytes (Participant 8)

RF-EMF did not affect gene expression in human lymphocytes.

T lymphocyte gene expression analysis was performed in collaboration with Participant 12 and Dr. Daniel Remondini in Bologna. The results suggest that no differences in gene expression are found between

quiescent T lymphocytes exposed to RF-EMF (DTX only) in comparison with sham-exposed cells. This finding did not suggest any significant interaction of RF-EMF with gene profile expression.

3.2.4.5 Brain cells of different origin (Participant 9)

RF-EMF exposure did not affect expression and activity of the inducible nitric oxide synthase (iNOS or NOS2) in nerve cells.

A basal level of NOS_2, probably due to the SVF deprivation, was detected in C6 cells, although inter-experiment's variation could be seen. A 48-hour treatment with LPS plus CK increased the expression of the enzyme by a factor 5 (Figure 126)

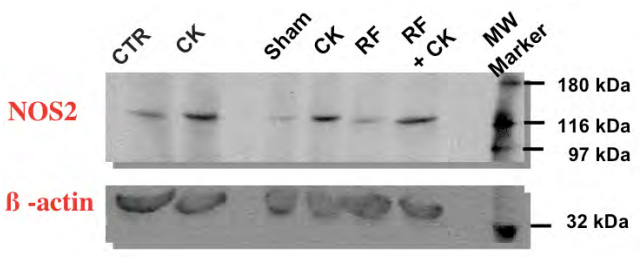


Figure 126. Representative blot for the detection of NOS_2 (upper blot) and ß-actin (lower blot) proteins.

Sham/sham experiments showed that a 15-20% inter-incubator variation had to be expected, so that a more than a 30% difference between sham- and RF-exposed data would be considered as a significant biological effect for both NOS_2 expression and nitrite accumulation (Figure 127).

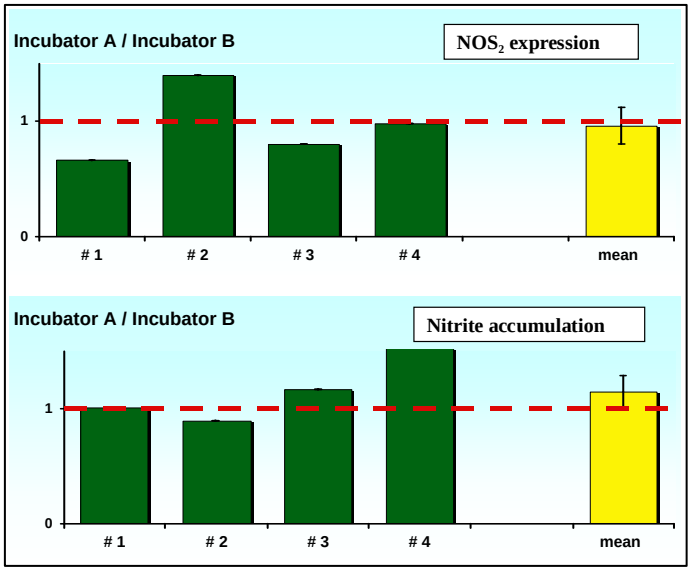


Figure 127. Expression of NOS_2 protein and nitrite accumulation (culture medium) in C6 cells in four sham/sham experiments (#1 to #4). Data are given as the ratio ± SEM between the levels found in the incubator A used for RF exposure and incubator B used for sham-exposure.

Exposure to GSM-900 at 0.2 W/kg and 2 W/kg for 48 hours was shown to not alter the expression of NOS_2 compared to sham exposure. Co-exposure to GSM and LPS plus cytokine was ineffective in modifying the effect of LPS plus cytokine treatment on NOS_2 expression (Figure 128).

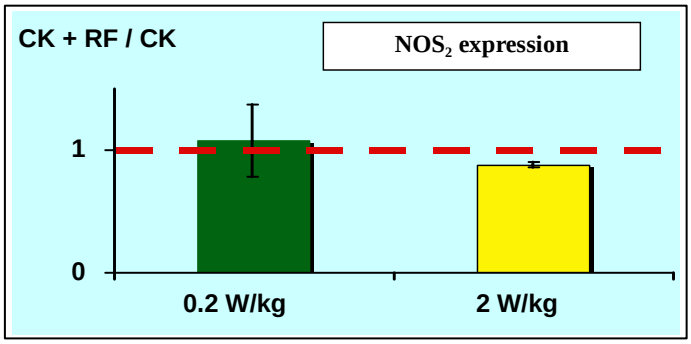


Figure 128. Expression of NOS_2 protein in C6 cells exposed to frame-GSM-900 at 0.2 and 2 W/kg for 48 hours. Data are given as the ratio ± SEM between the levels found in samples treated with CK+LPS (CK) and exposed to RF and those treated with CK alone.

Nitrite accumulation in culture medium was used as a marker of NOS_2 activation. No nitrite accumulation was shown in sham-exposed samples. Although inter-experiment variability, treatment with the cocktail of LPS plus CK led to a significant nitrite accumulation ($p<0.001$). As shown in Figure 129, a mean 20-fold increase in nitrite accumulation was measured after 48 hours of LPS plus CK treatment.

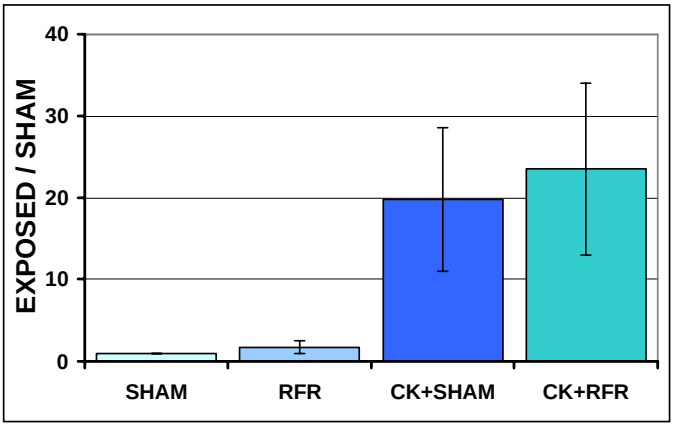


Figure 129. Nitrite accumulation in culture medium of C6 cells sham-exposed or exposed to GSM-900 at 0.2 W/kg and /or treated with CK+LPS (CK). In all cases, treatment duration was 48 hours. Data are given as the ratio ± SEM between the levels found in treated- versus sham-exposed samples.

No significant effect of GSM-900 exposure was detected on nitrite accumulation. When co-exposures to GSM-900 and LPS + CK treatment were performed, no modulation of chemically-induced NOS_2 expression. Overall, exposure to GSM-900 did not modulate CK+LPS-induced nitrite accumulation (Figure 130).

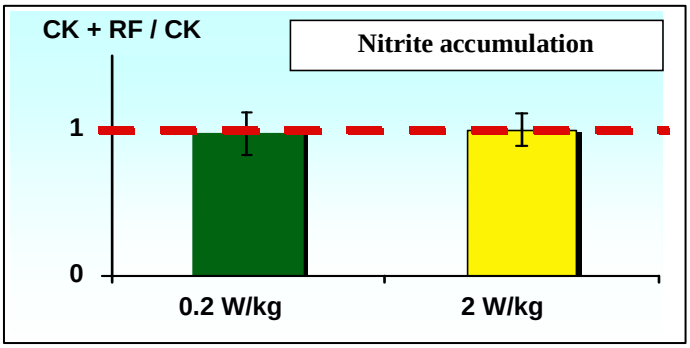


Figure 130. Nitrite accumulation (culture medium) in C6 cells exposed to frame-GSM-900 at 0.2 W/kg and 2 W/kg for 48 hours. Data are given as the ratio ± SEM between the levels found in samples treated with CK+LPS (CK) and exposed to RF and those treated with CK alone.

No evidence of an effect of RF-EMF (GSM-900) exposure on spontaneous expression and activity in C6 cells was obtained from our experiments. However, one can note that only strong treatments (serum deprivation plus long duration of chemical treatment) are shown to increase NOS_2 expression in C6 cells. It is noteworthy that most papers in the literature looked at the mRNA but not at the *protein* level as we did in the present work. Two SAR levels of GSM-900 were tested. Even at the highest SAR level of 2 W/kg corresponding to the public exposure limit recommended by the EU Commission, GSM-900 exposure was not shown able to influence NOS_2 expression or activity in activated C6 cells. Taken together, RF-EMF at a low SAR level were not identified as a stimulus for C6 cells activation.

RF-EMF (GSM-900 signals) did not affect heat shock protein expression in nerve cells.

When used as a positive control, heat shock (43°C for 20 min) increased expression of hsp70 in all nerve cell cultures, i.e. human U87 astrocytoma cells, rat C6 glioma cells and human SH-SY5Y neuroblastoma cell lines. However, when exposed to GSM-900 for 24 hours, none of the cell line showed a significant change in expression of hsp70 (Figures 131, 132). Altogether, our data show that exposure to ELF-EMF does not seem to be able to induce Hsp70 expression in rat and human nerve cells.

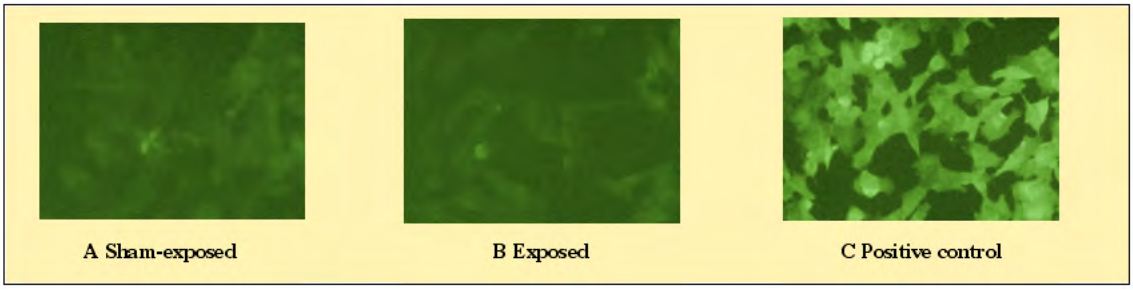


Figure 131. Fluorescent Hsp70 immunolabelling after sham-exposure or exposure of human SH-SY5Y neuroblastoma cells to frame GSM-900 signal at 2 W/kg for 24 hours (A and B) or to heat shock (43°C, 20 min) (C).

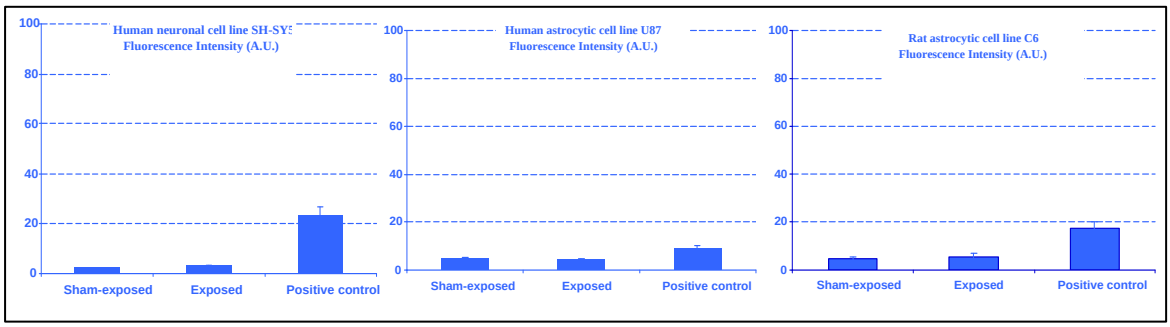


Figure 132. Effect of GSM-exposure (2 W/kg, 24h) or Heat Shock exposure (43°C, 20 min) (Positive control) on hsp70 expression in three different cell lines. Results are expressed in Fluorescence Intensity (A.U). Data from 3 independent experiments are presented as the Mean ± SEM

GSM-900 microwave exposure did not affect hsp27 expression in human endothelial cell line EA.hy926.

The hsp27 expression was measured using the immunofluorescence technique. Qualitative analysis did not allow for detecting any difference in fluorescence intensity in RFR exposed cells versus sham cells. Our quantitative results obtained after fluorescence image analysis of hsp27 expression in EA-hy926 revealed that no significant difference was observed between sham-or exposed cells in both cell lines (Figures 133, 134)

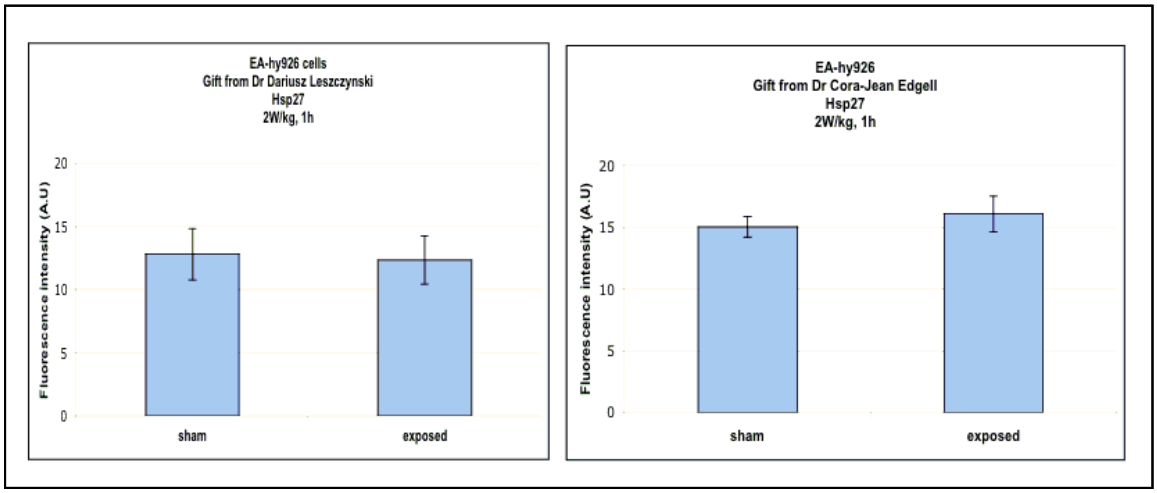


Figure 133. Effect of GSM-exposure (2 W/kg, 1 hour) on hsp27 in EA-hy926 cell lines given by Participant 6 and Dr Cora-Jean Edgell. Results are expressed as the fluorescence Intensity (A.U). Data from 5 independent experiments are presented as the Mean ± SEM.

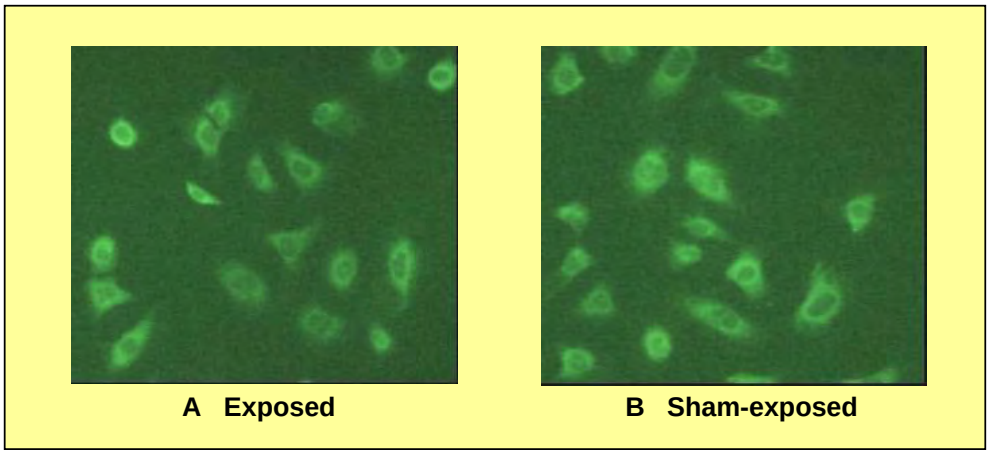


Figure 134. Fluorescent hsp27 immunolabelling after exposure(A) or sham-exposure(B) of EA-hy926 cell lines given by Participant 6 to GSM-900 signal at 2 W/kg for 1 hour.

A third method using Elisa test will allow us to quantify precisely if RFR are able to induce changes in hsp27 expression. However, with our method we were unable to confirm previous data on hsp27 expression in endothelial cell lines (Leszczynski et al., 2002). Therefore, we can not conclude that RFR induce stress response.

No conclusive data was obtained on the effect of RF-EMF exposure on Hsp27 expression in rat brain.

Table 28 shows results obtained on hsp27 expression in rat brains in the pilot experiment.

Results obtained on samples treated without perfusion show non-specific labelling disturbing the analysis. Results obtained on perfused brains show acceptable background noise. Image analysis obtained on perfused brains reveal conflicting and opposite results within groups preventing to draw conclusions.

Table 28. Hsp27 expression in rat brains

Experimental procedure	Background noise	Hsp27 labelling	Exposure conditions
Perfusion	-	+	Control
Perfusion	+	-	Control
Perfusion	-	-	Sham-exposed
Perfusion	-	+	Sham-exposed
Perfusion	-	-	Exposed
Perfusion	+	+/-	Exposed
Without perfusion	+	+	Control
Without perfusion	+	+	Control
Without perfusion	+	+/-	Sham-exposed
Without perfusion	+	++	Sham-exposed
Without perfusion	+	+	Exposed
Without perfusion	+	+/-	Exposed

– : negative labelling; + : positive labelling; +/- : negative or positive labelling depending of the area; +/+ : clear positive labelling

This pilot study did not allow us to draw conclusion on results obtained *in vitro* on hsp27 expression but it gave information on technical methodologies and on the number of animals to use.

RF-EMF (GSM-900) exposure weakly affected gene expression in immune cells.

This investigation was carried out in cooperation with Participant 12. Criteria for the selection of significantly altered gene expression was an exposed over sham ratio less than 0.5 for a significant decrease and more than 2.0 for a significant increase. Using these criteria, over 15588 human genes were detected, changes in expression of about 50 genes were significant corresponding to 0.3% of total number of detectable genes. Genes shown to be altered after RF-EMF exposure (increase or decrease) are known to be involved in signal transduction, ion electron transport, metabolism of energy and proteins, cell proliferation, apoptosis or differentiation, immune answer, inflammation, stress answer, extracellular matrix, cytoskeleton, adhesion and DNA repair. The largest modification in RNA expression corresponded to genes related to signal transduction (linked with GTP or calcium) and energy metabolism. Only a few genes involved in apoptosis or stress response were detected and show no significant sensitivity to RFR exposure. Concerning our purpose to investigate modification of genes involved in inflammatory response and processes, one gene corresponding to a component of major histocompatibilty complex class II and another acting as plasminogen activator were altered by RF-EMF. Finally, the largest increase of expression (30 fold increase), after mobile phone exposure, concerned genes described to participate in amine oxidase (copper containing) activity. This enzyme is involved in cell growth and proliferation but also in immune regulation.

3.2.4.6 Human endothelial cell lines EA.hy926 and EA.hy926v1 (Participant 6)

It has been suggested that high-throughput screening techniques (HTST) of transcriptomics and proteomics could be used to rapidly identify broad variety of potential molecular targets of RF-EMF and generate variety of biological end-points for further analyses (Leszczynski 2001). Combination of data generated by transcriptomics and proteomics in search for biological effects is called the “discovery science”. This term has been coined-in by Aebersold and co-workers (Aebersold et al. 2000) to define the new approach that will help in revealing biological mechanisms, some of which might be unpredictable using the presently available knowledge. This approach seems to be particularly suited for elucidation RF-EMF health hazard issue because it might reveal effects that are not possible to predict based on the present knowledge about the biological effects of RF-EMF. However, before committing large funds that are needed for HTST studies it is necessary to determine whether indeed this approach will be successful in unravelling physiologically significant biological events induced by RF-EMF. Due to their high sensitivity HTST are able to pick-up very small changes in protein or gene expression which changes might be of insufficient magnitude to alter cell physiology. Thus, although using HTST it might be possible to find biological effects induced by RF-EMF these effects might be of limited or no significance at all, from the physiological stand point. Therefore, to determine the usefulness of HTST approach to the issue of bio-effects induced by RF-EMF, we have performed a 5-step feasibility study and have shown that HTST might indeed help to identify experimental targets for physiological studies of RF-EMF-induced biological responses (Figure 135).

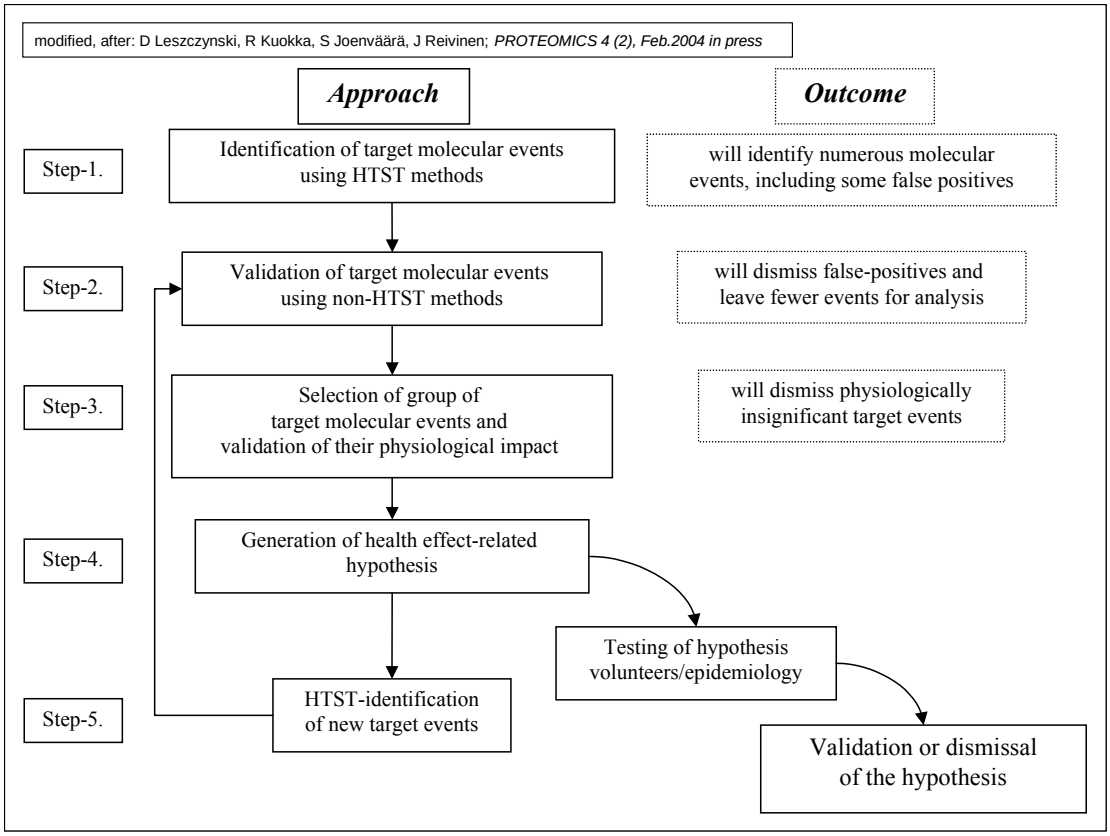


Figure 135. Scheme of experimental procedure which execution will elucidate new RF-EMF induced molecular events that might affect cell physiology. Events of magnitude sufficient to alter cell physiology could be then examined for their potential impact on the organ/whole body physiology in attempt to predict the extent of eventual health hazard.

A. The 5-step feasibility study

Step-1: HTST-identification of target molecular event

Firstly, we have determined the extent of cell response to RF-EMF (Leszczynski et al. 2002). This has been done by analysing global changes in the pattern of protein phosphorylation. As an experimental model we have used cultures of human endothelial cell line EA.hy926 (Edgell et al. 1983). Cells were exposed for 1 hour to 900MHz GSM mobile phone simulating signal at an average specific absorption rate (SAR) of 2.4 W/kg (Deli et al. 1995) that is slightly above the European safety limit (SAR=2.0 W/kg). In order to be able to determine changes in protein phosphorylation, the ^{32}P-labelled orthophosphate was present in the cell cultures during the 1 hour RF-EMF exposure period. Immediately after the end of exposure cells were harvested; proteins extracted and separated using standard two-dimensional electrophoresis (2-DE). Using PDQuest software (Bio-Rad, UK), some 1266 different protein spots were identified in silver-stained 2-DE gels (Figure 136A). Using autoradiography it was possible to determine that among the 1266 proteins spots, in non-irradiated control exposed cells, were detected some 110 phosphoproteins (Figure 136B), whereas in exposed cells were detected some 372 phosphoproteins (Figure 136C). The observed broad change in the pattern of global protein phosphorylation has suggested that cells respond to RF-EMF and that possibly any of the hundreds of phosphoproteins that have altered their phosphorylation status could, at least potentially, affect cell physiology. By using western blot or mass spectrometry, to identify the phosphoproteins present in the 2DE spots, it might be possible to find variety of protein targets that could be used in examining effects of mobile phone radiation on cell physiology. With this approach, the selection of molecular targets for further studies would not be based only on deduction of potentially affected events but on the knowledge of the identities of proteins that indeed respond to RF-EMF. Thus, in the continuation of Step-1, using

simple western blot screening with antibodies directed against various stress response proteins, we have identified hsp27 as one of the phosphoproteins responding to RF-EMF. Hsp27 is continuously expressed in endothelial cells (Edgell et al. 1983). In 2DE-western blots it appeared as two spots of 27kDa molecular weight but with different isoelectric points (pI=5.7 and pI=6.1) (Figure 136D). Only the $hsp27_{pI=5.7}$ isoform was phosphorylated and, following exposure to RF-EMF, the size of $hsp27_{pI=5.7}$-spot has increased (Leszczynski et al. 2002).

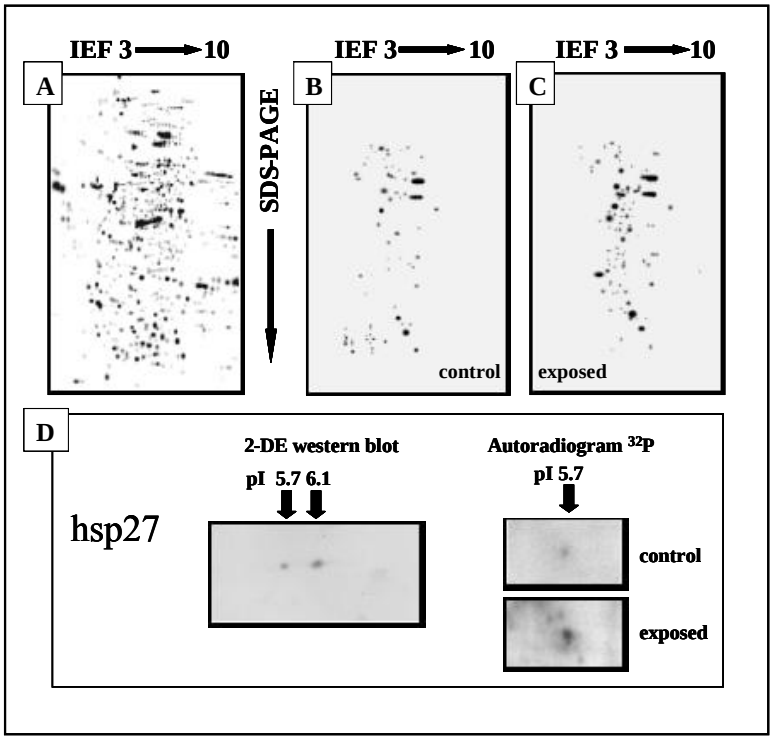


Figure 136. Identification of target molecular event for further studies – Step-1. Pattern of protein expression in EA.hy926 human endothelial cell line as determined by 2DE (panel A). Pattern of expression of ^{32}P-labeled phosphoproteins in control (panel B) and in exposed (panel C) cells. Hsp27 protein was identified, using 2DE western blot, as existing in EA.hy926 cells in two isoforms with different pI values (panel D-left). The pI 5.7 form was phosphorylated and its phosphorylation level has increased after RF-EMF exposure (panel D-right). For experimental details see Material and Methods section in Leszczynski et al. 2002.

Step-2: Validation of target molecular event

The change in phosphorylation status of hsp27 was confirmed in several ways to assure the validity of this observation (Leszczynski et al., 2002):

- immunoprecipitation of phosphorylated hsp27 (Figure 137A),
- immunoprecipitation of p38MAPK (Figure 137B), an up-stream kinase indirectly involved in phosphorylation of hsp27,
- inhibition of hsp27 phosphorylation by introduction to cell cultures of inhibitor of hsp27-up-stream kinase p38MAPK (SB203580) and determining hsp27 phosphorylation status by immunoprecipitation (Figure 137C).

Thus, in the Step-2 was confirmed that hsp27 is the valid molecular target event of the RF-EMF and that it is justified to further examine impact of this change on cell physiology.

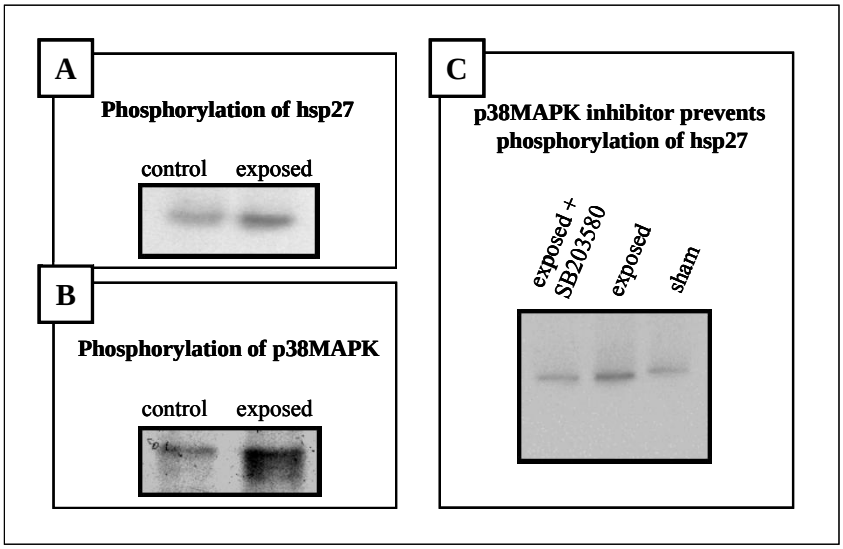


Figure 137. Validation of the target molecular event – Step-2. Increase in phosphorylation of hsp27 was confirmed by immunoprecipitation (panel A). Effect of p38MAP kinase on the RF-EMF-induced hsp27 phosphorylation was confirmed by determining, by immunoprecipitation, that p38MAPK is also activated by the RF-EMF exposure (panel B). As expected, presence of p38MAPK inhibitor, SB203580, in the culture medium during the exposure to RF-EMF, has prevented phosphorylation of hsp27 (panel C). For experimental details see Material and Methods section in Leszczynski et al. 2002.

Step-3: Cellular response – validation of the physiological event

Observed by us phosphorylation and increase in expression of hsp27 (Leszczynski et al. 2002) is a well-established mechanism of cell response to a broad variety of stress stimuli (Rogalla et al. 1999). Therefore, the observed by us doubling of Hsp27 expression and 2- to 7-fold increase in amount of phosphorylated hsp27 in cells (Leszczynski et al. 2002) have suggested that EA.hy926 cells have recognised RF-EMF as an external stress factor and that they have launched an hsp27-dependent counter response. Phosphorylation of hsp27 has been shown to regulate polymerisation of F-actin and stability of made of this protein - stress fibers (Landry and Huot 1995). Thus, we have examined status of the stress fibers in exposed cells by staining F-actin with AlexaFluor-labelled phalloidin. As shown in Figure 136A, RF-EMF exposure has caused increase in cellular staining with phalloidin what indicates increase in stability of F-actin stress fibers. The stability of stress fibers, as determined by the pattern of staining with phalloidin-AlexaFluor, increased after 1 hour irradiation and did not decline during the 1 hour of post-irradiation incubation. Induction of the stability of stress fibers caused cells to shrink and visible cell shrinking was observed among the cells brightly stained with AlexaFluor-phalloidin (Figure 138A; middle and right panels). The increase in the stability of stress fibers was prevented in the presence of p38MAPK inhibitor SB203580 (Figure 138B). Also it was possible to observe that in cells expressing high levels of hsp27 (Figure 138C), the cell edges were brightly stained with phalloidin-AlexaFluor, what indicates re-localisation of F-actin stress fibers to cell ruffles whereas in cells expressing low levels of hsp27, network of stress fibers was seen throughout the whole cytoplasm but not in the ruffles. Such behaviour of hsp27 and stress fibers in cells exposed to RF-EMF is in agreement with the general pattern of cellular response to stimuli that activate hsp27-dependent stress response (Landry and Huot 1995).

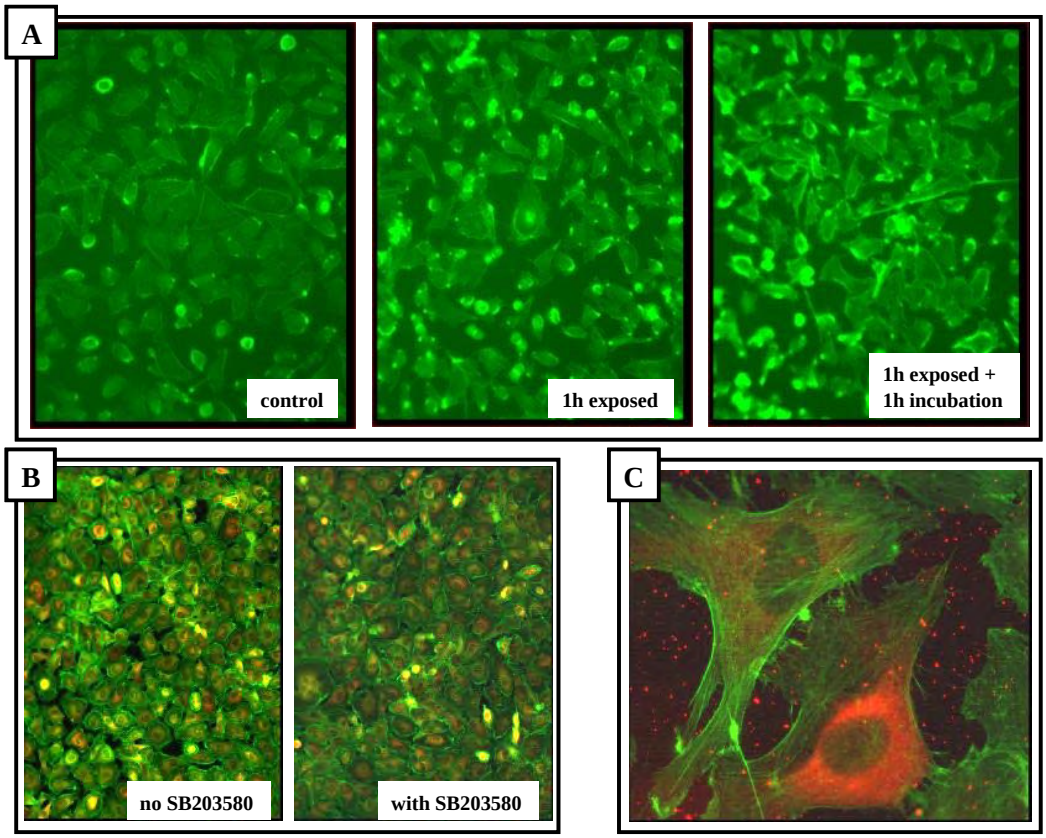


Figure 138. Cellular response to RF-EMF – validation of physiological event – Step-3.Exposure of cells to RF-EMF has caused increase in cell staining with AlexaFluor-labelled phalloidin (panel A). This suggests the increase in the expression/stability of F-actin, protein that forms cellular stress fibers. Rounding up is visible among the cells expressing highest F-actin content (the brightest staining with AlexaFluor-phalloidin). This effect persisted during the 1-hour post-exposure incubation of cells in control conditions. Presence of p38MAPK inhibitor, SB203580, in cell culture medium has prevented increase in AlexaFluor-phalloidin staining (panel B). Large magnification of cells shown in panel C demonstrates difference in distribution of AlexaFluor-phalloidin stained stress fibers (green colour) in cells with high (cell on the right) and low (cell on the left) content of hsp27 protein (indirect immunohistochemical staining; red fluorescence).

Step-4: Generation of hypothesis based on molecular and physiological events

The above results (Leszczynski et al. 2002) have formed basis and support for our working hypothesis (Figure 132, Step-4). Stabilisation of stress fibers and caused by it cell shrinking, when occurring in endothelial cells lining brain's capillary blood vessels, might be of importance for the functioning of blood-brain barrier. Stabilisation of stress fibers and cytoplasmic distribution of F-actin was previously shown to cause: (i) cell shrinkage (Landry and Huot 1995; Piotrowicz and Levin 1997a), that might lead to opening of spaces between cells, (ii) increase in the permeability and pinocytosis of endothelial monolayer (Deli et al. 1995; Lavoie et al. 1993), (iii) increase in formation of the so called "apoptosis-unrelated" blebs on the surface of endothelial cells (Becker and Ambrosio 1987), which eventually might obstruct blood flow through capillary blood vessels, (iv) stronger responsiveness of endothelial cells to estrogen and, when stimulated by this hormone, to secrete larger than normally amounts of basic fibroblast growth factor (bFGF) (Piotrowicz et al. 1997b) which might, in endocrine manner, stimulate de-differentiation and proliferation of endothelial cells and possibly led to the associated with cell's proliferative state - cell shrinkage and unveiling of basal membrane. Also, the activated (phosphorylated) hsp27 has been shown to inhibit apoptosis by forming complex with the apoptosome (complex of Apaf-1 protein, pro-caspase-9 and cytochrome *c*), or some of its components, and preventing proteolytic activation of pro-caspase-9 into active form of caspase-9 (Pandey et al. 2000; Concannon et al. 2001). This, in turn, prevents activation of pro-caspase-3 which is activated by caspase-9. Thus, induction of the

increased expression and phosphorylation of hsp27 by the RF-EMF exposure might lead to inhibition of the apoptotic pathway that involves apoptosome and caspase-3. This event, when occurring in RF-EMF exposed brain cells that underwent either spontaneous or external factor-induced transformation/damage, could support survival of the transformed/damaged cells. Therefore, based on the known cellular role of over-expressed/phosphorylated hsp27 we have proposed a hypothesis (Leszczynski et al. 2002) that: the activation (phosphorylation) of hsp27 by mobile phone radiation might be the molecular mechanism (i) regulating increase in blood-brain barrier permeability, which would explain, observed in some animal experiments, increase in blood-brain barrier permeability, and (ii) regulating apoptosis through interference with the cytochrome c/caspase-9/caspase-3 pathway (Figure 139). Thus, it is possible that the RF-EMF might have effect on cytoskeleton-related and on the apoptosis-related cell functions. This notion supports and justifies further examination of cytoskeleton and apoptosis related properties of RF-EMF exposed endothelial cells.

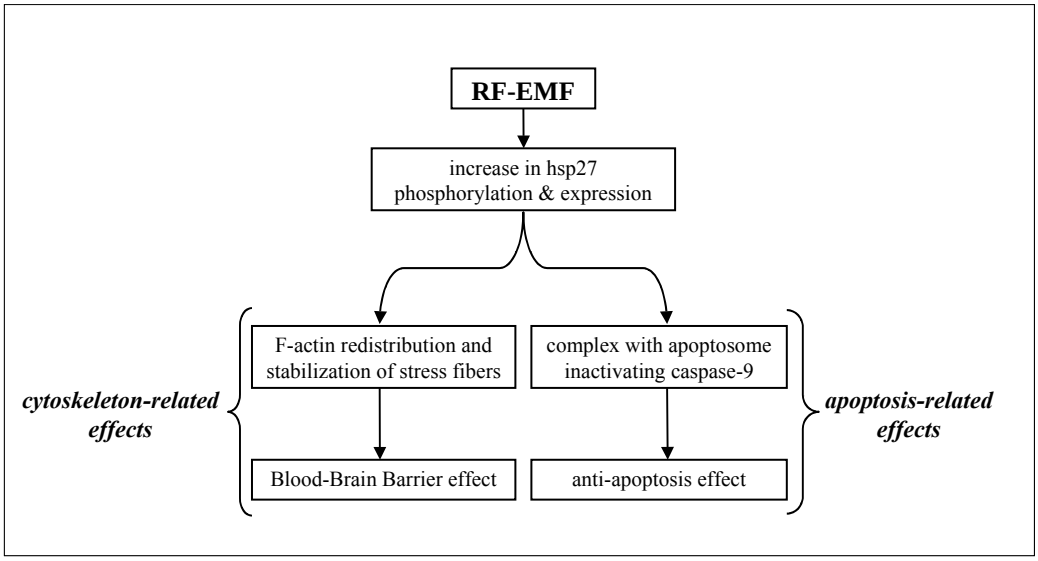


Figure 139. Hypothesis based on the molecular and physiological events – Step-4. Based on the known functions of hsp27 we have proposed that RF-EMF induced hsp27 phosphorylation might affect cell cytoskeleton and cell apoptosis. For full scheme and description of hypothesis see reference Leszczynski et al. 2002.

Step-5: HTST-identification of new target events, with support of hypothesis

Further experiments using HTST have revealed additional information pertinent to the cytoskeleton and apoptosis related properties of RF-EMF exposed endothelial cells.

The suggested changes in the cytoskeletal proteins were detected using 2-DE separated proteins. Approximately 1300 protein spots were detected 2-DE. Comparison of the control and exposed samples revealed some 49 protein spots which were statistically significantly (student T-test, $p<0.05$, $n=10$) affected by the exposure (increased or declined expression). Few of the spots were selected for the mass spectrometry identification using the following criteria: spots needed to be (i) enough separate from the adjacent spots, (ii) sufficiently large and (iii) well focused in all dimensions. Cytoskeletal proteins vimentins (Figure 140) and tubulin (not shown) were identified by mass spectrometry among the proteins that responded to RF-EMF. The suggested interference with apoptosis was further examined using cDNA Expression Arrays (Clontech) and screening expression of 3600 different genes. Among the genes that were down-regulated in cells exposed to RF-EMF were numerous genes encoding proteins of Fas/TNFα-apoptotic pathway (Figure 141). This pathway was suggested as target for the RF-EMF induced phosphorylated hsp27. Therefore, concomitantly observed increase in hsp27 phosphorylation, that is anti-apoptotic event, and down-regulation of proteins of Fas/TNFα apoptotic pathway suggest that further studies aiming at elucidation of RF-EMF effect on cell apoptosis are justified.

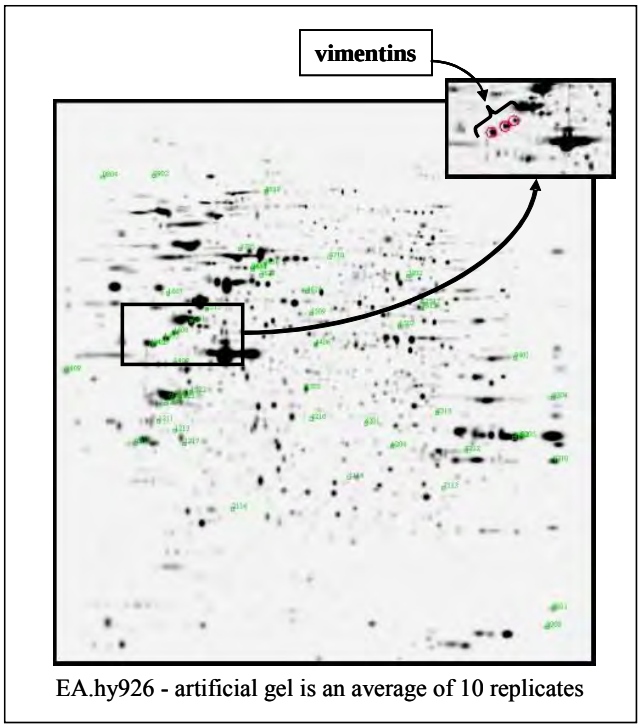


Figure 140. HTST-identification of new target events – cytoskeleton-related – Step-5. Using larger 2-DE gels (20x20 cm) and 10 replicates of each run we have identified some 49 proteins that, in statistically significant fashion, have altered their expression following RF-EMF exposure. Among the mass spectrometry identified spots were cytoskeletal proteins vimentins (inset) and tubulin (not shown).

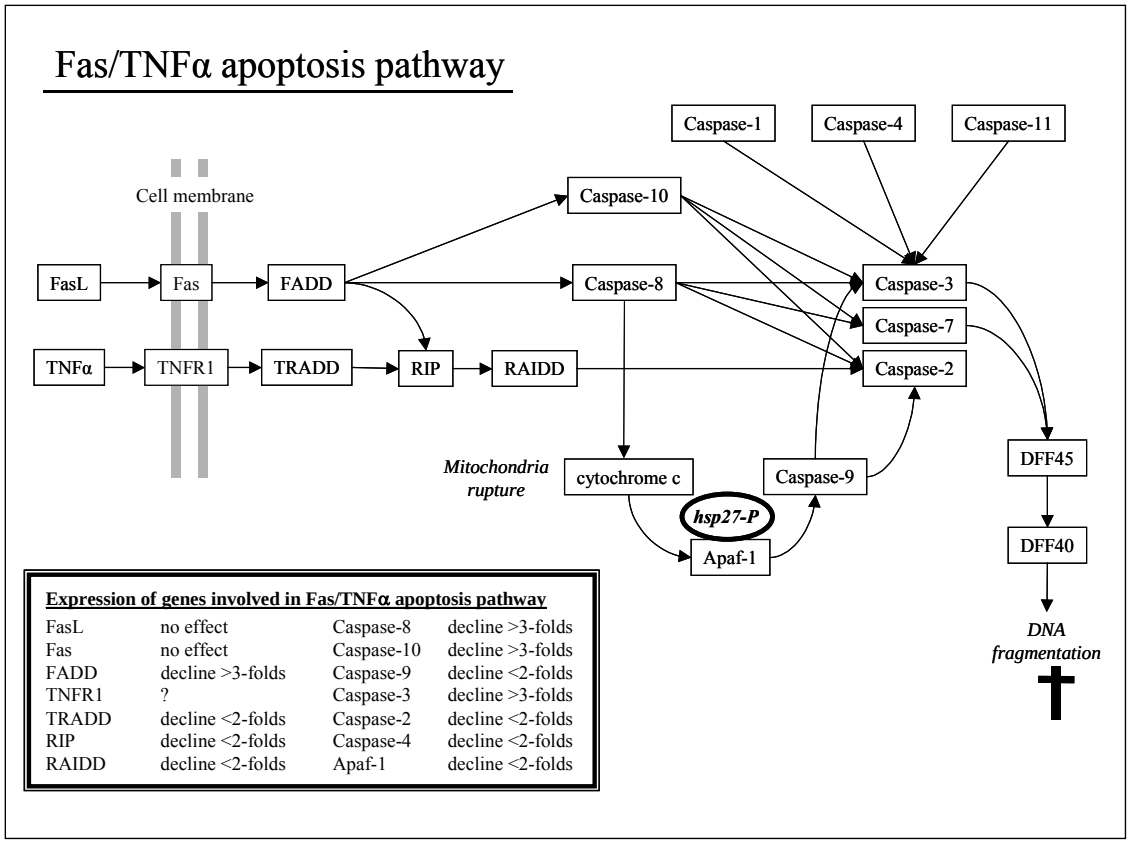


Figure 141. HTST-identification of new target events – apoptosis-related – Step-5. Analysis of RF-EMF-induced expression changes, using cDNA Expression Array for 3600 tumour-related genes, has revealed that the majority of genes that encode proteins forming Fas/TNFα apoptotic pathway are down-regulated (Table inset).

B. Genotype-dependent cell response to 900 MHz GSM radiation

We have compared response to mobile phone radiation of two human endothelial cell lines: fast proliferating EA.hy926 (Edgell et al., 1983) and its slow proliferating variant EA.hy926v1 (derived by sub-cloning from the EA.hy926 cell line).

Proteomics approach

Using 2-DE and MALDI-MS proteomics approach we have determined what proteins respond to the mobile phone radiation. Using PDQuest 6.2 software (Bio-Rad, UK) the 2-DE artificial gels (Figure 135) were generated from 10 independent protein samples from ten independent replicates of controls and irradiated cell cultures. The protein expression pattern in ten replicate control samples was then compared with the protein pattern in ten replicate irradiated samples. The normalised spot volumes of the proteins from control and exposed sample gels were statistically analysed using student t-test at the confidence level of 95%. The most striking observation was that the comparison in-between the two cell lines showed that their protein expression patterns are very different in spite of the closely related origin of both cell lines (Figures 142A, 142B). Only approximately half of all of the protein spots could be matched confidently between the cell lines. This difference in protein expression pattern might explain the observed differences in the growth rate between the cell lines. Because of the observed differences in the protein expression and proliferation between the cell lines, it was not a surprise that the response to the mobile phone radiation also varied between EA.hy926 and EA.hy926v1 cell lines. The comparison of the exposed and control samples has shown several tens of protein spots with radiation-induced statistically significant change in expression levels (t-test $p<0.05$). In EA.hy926 cell line there were 38 of protein spots which expression was altered by the radiation exposure (Figure 142A) whereas in EA.hy926v1 cell line there were 45 differentially expressed protein spots (Figure 142B). The identity of

the all radiation-responding protein spots is being determined by MALDI-MS and will be reported in due time.

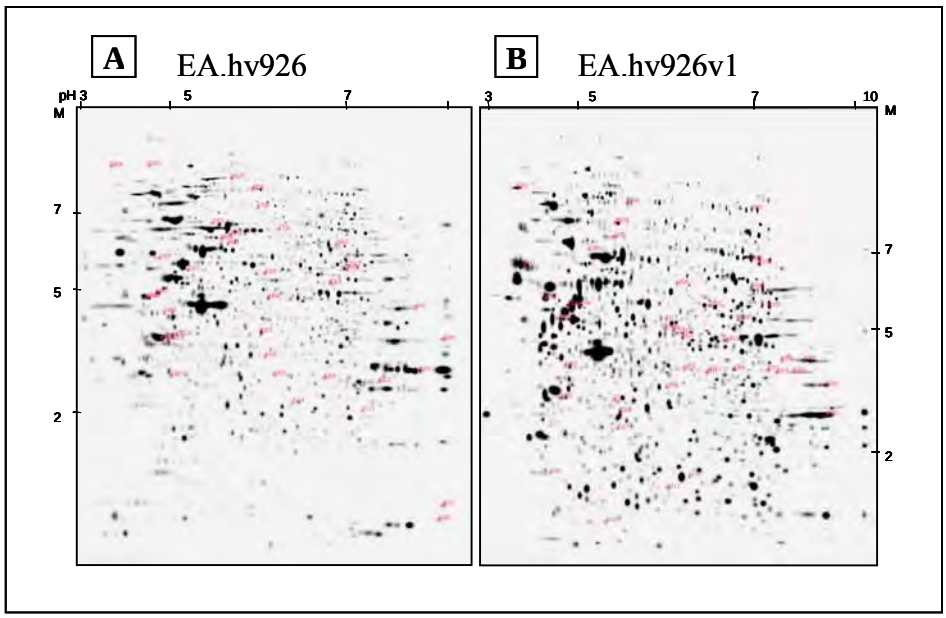


Figure 142. 2-DE gels of proteins extracted from human endothelial cell lines; EA.hy926 (A) and EA.hy926v1 (B). The 1st dimension IEF using pH gradient 3-10 NL, 2nd dimension 8% SDS-PAGE. Statistically significantly (t-test $p<0.05$) differing spots in the cell lines are numbered using PDQuest SSP numbers.

(A) EA.hy926 cell line - 38 statistically significantly differing spots. Four spots: vimentin (1402 and 1405), isocitrate dehydrogenase 3 (NAD+) alpha (4305), and heterogeneous nuclear ribonucleoprotein H1 (4406), were identified using mass spectrometry.

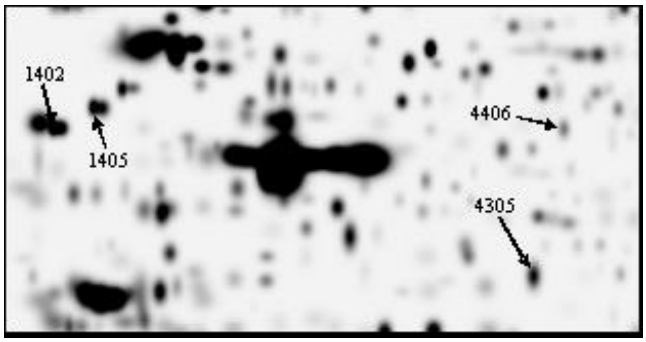
(B) EA.hy926v1 cell line; 45 statistically significantly differing spots.

Few of the protein spots, which expression was statistically significantly altered by the irradiation, were identified using MALDI-MS (Figure 143). In order to increase probability of a single protein present in the single spot, the protein spots that were selected for MALDI-MS analysis had to fulfil the following requirements: (i) spots were well separated from other spots in both 2-DE dimensions, (ii) spots were sufficiently large (Figure 143A). The MALDI-MS analysis service was purchased from the Protein Chemistry Laboratory of the Institute of Biotechnology at the Helsinki University, Finland. The selected spots were reduced with DTT and alkylated with iodoacetamide before overnight digestion with a sequence-grade modified trypsin (Roche, France). The peptide mixture was concentrated and desalted using Millipore ZipTip™ µ-C18 pipette tips. The peptide mass fingerprints were measured with Bruker Biflex™ MALDI-ToF mass spectrometer in a positive ion reflector mode using α-cyano-4-hydroxycinnamic acid as a matrix. The database searches were performed using ProFound and Mascot searches. The protein spots that were identified with MALDI-MS were as follows (Figure 143):

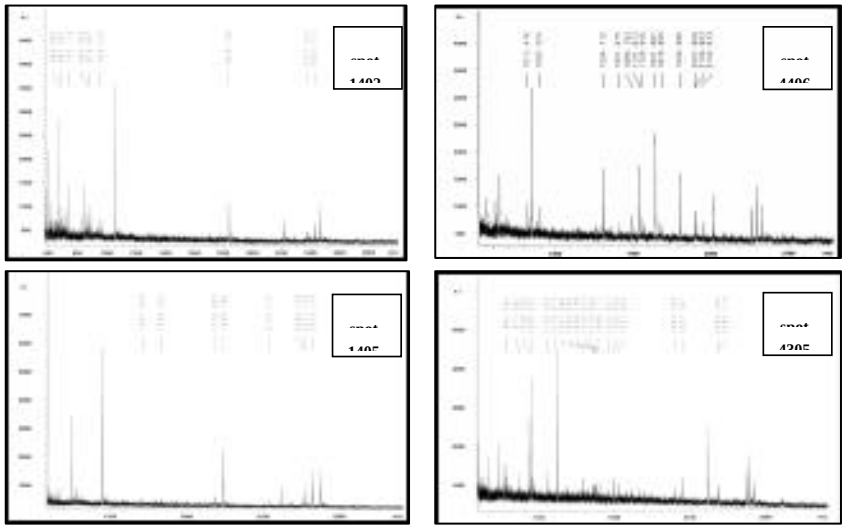
- Protein spot 4305 - isocitrate dehydrogenase 3 (NAD+) alpha (Kim et al. 1995) is a subunit of the mitochondrial enzyme, which catalyses the conversion of isocitrate to 2-oxoglutarate in the citric acid cycle. The expression level of this protein was moderately down-regulated in the exposed samples having a ratio exposed vs. control 0.72 with the p-value of 0.03. The down-regulation of this protein might affect cellular energy production.
- Protein spot 4406 - heterogeneous ribonucleoprotein H1 (Honore et al. 1995) is a component of the heterogeneous nuclear ribonucleoprotein (HNRNP) complexes which provide a substrate for the processing events which pre-mRNAs go through before becoming functional mRNAs in the cytoplasm. The expression level of this protein is slightly down-regulated in the exposed samples with a ratio exposed vs. control 0.61 with the p-value 0.03. The potential down-regulation of this protein might affect protein translation process.

- Protein spots 1402 and 1405 - vimentin (Ferrari et al. 1986) is a protein component of class III-intermediate filaments. In EA.hy926 cells it was found to be expressed in at least two different iso-forms differing in molecular weight and isoelectric point. Both vimentin iso-forms were up-regulated; spot 1402 (experimental MW/pI ca. 47 kDa/4.4) by 2.5-fold with p-value of 0.006 and spot 1405 (experimental MW/pI ca. 48kDa/4.8) by 2.2-fold with p-value of 0.02.

A



B



C

SSP	Protein name	Swiss-prot ID code	Sequence coverage	Swiss-prot annotations
4305	Isocitrate dehydrogenase 3 (NAD+) alpha	P50213	21%	component of enzyme complex catalysing conversion of isocitrate to 2-oxoglutarate
4406	Heterogeneous nuclear ribunocleoprote in H1	P31943	34%	component of HNRNP complex providing a substrate for pre-mRNA processing
1402	vimentin	P08670	19%	class III-intermediate filament
1405	vimentin	P08670	14%	class III-intermediate filament

Figure 143. (A) Fragment of the 2-DE gel of EA.hy926 cells with marked spots that were identified with MALDI-MS. (B) MALDI-MS spectra showing peptide finger prints of the four identified protein spots. (C) Table summarising the properties of the identified proteins**.**

Alterations in the vimentin expression suggest that some form of cytoskeletal response might be taking place in cells exposed to the mobile phone radiation. This notion agrees with our earlier observation of the effect of the mobile phone radiation on the stability of F-actin stress fibers (Leszczynski et al. 2002; Leszczynski et al. 2004). Changes in the vimentin expression observed in 2-DE were further confirmed by SDS-PAGE and western blotting and by cell staining using indirect immunofluorescence method. For SDS-PAGE/western blotting a standard protocol was used. Briefly, the cell lysates were separated using 7.5% SDS-PAGE, blotted to PVDF-membrane, blocked with 5% non-fat dry milk and exposed to the primary vimentin antibody (Zymed, USA) and the secondary antibody containing a HRP-conjugate (Dako, Denmark). The signal was detected using enhanced chemiluminescence (Pierce, UK). For immunocytochemistry cell were fixed in 3% paraformaldehyde, membranes were permeabilised in 0.5% Triton X-100 and as a primary antibody was used vimentin antibody (Zymed, USA) and the secondary antibody was TRICT-conjugated (Dako, Denmark). The images were captured using a Leitz fluorescence microscope and computerised image acquisition system (Metafer, Germany).

SDS-PAGE and western blot have confirmed that EA.hy926 cells express two iso-forms of vimentin. The higher molecular weight form (experimental MW ca. 57 kDa) was present both in control and in irradiated cells and its expression was not affected by the irradiation (Figure 144A). The lower molecular weight vimentin (experimental MW ca. 48 kDa) was not detectable in the non-irradiated cells but was expressed in the irradiated cells (Figure 144B). Indirect immunohistochemistry staining of vimentin has shown the change in the distribution pattern of the vimentin filaments after the exposure to the mobile phone radiation (Figure 143C, 143D). Together, the observed changes in the vimentin expression suggest that the mobile phone radiation might potentially alter cell physiology by affecting cellular cytoskeleton.

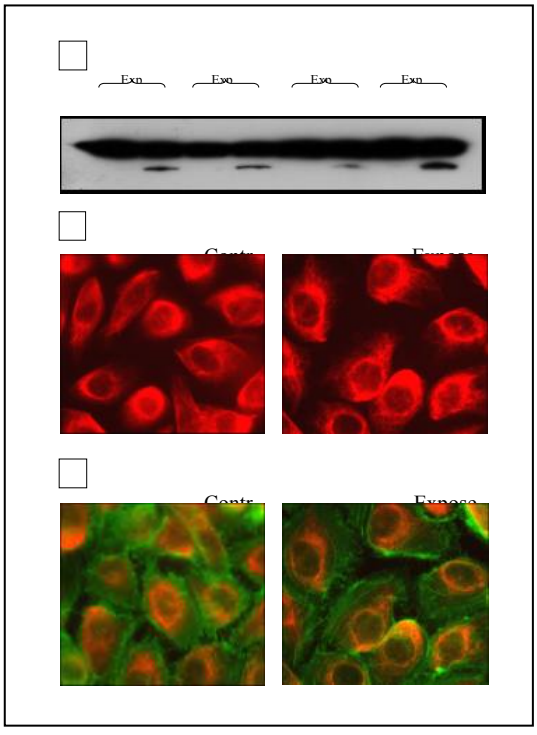


Figure 144. (A) Western blot-detected expression of vimentin in non-irradiated EA.hy926 cells (C-lanes) and in exposed cells (RF-lanes). MW ca. 57 kDa and ca. 48 kDa. Four separate experiments are shown. (B) Immunostaining of vimentin in non-exposed and in exposed EA.hy926; red colour – vimentin. Note diffuse-like staining for vimentin in non-exposed cells as compared with more filament-like expression in exposed cells.(C) Immunostaining of vimentin (red colour) and F-actin stress fibers (green colour) in non-exposed and in exposed EA.hy926. F-actin was detected with phalloidin-AlexaFluor. Note diffuse-like staining for both vimentin and F-actin in non-exposed cells as compared with more filament-like expression in exposed cells.

Transcriptomics approach

Using cDNA Expression arrays (Clontech, USA) we have determined that number of genes increased/declined expression in both cell lines following the exposure to mobile phone radiation (900 MHz GSM). Most strikingly, genes that were up regulated in one of the cell lines were down-regulated or not affected in the other cell line (Tables 29, 30, and 31). It suggests that the cell response might depend on the genotype.

Table 29. Genes which were up-regulated in EA.hy926 cell line following to the exposure to the 900 MHz GSM.

Gene name	**EA.hy926 (*fast proliferating*)**		**EA.hy926v1 (*slow proliferating*)**	
Increased expression in EA.hy926	**Ratio RF/sham**	**Difference RF-sham**	**Ratio RF/sham**	**Difference RF-sham**
proliferating cell nucleolar antigen P120; NOL1	**29,71**	**18734**	**0,38**	**-6343**
Homo sapiens mRNA for beta 2-microglobulin	**14,70**	**3895**	**0,50**	**-5312**
MCM7 DNA replication licensing factor; CDC47 homolog; p1.1-MCM3	**12,18**	**9029**	**0,98**	**-172**
zinc-finger protein (ZNFPT7) (fragment).	**8,43**	**15053**	**0,46**	**-2512**
chloride conductance regulatory protein ICLN; nucleotide-sensitive chloride channel 1A; chloride ion current inducer protein (CLCI); reticulocyte PICLN	**5,37**	**16944**	**0,79**	**-1686**
HHR23A; UV excision repair protein protein RAD23A	**4,42**	**20499**	**0,66**	**-7485**
ferritin heavy chain (FTH1); FTHL6	**4,30**	**6961**	**0,93**	**-2435**
CD166 antigen precursor (activated leukocyte-cell adhesion molecule) (ALCAM).	**4,09**	**3184**	**0,70**	**-2869**
nucleolar phosphoprotein B23; nucleophosmin (NPM); numatrin	**3,89**	**50540**	**0,97**	**-1699**
annexin IV (ANX4); lipocortin I; calpactin II; chromobindin 9; phospholipase A2 inhibitory protein	**3,68**	**7176**	**4,20**	**15254**
N4-(beta-N-acetylglucosaminyl)-L-asparaginase precursor (EC 3.5.1.26) (glycosylasparaginase) (aspartylglucosaminidase) (N4-(N-acetyl-beta-glucosaminyl)-L-asparagine amidase) (AGA).	**3,63**	**13266**	**0,55**	**-1060**
glial growth factor 2 precursor (GGFHPP2); neuregulin; heregulin-beta3 + neu differentiation factor + heregulin-alpha	**3,53**	**21908**	**0,70**	**-3070**
serine/threonine protein phosphatase PP1-alpha 1 catalytic subunit (PP-1A)	**3,31**	**7521**	**1,08**	**1402**
flavin reductase (EC 1.6.99.1) (FR) (NADPH-dependent diaphorase) (NADPH-flavin reductase) (FLR) (biliverdin reductase B) (EC 1.3.1.24) (BVR-B) (biliverdin-IX beta-reductase) (green heme binding protein) (GHBP)	**3,17**	**15395**	**0,77**	**-2827**
cytochrome c	**2,97**	**12337**	**0,82**	**-2047**
DNA-directed RNA polymerase II 19 kD polypeptide (EC 2.7.7.6) (RPB7).	**2,88**	**12043**	**0,88**	**-1360**

Table 30. Genes which were down-regulated in EA.hy926 cell line following to the exposure to 900 MHz GSM.

Gene name	EA.hy926 (*fast proliferating*)		EA.hy926v1 (*slow proliferating*)	
Decreased expression in EA.hy926	**Ratio RF/sham**	**Difference RF-sham**	**Ratio RF/sham**	**Difference RF-sham**
pyruvate kinase M2 isozyme (PKM2)	**0,33**	**-6273**	**0,90**	**-2490**
RAD51-interacting protein	**0,31**	**-3405**	**0,79**	**-510**
glutathione S-transferase mu1 (GSTM1; GST1); HB subunit 4; GTH4	**0,29**	**-3486**	**1,59**	**599**
glutathione S-transferase A1 (GTH1; GSTA1); HA subunit 1; GST-epsilon	**0,29**	**-4095**	**0,36**	**-2545**
early growth response protein 1 (hEGR1); transcription factor ETR103; KROX24; zinc finger protein 225; AT225	**0,28**	**-4223**	**0,54**	**-1932**
caspase-3 (CASP3); apopain precursor; cysteine protease CPP32; YAMA protein; SREBP cleavage activity 1; SCA-1	**0,27**	**-5844**	**1,04**	**79**
calpain 2 large (catalytic) subunit; M-type calcium-activated neutral proteinase (CANP)	**0,27**	**-3464**	**0,95**	**-1210**
ras-related C3 botulinum toxin substrate 1; p21-rac1; ras-like protein TC25	**0,26**	**-6905**	**1,10**	**730**
alpha-actinin 1 cytoskeletal isoform; F-actin cross linking protein	**0,25**	**-5276**	**0,82**	**-2597**
ras-related protein RAB-11B; YPT3	**0,24**	**-2870**	**0,79**	**-3643**
DNA ligase I; polydeoxyribonucleotide synthase (ATP) (DNL1) (LIG1)	**0,22**	**-2969**	**0,38**	**-2497**
ATP synthase lipid-binding protein P2 precursor (EC 3.6.1.34) (ATPase protein 9) (subunit C)	**0,19**	**-4520**	**0,80**	**-1486**
EDF-1 protein	**0,14**	**-6951**	**0,68**	**-2931**
coatomer delta subunit; delta-coat protein; delta-COP; archain (ARCN1)	**0,14**	**-4280**	**n/a**	**n/a**
nuclear transport factor 2 (NTF-2) (placental protein 15) (PP15).	**0,14**	**-2836**	**0,65**	**-2155**
fascin (actin bundling protein).	**0,14**	**-16072**	**0,54**	**-6852**
neurogranin (NRGN); RC3	**0,11**	**-18728**	**0,86**	**-2175**
MYLE	**0,11**	**-4959**	**0,81**	**-539**
sepiapterin reductase (EC 1.1.1.153) (SPR).	**0,10**	**-3467**	**0,63**	**-1123**
caspase-8 precursor (CASP8); ICE-like apoptotic protease 5 (ICE-LAP5); MORT1-associated CED-3 homolog (MACH); FADD-homologous ICE/CED-3-like protease (FADD-like ICE; FLICE); apoptotic cysteine protease MCH-5	**0,05**	**-3359**	**1,90**	**1080**

Table 31. Genes which were affected in EA.hy926v1 cell line following to the exposure to 900 MHz GSM.

Gene name	EA.hy926v1 *(slow proliferating)*		EA.hy926 *(fast proliferating)*	
Increased expression in EA.hy926v1	**Ratio RF/sham**	**Difference RF-sham**	**Ratio RF/sham**	**Difference RF-sham**
procollagen C-proteinase enhancer protein precursor.	**9,58**	**10373**	**1,09**	**5589**
HOMER-3.	**9,45**	**3027**	**0,98**	**-25**
T-lymphoma invasion and metastasis inducing TIAM1	**5,55**	**6554**	**0,01**	**-1335**
elafin precursor (elastase-specific inhibitor) (ESI) (skin-derived antileukoproteinase) (SKALP).	**5,33**	**10720**	**1,53**	**12919**
mitochondrial matrix protein P1 precursor; p60 lymphocyte protein; chaperonin homolog; HUCHA60; heat shock protein 60 (HSP-60); HSPD1	**4,43**	**8459**	**1,00**	**-89**
proteasome component C8; macropain subunit C8; multicatalytic endopeptidase complex subunit C8	**4,06**	**5029**	**1,10**	**5254**
special AT-rich sequence binding protein 1 (SATB1); MAR/SAR DNA-binding protein	**3,48**	**11151**	**0,99**	**-222**
HLA class I histocompatibility antigen C-4 alpha subunit (HLAC)	**3,12**	**2805**	**0,64**	**-1344**
ras-related protein RAP-1B; GTP-binding protein SMG p21B	**3,04**	**4820**	**63,80**	**2512**
phospholipase A2; tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta polypeptide (YWHAZ); 14-3-3 protein zeta/delta; protein kinase C inhibitor protein 1 (KCIP1); factor activating exoenzyme S (FAS)	**2,93**	**4033**	**1,14**	**767**
Decreased expression in EA.hy926v1				
tuberin; tuberous sclerosis 2 protein (TSC2)	**0,33**	**-7082**	**0,42**	**-16846**
KIAA0115; dolichyl-diphosphooligosaccharide protein glycosyltransferase 48-kDa subunit precursor; oligosaccharyl transferase 48-kDa subunit; HA0643	**0,27**	**-3494**	**0,19**	**-1968**
sodium channel beta-1 subunit precursor (SCN1B)	**0,27**	**-2842**	**0,00**	**-1846**
embryonic growth/differentiation factor 1 (GDF1) + UOG-1	**0,21**	**-3542**	**0,00**	**-702**
SH3P18 SH3 domain-containing protein	**0,05**	**-3389**	**0,00**	**-949**

C. Comparison of the effect of CW and modulated RF-EMF on protein expression

Using cICAT method combined with liquid-phase chromatography and mass spectrometry we have compared protein expression changes in cells exposed either to continuous wave or to radiofrequency modulated ("talk" signal) RF-EMF (1800 MHz GSM). The cICAT reagent labelled samples were analysed using an automated mass spectrometric approach in which those proteins showing abundance differences between the two conditions being compared were selectively identified. In total, 58 unique proteins were identified and determined to show significant changes in abundance using this approach. These proteins were selected for identification by MS/MS analysis based upon the criteria that the measured abundance ratios ($C^{13}(0)/C^{13}(9)$) were either >1.7 or <0.60. The average abundance ratio for all detected cICAT reagent labelled peptide pairs (n=1476) was 1.26 +/-0.38, indicating that the vast majority of proteins within the two samples did not change in abundance. Peptides detected as singlets (i.e. having no corresponding $C^{13}(0)$ or $C^{13}(9)$ signal) were also selected for MS/MS analysis. The threshold abundance ratio values were selected based on the following criteria: 1) In relation to previously described errors of quantitative measurements using the ICAT reagent, these values represent conservative estimates of significant abundance changes; 2) These values are significant outliers relative to the average and median $C^{13}(0)/C^{13}(9)$ values for this dataset. The average $C^{13}(0)/C^{13}(9)$ value for all detected cICAT reagent labelled peptides (n = 1476) was 1.26 +/-0.38, and the median was 1.19. The threshold values for significant changes in abundance are therefore well outside the standard deviation for this dataset. Furthermore, the fact that the average and median values are close to one indicates the accuracy of the quantitative measurements used here, as it is expected that the majority of proteins will be constitutively represented, giving ratios close to one.

In conclusion it appears that the "talk" signal has caused increase in expression of a variety of proteins whereas CW did not (Table 32). It suggests that the modulation might have impact on cell response to RF-EMF.

Table 32. List of proteins induced by "talk" signal but not by the CW signal.

Protein name	Accession No.	$C^{13}(0)/C^{13}(9)$	Confidence score
serine-threonine protein kinase	NP_055212	1.9	0.996
GP:AF090929_1		1.8	0.912
RING finger protein 20	AAK58539	2.0	0.958
Homo sapiens cDNA FLJ20303	AK000310	2.4	0.957
hypothetical protein FLJ20420	NP_060282	2.0	0.984
fatty-acid synthase	PIR:G01880	2.2	0.993
hypothetical protein DKFZp434G171.1	T42678	1.9	0.855
hypothetical protein DKFZp564N1563.1 (2)	T46270	0.5	0.921
Serine/threonine protein phosphatase 2A	Q15173	1.8	0.950
Beta-adaptin 1	Q10567	2.0	0.813
Actin-like protein 2	O15142	2.0	0.993
Sarcoplasmic/endoplasmic reticulum calcium ATPase 2 (2)	P16615	1.9	0.944
CD59 glycoprotein precursor (6)	P13987	2.4	0.995
Chloride intracellular channel protein 1	O00299	2.7	0.997
Cellular nucleic acid binding protein (2)	P20694	2.2	0.980
Cofilin, non-muscle isoform	P23528	1.7	0.990
Coatomer alpha subunit	P53621	2.4	0.997
Coatomer beta subunit	P53618	2.0	0.993
Cleavage and polyadenylation specificity factor	Q9P2I0	1.9	0.994
Cyclophilin A (3)	P05092	2.2	0.975
Destrin (Actin-depolymerizing factor)	P18282	1.7	0.990
Aspartyl aminopeptidase	Q9ULA0	1.8	0.993
D-dopachrome tautomerase	P30046	1.7	0.952
Elongation factor 2	P13639	2.0	0.982
Alpha enolase	P06733	2.1	0.981
Fatty acid synthase (2)	P49327	2.1	0.975
Filamin A (2)	P21333	2.5	0.975
FL cytokine receptor precursor	P36888	1.9	0.970
Follistatin-related protein 1 precursor Q12841	Q12841	1.7	0.942
PROTEIN KINASE C SUBSTRATE	P14314	1.7	0.984
Transducin beta chain 1	P04901	1.9	0.993
Transducin beta chain 2	P11016	1.8	0.964
Guanine nucleotide-binding protein beta subunit-like protein (3)	P25388	2.9	0.998
Stress-induced-phosphoprotein 1	P31948	2.2	0.993
Pyruvate kinase, M1 isozyme	P14618	1.9	0.983
LAM2_HUMAN, partial CDS	AAC34573	0.6	0.994
Galectin-1 (3)	P09382	1.9	0.996
Myosin heavy chain, nonmuscle type A	P35579	0.6	0.996
Myoferlin (2)	Q9NZM1	2.9	0.940
NHP2-like protein 1 (3)	P55769	3.6	0.980
Nitric-oxide synthase, endothelial (2)	P29474	2.0	0.990
Purine nucleoside phosphorylase (2)	P00491	1.9	0.997
40S ribosomal protein S27a	P14798	3.0	0.974
Heterogeneous nuclear ribonucleoprotein D0	Q14103	3.0	0.984
Heterogeneous nuclear ribonucleoprotein K	Q07244	2.6	0.880
Heterogenous nuclear ribonucleoprotein U	Q00839	2.1	0.992
ribosomal protein S2 (2)	P15880	1.8	0.940
40S ribosomal protein S3a	P49241	2.1	0.991
Splicing factor, arginine/serine-rich 9	Q13242	2.0	0.986
Tubulin beta-2 chain	P05217	2.8	0.998
T-complex protein 1, theta subunit (2)	P50990	4.9	0.995
Transcription intermediary factor 1-beta	Q13263	2.3	0.995
Thioredoxin	P10599	2.1	0.935
Hypothetical UPF0123 protein BK223H9.2	Q9UH06	2.1	0.994
Splicing factor U2AF 35 kDa subunit (2)	Q01081	2.4	0.945
Ubiquitin-activating enzyme E1	P22314	1.8	0.963
Zinc finger protein 147	Q14258	3.7	0.995
Nuclear pore complex protein Nup133	Q8WUM0	2.3	0.914

3.2.4.7 Whole-genome analysis of various cell lines exposed to RF-EMF (Participant 12)

Altogether, 58 whole-genome analyses of 10 different cell lines (sham-exposed cells and control cells) were performed (Table 1). After primary data analysis, we only worked on genes which were reproducibly regulated in several experiments (see materials and methods) and which belonged to certain gene families (Table 33). We defined gene families which are potentially relevant for the cellular answer on EMF exposure: signal transduction, ion/electron transport, metabolism of energy/proteins, cell proliferation/apoptosis, immune answer/inflammation and extracellular matrix/cytoskeleton. Each gene family was sub-divided in subgroups again, e.g. GTP proteins in the signal transduction family (Tables 33, 11). In a first step, we did not go into single genes, but simply counted genes up- or down-regulated in the different gene families. The total number of regulated genes in a certain gene family is not very meaningful, because the sizes of the gene families are of course very different. Therefore, the total numbers of genes on the human array belonging to a gene family are shown in the first column of Tables 33 and 11. Although a single gene might appear in different categories (e.g. all small G proteins are GTP binding proteins), the tables give a good overview on what might happen in the cells after EMF exposure on the molecular level.

Although appearing regulated in all experiments, mitochondrial genes, ribosomal genes and cell cycle genes especially showed a high rate of regulation in the some RF-EMF experiments (U937 human monocytic cells and microglia cells, Participant 9; HL-60 human hematopoietic leukemia cells, Participant 2).

Moreover, the bio-statistical analysis of RF-EMF experiments (Participant 8, Dr. Remondini) allowed some interesting conclusions from the experiments with HL-60 cells (Participant 2), endothelial cells (Participant 6) and U937 cells (Participant 8, Table 34). Again, the regulation of mitochondrial and ribosomal genes was evident with this analysis. Most of the regulated genes in endothelial cells appear in the groups of ATP-associated genes (energy metabolism), transcription, and cytoskeleton. Remarkably, compared to ELF-EMF experiments, we find more up-regulated genes in RF-EMF experiments (Table 34, see also Table 12). However, the results do have to be interpreted in more detail, because down-regulation of a special gene does not mean that the respective process is down-regulated as well (for example, down-regulation of Bcl-2 might lead to up-regulation of apoptosis).

For T-lymphocytes (Participant 8) and microglia cells (Participant 9) the bio-statisitical analysis did not reveal significant data.

In detail, the following genes were extracted by bio-statistics so far:

Actin associated proteins (belong to cytoskeleton):
- Caldesmon (tropomysin binding, actin binding. Activation of ERK MAP kinases lead to phosphorylation of caldesmon. Regulatory protein of the contractile apparatus): down-regulated (endothelial cells, Participant 6).
- Gamma-actin: down-regulated ((endothelial cells, Participant 6, and U937 cells, Participant 8)
- “coactosin-like”: down-regulated (endothelial cells, Participant 6)
- “actin-binding”: down-regulated (endothelial cells, Participant 6)
- “procollagen-proline 2”: down-regulated (endothelial cells, Participant 6)
- “actin modulating activity”: up-regulated (endothelial cells, Participant 6)
- “actin-binding, calcium ion binding”: down-regulated (endothelial cells, Participant 6)
- CD2-associated protein, actin binding: down-regulated (endothelial cells, Participant 6)
- Tropomodulin 3: actin binding down-regulated (endothelial cells, Participant 6)

Calcium (Ca2+)-associated proteins:
- Ca: “hypothetical protein” (actin-binding): down-regulated (endothelial cells, Participant 6)
- “hypothetical protein”: down-regulated (endothelial cells, Participant 6)
- voltage-gated Ca channel: up-regulated (perhaps up-regulated, because Ca goes down? Endothelial cells, Participant 6)

Cytoskeleton (compare also actin and calcium-associated proteins):
- “hypothetical protein”: down-regulated (endothelial cells, Participant 6)
- “protein phosphatase 4, caldesmon): down-regulated (endothelial cells, Participant 6)
- “SH3 protein interacting with Nck”: down-regulated (endothelial cells, Participant 6)
- “in kinesin complex”: down-regulated (endothelial cells, Participant 6)

Table 33. Numbers of genes regulated within different gene families

Gene Family	total number of clones in Human Unigene RZPD-2	partner 8 U937 monocytes RF up-regulated genes	partner 8 microglia cells RF up-regulated genes	partner 8 U937 monocytes RF down-regulated genes	partner 8 microglia cells RF down-regulated genes	partner 6 endothelial cellsPr1 RF 900MHz up-regulated genes	partner 6 endothelial cells RF 1800 MHz Exp1 up-regulated genes	partner 6 endothelial cells RF 1800 MHz Exp2 up-regulated genes	partner 6 endothelial cellsPr1 RF 900MHz down-regulated genes	partner 6 endothelial cells RF 1800 MHz Exp2 down-regulated genes	partner 6 endothelial cells RF 1800 MHz Exp2 down-regulated genes	partner 2 HL-60 RF ON/OFF up-regulated genes	partner 2 HL-60 RF continuous waves Exp1 up-regulated genes	partner 2 HL-60 RF continuous waves Exp2 up-regulated genes	partner 2 HL-60 RF ON/OFF down-regulated genes	partner 2 HL-60 RF continuous waves Exp2 down-regulated genes	partner 2 HL-60 RF continuous waves Exp2 down-regulated genes	Gene "Superfamily"
Signal	2528	149	40	##	45	190	176	92	160	149	149	91	153	162	190	155	72	signal transduction
GTP	560	37	15	45	14	42	58	24	49	40	51	20	43	51	49	38	18	signal transduction
Small G	235	16	5	20	3	14	17	10	23	15	19	8	17	18	21	18	7	signal transduction
Jak	23	2	0	1	1	1	4	2	1	4	0	1	4	2	1	1	0	signal transduction
Rab	80	3	1	11	3	6	6	4	6	4	9	0	5	5	6	3	5	signal transduction
Ras	66	4	2	7	0	4	4	1	6	4	7	2	6	6	7	2	2	signal transduction
wnt	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	signal transduction
phosphatase	334	24	6	21	7	24	31	17	23	20	25	19	18	26	19	29	9	signal transduction
protein kinase	304	19	6	19	4	19	24	16	27	15	16	18	16	25	23	11	13	signal transduction
phospholipase	72	6	1	7	1	6	4	5	1	2	4	1	3	7	7	5	2	signal transduction
calcium	715	40	6	39	14	56	51	27	45	30	45	20	45	44	45	35	13	signal transduction
calmodulin	131	8	2	6	1	8	6	1	11	8	9	6	5	8	11	10	4	signal transduction
channel	348	12	1	18	7	12	12	8	16	12	11	12	28	11	16	13	6	ion/electron transport
voltage-gated	164	3	0	6	2	7	5	3	3	5	4	5	12	9	7	2	3	ion/electron transport
electron transport	423	25	11	29	6	35	44	20	37	17	34	25	19	36	26	18	15	ion/electron transport
ion transport	501	22	8	32	11	35	21	13	25	18	21	26	39	29	27	37	14	ion/electron transport
metaboli	1241	80	21	81	15	98	124	68	91	71	86	66	87	110	114	64	39	metabolism of energy/proteins
ATP	1234	81	27	92	24	75	116	49	111	77	83	86	102	104	111	82	47	metabolism of energy/proteins
mitochon	574	50	10	63	19	55	61	32	67	46	55	49	51	76	64	66	24	metabolism of energy/proteins
ribosom	254	39	14	39	19	32	35	15	30	25	32	33	31	23	26	37	13	metabolism of energy/proteins
translation	168	21	2	13	9	18	14	12	25	14	18	18	12	12	21	14	17	metabolism of energy/proteins
transcript	1991	116	39	##	41	172	142	78	144	120	129	122	143	138	138	130	75	metabolism of energy/proteins
cell cycle	478	34	11	39	14	42	46	28	46	37	39	44	49	42	46	22	23	cell proliferation/apoptosis/differentiation
apoptos	373	29	8	26	10	34	36	12	18	31	29	18	30	39	32	30	10	cell proliferation/apoptosis/differentiation
differentiat	177	17	2	20	1	14	11	8	5	6	13	6	7	9	12	7	4	cell proliferation/apoptosis/differentiation
immun	390	19	5	26	7	22	44	21	25	19	24	19	31	30	29	17	18	Immune answer/inflammation/stress answer
inflamma	184	8	1	6	3	15	15	8	13	13	9	10	11	6	12	14	2	immune answer/inflammation/stress answer
stress	118	5	6	7	2	8	16	5	8	5	13	5	8	12	10	11	4	immune answer/inflammation/stress answer
peroxidase	32	2	2	7	0	3	4	2	5	3	16	1	4	4	4	5	3	immune answer/inflammation/stress answer
heat shock	188	2	2	4	2	3	5	4	6	6	2	3	3	2	4	6	1	immune answer/inflammation/stress answer
DNA repair	154	10	3	17	4	11	15	6	13	16	15	7	13	12	17	7	8	immune answer/inflammation/stress answer
early	8	0	0	1	0	0	1	0	0	1	2	1	0	0	2	1	1	immune answer/inflammation/stress answer
adhesion	573	30	5	28	9	42	44	19	42	28	38	19	34	31	32	28	14	extracellular matrix/cytoskeleton/adhesion
extracellular matrix	226	14	4	7	5	12	12	7	7	8	8	5	11	10	11	10	8	extracellular matrix/cytoskeleton/adhesion
cytosk	529	33	9	36	21	39	41	19	33	37	43	24	30	46	48	32	21	extracellular matrix/cytoskeleton/adhesion
junction	129	0	3	11	5	3	10	2	9	3	10	5	5	7	4	8	2	extracellular matrix/cytoskeleton/adhesion
actin	494	35	7	35	19	42	39	24	38	41	40	28	30	34	44	40	21	extracellular matrix/cytoskeleton/adhesion

Table 34. Numbers regulated genes in different expression profiling experiments (bio-statistical analysis by Dr. Remondini/Participant 8)

GeneFamily	total number of clones in Human Unigene RZPD-2	partner 6 endothelial cells RF up-regulated genes	partner 6 endothelial cells RF down-regulated genes	partner 2 HL-60 cell RF up-regulated genes	partner 2 HL-60 cell RF down-regulated genes	partner 8 U937 cells RF up-regulated genes	partner 8 U937 cells RF down-regulated genes	Gene "Superfamily"
Signal	2528	4	9	1	0	1	1	signal transduction
GTP	560	1	2	1	0	0	0	signal transduction
Small G	235	0	1	0	0	0	0	signal transduction
Rab	80	0	1	0	0	0	0	signal transduction
Ras	66	0	0	0	0	0	0	signal transduction
phosphatase	334	0	1	0	0	1	0	signal transduction
protein kinase	304	0	2	0	0	0	0	signal transduction
calcium	715	1	2	0	0	0	0	signal transduction
calmodulin	131	0	1	0	0	0	0	signal transduction
channel	348	1	0	1	0	1	0	ion/electron transport
voltage-gated	164	1	0	0	0	0	0	ion/electron transport
ion transport	501	1	0	0	0	0	1	ion/electron transport
electron transport	423	1	1	0	0	1	0	ion/electron transport
metaboli	1241	3	0	0	0	2	0	metabolism of energy/proteins
ATP	1234	1	4	0	0	0	1	metabolism of energy/proteins
mitochon	574	2	1	0	0	0	0	metabolism of energy/proteins
ribosom	254	0	1	0	0	1	4	metabolism of energy/proteins
translation	168	0	0	0	0	0	0	metabolism of energy/proteins
transcript	1991	1	6	3	0	2	0	metabolism of energy/proteins
cell cycle	478	0	3	1	0	0	0	cell proliferation/apoptosis/differentiation
apoptos	373	1	0	0	0	0	0	cell proliferation/apoptosis/differentiation
differentiat	177	0	1	1	0	0	0	cell proliferation/apoptosis/differentiation
immun	390	0	0	0	0	0	0	immune answer/inflammation/stress answer
DNA repair	154	0	1	0	0	0	0	immune answer/inflammation/stress answer
inflamma	184	0	0	0	0	1	0	immune answer/inflammation/stress answer
adhesion	573	0	1	0	0	1	0	extracellular matrix/cytoskeleton/adhesion
extracellular matrix	226	1	0	0	0	1	0	extracellular matrix/cytoskeleton/adhesion
cytosk	529	0	5	0	0	1	1	extracellular matrix/cytoskeleton/adhesion
actin	494	0	4	0	0	1	1	extracellular matrix/cytoskeleton/adhesion
junction	129	0	1	0	0	1	0	extracellular matrix/cytoskeleton/adhesion

3.2.4.8 Summary (Participant 1)

Our data indicate an effect of RF-EMF on gene and protein expression in various cell systems. This conclusion is based on the following findings:

- RF-EMF exposure at a SAR value of 1.5 W/kg caused a transient up-regulation of p21 and c-myc genes and a long-term up-regulation of the stress response gene hsp70 in embryonic stem cells deficient of the p53 gene (3.2.4.1)
- RF-EMF exposure at a SAR value of 2 W/kg reduced the expression of the receptor FGFR1 of fibroblast growth factor (FGF) in human neuroblastoma cells (NB69) and in neural stem cells of rats (3.2.4.2).
- RF-EMF exposure at a SAR value of 1.3 W/kg up- or down-regulated the expression of various genes and proteins in HL-60 cells and in endothelial cells of human origin (3.2.4.3, 3.2.4.6, 3.2.4.7).
- RF-EMF exposure at a SAR value of 2.4 W/kg activated the p38MAPK/hsp27 stress response pathway in endothelial cells of human origin (3.2.4.6).
- RF-EMF exposure at a SAR value of 2.4 W/kg changed the global pattern of protein phosphorylation in endothelial cells of human origin with possible consequences for the signal transduction pathway (3.2.4.6).
- RF-EMF exposure at a SAR value at 2 W/kg did not significantly affect gene expression in human lymphocytes, although a few genes among several thousand tested with the micro-array system were found altered in two human immune cell lines (3.2.4.4, 3.2.4.5).
- RF-EMF exposure at a SAR value of 2 W/kg did not affect the expression and activity of the inducible nitric oxide synthase (iNOS) and the expression of hsp27 and hsp70 in nerve cells (3.2.4.5).
- The increased expression of hsp27 in endothelial cells (EA.hy926) after RF-EMF exposure as described in 3.2.4.6 could not be reproduced in another laboratory where slightly different methods were used (3.2.4.5).

4.0 DISCUSSION

4.1 Results obtained after ELF-EMF exposure

4.1.1 Genotoxic effects

4.1.1.1 Human fibroblasts, lymphocytes, monocytes, melanocytes and muscle cells and granulosa cells of rats (Participant 3)

Intermittent ELF-EMF exposure generated DNA strand breaks in various but not all cell lines.

Our results show, that intermittent exposure to a 50 Hz magnetic field causes a reproducible increase in DNA strand breaks in cultured human cells. These findings are in accordance with some recent studies with whole-body exposure of rodents to ELF-EMF which revealed DNA single- and double-strand breaks in the brain (Lai and Singh 1997c; Singh and Lai 1998; Svedenstal et al. 1999a/b). However, the majority of the studies investigating genotoxic effects of 50/60 Hz electromagnetic fields (McCann et al. 1993, 1998; Murphy et al. 1993; Moulder 1998) have reported a negative outcome on genotoxicity. Our results from tests with continuous exposure of fibroblasts to EMF corroborate these findings. Subjecting cells continuously to a constant field probably may induce adaptive mechanisms, protecting the genome from harmful influences. A regular change of environmental conditions might interfere with such mechanisms and lead to DNA damage. The extent of damage would depend on the duration of exposure and the time of recovery.

It is highly unlikely, that the observed genotoxic damage is caused non-specifically by spots of increased temperature within the cell layer as a secondary effect of the electromagnetic field. If so, the damage would increase with a prolongation of on-time during the intermittent exposure and would be largest at continuous exposure. The largest effects, however, are obtained at 5 min on/10 min off cycles, and continuous exposure had no effect at all. Therefore, we conclude, that the observed induction of DNA-single and double strand breaks is a direct consequence of an intermittent exposure to ELF-EMF.

Environmental exposure to continuous ELF-EMF is rather exceptional. Different electrical household devices (hair dryer, razor, vacuum cleaner) reaching peak values up to 1 mT are often used for a short period of time (5-10 min), producing a variety of exposure levels. To date, we could make out only one study dealing with genotoxic effects of ELF-EMF at intermittent exposure. This was done by Nordenson et al. (1994), who found a significant increase of chromosome aberrations in human amniotic cells (50 Hz, 30 µT, 20 s on-off). However, these results have not been corroborated by other studies as yet.

Genotoxic effects of ELF-EMF varied with exposure time.

We observed a time dependent increase of DNA breaks up to 15 to 19 hours of ELF-EMF exposure and then a decline to a steady state level of about 1.5 fold of the base line. This unexpected finding can be explained, if the exposure activates DNA repair processes and this activation takes a time of 10 to 12 hours. After this time the DNA damage is repaired then at an enhanced rate, which leads to a reduction of DNA breaks albeit not to a normalisation. This explanation is experimentally supported by the observation, that the single strand DNA breaks (alkaline conditions) are repaired after approximately 30 minutes, and double strand breaks 7 to 9 hours after finishing the exposure. Removal of damaged DNA-bases by induced repair enzymes (glycosylases) may lead to a temporary increase of abasic sites in the DNA (Friedberg et al. 1995). Abasic sites (alkali-labile sites) result in DNA single strand breaks after alkaline treatment (Tice et al. 2000). The alkali-labile sites generated after ELF-EMF exposure are therefore detected as peak at hour 12 to 17 at Comet assay conditions of pH > 13, but not of pH 12.1, the latter not being able to cleave the alkali sensitive sites.

It is well known, that the repair of single strand breaks is a fast and almost error free process, while the repair of more complex DNA damage (i.e. DNA double strand breaks) by homologous recombination, single strand annealing or non-homologous end joining require more time and are error prone in part (Van den Bosch et al. 2002). Therefore DNA double strand breaks may affect the integrity of the genome leading to cell death, uncontrolled cell growth or cancer (Van Gent et al. 2001).

Our results show, that intermittent exposure to a 50 Hz magnetic field causes a time dependent increase in micronuclei in cultured human fibroblasts. These findings are in accordance with Simko et al. (1998a/b), who could demonstrate an ELF-EMF-induced formation of micronuclei in human amnion and in human squamous cell carcinoma cells. In contrast, the greater part of studies performed as yet using different cell types did not point to direct clastogenic effects of ELF-EMFs (Livingston 1991; Scarfi et al. 1991, 1994; Paile et al. 1995), but they propose epigenetic or co-clastogenic mechanisms in combination with other genotoxic exposures (Lagroye et Poncy 1997; Cho and Chung 2003; Simko et al. 2001b). Micronucleus formation can either result from chromosomal non-disjunction due to damage of kinetochore proteins or from acentric fragments secondary to DNA double strand breaks. Since we did not use kinetochor antibodies to differentiate between these two possible mechanisms, the cause for the micronuclei induction remains an open question. At extended exposure times micronucleus frequencies reached a constant level, which is not in contrast to the results found in Comet assay since micronuclei cannot be repaired.

ELF-EMF produced DNA strand breaks in human fibroblasts in a dose dependent way.

We could demonstrate a dose dependent relationship between alkaline and neutral Comet assay tailfactors and applied magnetic flux density. The guidelines of the International Commission on Non-Ionizing Radiation Protection (ICNIRP 1998) are 500 µT during workday for occupational exposures and 100 µT for 24 h/day for the general population. The on-set of genotoxic effects in our tests was at a magnetic flux density as low as 35 µT at 15 hours and 70 µT at 24 hours of exposure, being well below these proposed guideline values. Moreover, these guidelines are dealing with continuous EMF exposure. No proposal how to handle intermittent exposures has been made by the ICNIRP as yet.

Generation of DNA strand breaks in human fibroblasts through ELF-EMF was related to the age of the donors.

Our findings of significant differences in basal DNA single and double strand break levels in fibroblasts of donors of different age are consistent with studies of several species and tissues (Mullaart et al. 1988; Holmes et al. 1992; Zahn et al. 1996; Diem et al. 2002). In addition, we here report differences in response to ELF-EMF exposure in relation to donor age, which point to a higher susceptibility of older donors to the genotoxic action of ELF-EMF. This could be interpreted by a later on-set of DNA repair. These findings are in agreement with age-related increases of DNA damage and mutations as a result of a reduced DNA repair capacity (Wolf et al. 2002; Bohr 2002; Cabelof et al. 2002; Ben Yehuda et al. 2000; Goukassian et al. 2000). Observations of an altered gene activity during ageing were reported for the rat brain, heart, and liver (Salehi et al. 1996; Goyns et al. 1998) and human fibroblasts (Linskens et al. 1995). This decline may be due to a reduction in chromatin associated RNA polymerase II activity (Rao and Loeb 1992), to mutation-induced changes in binding activity of transcription factors (Sheerin et al. 2001), or due to a decline in protein synthesis secondary to a decrease in the amount and activity of certain elongation factors (Shikama et al. 1994). Changes in the availability of proteins or enzymes may be critical if proteins of DNA repair machinery are affected.

Effects of ELF-EMF were cell type specific.

Our results point to cell type specific reaction and to differences in sensitivity of different tissues to ELF-EMF exposure. We could identify three responder (human fibroblasts, human melanocytes, transformed rat granulosa cells) and three non-responder cell types (human lymphocytes, monocytes and skeletal muscle cells). Up to date a plausible mechanism for these findings is mere speculation, but these data propose an epigenetic, indirect action of intermittent ELF-EMF. The observed cell specific response can not be explained by age-related effects, since the non-responding skeletal muscle cells are derived from the oldest donor.

In our experiments we exposed dividing and quiescent lymphocytes to ELF-EMF, and in both cases no induction of DNA strand breaks could be observed. Isolated monocytes did not respond either. The other cell types used were cells in the log-growing phase and some of them showed genotoxic effects, whereas dividing skeletal muscle cells did not react. Therefore, it is not likely that the observed effects could be due to differences in response between proliferating and non-proliferating cells. In addition, the observed effects can not be attributed to differences between adherent cells or suspension cultures, since there are non-responder cell types in both cases.

Based on the results with human fibroblasts, which suggest an induction of DNA repair upon intermittent ELF-EMF exposure, we speculate, that the effects reported here, may reflect differences in DNA repair

capacities between different tissues. This explanation, however, requires further assessment, e. g. evaluation of repair kinetics.

Generation of DNA strand breaks in human fibroblasts through ELF-EMF and their repair were modified by UVC or heat stress.

During ELF-EMF exposure UVC induced DNA-damage was repaired very slowly, although the maximum at 15h ELF-exposure could not be detected any more. The results were similar with the neutral Comet assay, but DNA damage (DNA double strand breaks) was repaired within a shorter time. These results suggest that ELF-EMF-exposure might impair and/or delay the onset of repair of DNA damage.

In regard to studies on repair kinetics, the exposure time dependent extent of DNA damage implicated an induction of DNA repair upon intermittent ELF-EMF exposure. We concluded that pre-exposure to intermittent ELF-EMF would have a protective effect and reduce genotoxic actions of additional exposures. In contrast to our assumption, pre-exposure to intermittent ELF-EMF for 20 hours resulted in an additive genotoxic effect of combined exposures and a reduced repair rate of UVC or heat stress induced DNA damage. A protective effect of ELF-EMF exposure could not be confirmed by these results. In contrast, they suggest an impairment or delay of DNA repair mechanisms due to ELF-EMF exposure.

Recently, Robison et al. (2002) have demonstrated that pre-exposure to ELF-EMF for 4 to 24 hours can decrease DNA repair rate and protect human HL-60 cells from heat induced apoptosis. Miyakoshi et al. (2000) showed that strong ELF-EMF for 2 hours can potentiate X-ray-induced DNA strand breaks in human malignant glioma cells, whereas others (Whitson et al. 1986; Frazier et al. 1990; Cantoni et al. 1996) found no evidence that ELF-EMF could inhibit repair of DNA damage induced by ionising radiation or UV light using different human cell types. However, in these experiments ELF-EMF exposure was not performed prior to UV or X-ray exposure, but afterwards. ELF-EMF preconditioning of cells may evoke different reactions. In addition, responses of the cells could differ with ELF-EMF exposure duration, applied exposure protocol (continuous vs. intermittent) or used cell type. Anyhow, these experiments may not overrule our theory of an induction of DNA repair upon ELF-EMF exposure, since repair processes are very complex and different mechanisms may engaged in the repair of UV or thermal stress induced DNA damage.

Generation of DNA strand breaks in human fibroblasts through ELF-EMF was dependent on the genetic background of cells.

We concluded that the cupola-shaped time dependent pattern of DNA breaks in the Comet assay mirrors the action of repair processes. This is supported by the more than two fold increased rate of DNA breaks in DNA repair deficient fibroblasts from a patient with Ataxia Telangiectasia after 24 hours of exposure. The increased DNA breaking rate seen in fibroblasts from this patient and in fibroblasts from older donors points to the significance of the genetic background regarding the response to ELF-EMF-exposure.

Generation of DNA strand breaks in human fibroblasts by ELF-EMF was dependent on the frequency of ELF-EMF.

Although intermittent ELF-EMF induced DNA strand breaks in the Comet assay at a broad frequency range between 3 and 550 Hz, there are noteworthy peak effects at 50 Hz and 16.66 Hz, these representing the commonly used frequencies of alternating current in Europe. However, this has been tested as yet at intermittent 5 min on /10 min off cycles only and may be different under changed intermittent conditions.

ELF-EMF generated chromosomal aberrations in human fibroblasts.

Structural chromosome aberrations result from breakage and abnormal rearrangement of chromosomes. They can be classified either to stable or unstable aberrations, depending upon their ability to persist in dividing cell populations. Unstable aberrations are ring chromosomes, dicentric chromosomes or acentric fragments, whereas stable aberrations, which result from repair processes, consist of balanced translocations or other symmetrical rearrangements. At exposure conditions producing maximum effects in micronucleus test and in Comet assay, we observed significant increases in gaps, breaks, ring chromosomes, dicentric chromosomes and acentric fragments, but not of translocations. These results are in accordance with studies performed by Nordenson et al. (1994) and Khalil and Quassem (1991), who applied intermittent or pulsed field ELF-EMF exposure. Several other studies performed at continuous ELF-EMF exposure could detect an increase in chromosomal damage (Jacobson-Kram et al. 1997; Galt et al. 1995; Scarfi et al. 1991; Paile et al. 1995).

The fate of a cell carrying a chromosomal aberration is crucial for the assessment of a possible cancer risk. Cells with unstable aberrations like rings, dicentrics or acentric fragments will be committed to apoptosis or cell death, whereas cells with repairable DNA damage like chromosomal gaps or breaks may survive. The repair process itself can lead to translocations, thereby creating a stable mutation. Surprisingly this could not be detected in 24-times 1,000 metaphases, when each chromosome had been separately painted.

Although no significant differences in cell numbers could be detected between ELF-EMF exposed and sham-exposed cells, a possible elimination of cells carrying non-stable chromosomal aberrations is not contradictory to these previous findings. Cell numbers were assessed directly after ELF-EMF exposure termination, whereas for evaluation of chromosomal aberrations, cells were maintained in culture. In addition, the total fraction of cells with non-stable aberrations in exposed cells was 0.8%. The method used for assessment of cell numbers (Coulter counter) is to imprecise to detect such o low number of cells.

Since experimental analyses have shown, that DNA double strand breaks are the principal lesions to produce chromosomal aberrations (Bryant 1998; Natarajan and Obe 1978; Obe et al. 1992), the induction of micronuclei and chromosomal aberrations is in good agreement with the previous demonstration of DNA strand breaks.

ELF-EMF did not influence the mitochondrial membrane potential.

Hitherto, response of $\Delta\Psi_m$ to ELF-EMF exposure has not been assessed and no data on EMF-induced modifications of the membrane potential of cells are available. Effects of electric fields on membrane ATPases (optimal ranges: 5 - 30 Vcm^{-1}, 10 Hz - 1 MHz) have been reported by several groups (Tsong 1992). Short pulses of electric field (100 µs decay time) of several $kVcm^{-1}$ have been used to trigger ATP synthesis in rat liver submitochondrial particles. The electric field-induced ATP synthesis was abolished by inhibitors of the F_0F_1-ATPase, oligomycin, *N,N´*dicyclohexylcarbodiimide, venturicidin and aurovertin, but occurs independently of components of the mitochondrial electron transport chain. In low field experiments (<75 Vcm^{-1}) Tsong showed a dependence of ATP yield on the field strength and frequency of the alternating current (AC) field. Effects of AC fields on the activity of Na^+,K^+-ATPase, the enzyme principally responsible for establishing ion gradients across the cell membrane, have also been reported by Blank (1992). Under normal conditions *in vitro*, the enzyme in weak electric fields has a decreased ability to split ATP (100 Hz, threshold for effects of 5 μVcm^{-1} estimated by extrapolation). When the enzyme activity was inhibited to less than half its optimal level by ouabain or low temperature, an increase in ATP splitting was observed. The greatest effects appear to be in the extremely low frequency range that includes 50 Hz.

Our data do not indicate an influence of intermittent ELF-EMF exposure of human fibroblasts on $\Delta\Psi_m$. Although these results cannot rule out rapid alterations of $\Delta\Psi_m$, we consider it to be unlikely that ELF-EMF-induced formation of DNA strand breaks is mediated via significant intracellular changes which affect $\Delta\Psi_m$.

4.1.1.2 Human fibroblasts and granulosa cells of rat (Participant 7)

The genotoxic effects induced by ELF-EMF are not reflected by physiological functions like volume regulation and free cytoplasmic Ca^{2+}-concentration.

The experiments have been performed on two different cellular levels, the genomic and the cellular level using cultured granulosa cells of rat (GFSHR-17) and human fibroblasts. On the genomic level the neutral and alkaline Comet assay has been applied to evaluate ELF-EMF (5min on/10 min off, 1 mT) induced effects on DNA single- and double-strand breaks. In parallel, the effects were compared with those obtained on the cellular level by analysis of volume regulation (Ngezahayo et al., 2003) and cytoplasmic free Ca^{2+} concentration (Pilger et al., submitted). Since Participant 3 observed no effect during permanent ELF-EMF exposure on the occurrence of DNA strand-breaks, but for intermittent exposure (Ivancsits et al. 2002a; Ivancsits et al. 2003b), we followed this exposure protocol.

The results of the alkaline Comet assay indicate that intermittent exposure to ELF-EMF induced a significant increase of single- and double-strand breaks in rat granulosa cells (Figure 26) with a maximum after exposure for 16h to 18h. The maximum is followed by a decline of DNA damage in the time range

of a few hours which can be attributed to the onset of DNA repair mechanisms (Ivancsits et al. 2002a). It should be noted that a similar time course of DNA damage was observed at an exposure frequency of 16 2/3 Hz for rat granulosa cells. The time course of DNA damage on rat granulosa cells is similar as reported for cultured human fibroblasts (Ivancsits et al. 2002a; Ivancsits et al. 2003b), CHO and HeLa cells, but appears to be more pronounced. Therefore, it seems to be reasonable to suggest that the sensitivity of cultured cell lines to ELF-EMF (intermittently applied) depends significantly on the cell type.

In addition the frequency dependence of DNA damage was studied. The quantity of DNA single-strand breaks appears to depend on the frequency of the applied ELF-EMF with a maximum at lower frequency within the applied sequence of frequencies (8 Hz, 16 2/3 Hz, 50 Hz, 1000 Hz) (Figure 27). Surprisingly, virtually no frequency dependence is found for the results of the neutral Comet assay (Figure 28).

The important question arises whether the ELF-EMF effects observed on the genomic level are reflected in a change of the macroscopic cellular behaviour, especially in basic regulatory physiological functions. As marker of physiological cell functions the regulatory volume decrease/increase of rat granulosa cells was considered. In response to a constantly applied hypotonic shock rat granulosa cells swell due to water influx like an osmometer and shrink thereafter to the original cell volume within the time scale of an hour. According to this physiological regulatory behaviour a hypertonic shock causes cell shrinkage. The results show no significant influence of ELF-EMF exposure at the additional stress condition caused by a non-isotonic bath medium (Figure 69). It could be argued that, since for technical reasons the regulation of cell volume was studied 10 min after the end of ELF-EMF exposure for 18h, the DNA repair mechanisms act significantly and thereby bias the results. But the time span for DNA repair after an exposure time of 18h occurs in the range of hours (Figures 16, 17), whereas the experiments focused to volume regulation were started 10 min after end of ELF-EMF exposure. Therefore it can be concluded that a significant increase of DNA single- and double-strand breaks by ELF-EMF exposure is virtually not reflected in a change of regulatory volume decrease/increase of granulosa cells. As second macroscopic cellular parameter the time course of free cytoplasmic Ca^{2+}-concentration ($[Ca^{2+}]_i$) was studied by fluorescence-spectroscopy after ELF-EMF exposure for 5h, 6h, 7h, 8h, and 18h. $[Ca^{2+}]_i$ was recorded in the absence (Figure 67, Table 9) and presence of a further stress factor, the exposure to H_2O_2 containing bath media (Figure 68, Table 10)). Also for this cellular parameter no significant influence of ELF-EMF exposure could be observed. In co-operation with Participant 3 a further cell-culture system, human fibroblasts, were used. In parallel to the results showing ELF-EMF induced DNA strand-breaks, the corresponding free Ca^{2+}-concentration was recorded. Also for this cell system the observed ELF-EMF induced DNA strand-breaks are not reflected in a change of the cellular level of free cytoplasmic Ca^{2+} (Figures 66, 67) or the mitochondrial potential (see also ref. Pilger et al., submitted). In contrast to our findings Tonini et al. (2001) reported a fast, within the time range of minutes, and significant increase of $[Ca^{2+}]_i$ in a cultured neuroblastoma cell line by ELF-EMF exposure at 50- to 60-Hz and 0.12 μT (0.24 μT). Surprisingly, the observations were made at continuous ELF-EMF exposure. The various findings could be related to the specific cell type, the different set-up used for ELF-EMF exposure or the method applied for analysis of $[Ca^{2+}]_i$.

4.1.1.3 Mouse embryonic stem cells (Participant 4)

ELF-EMF did not induce the formation of DNA strand breaks in embryonic stem cells.

The potential to induce primary DNA damage by ELF-EMF was analysed by the Comet assay, as a consequence of up-regulation of the DNA-damage inducible gene GADD45 after ELF-EMF exposure (4.1.3.1). In addition, it was shown by Participant 3 that the exposition of human fibroblasts to ELF-EMF results in the increase in DNA breaks suggesting a possible direct mutagenic effect (Ivancsits et al., 2002). A correlation has been described between up-regulation of GADD45, bcl-X_L, and increased DNA damage as determined on the basis of the alkaline Comet assay in human preneuronal cells (Santiard-Baron et al. 2001). However, we did not observe significant effects of 6h or 48h intermittent ELF-EMF exposure on single- and double-strand DNA break induction in the alkaline and neutral Comet assay. One reason for our negative results (as compared to the data presented by Participant 3 on human fibroblasts, e.g.) could be the different intermittency scheme of exposure, which was applied (5 min on/30 min off by Participant 4 vs. 5 min on/10 min off by Participant 3). Actually Ivancsits et al. did not observe effect by using an intermittency scheme of 5 min on/25 min off, but only for shorter off time durations. However, by RF-EMF exposure of ES cells using 5 min on/30 min off cycles, we found a low, but significant

increase in double-strand DNA breaks, suggesting that the EMF frequency has significance for the DNA damaging effects.

4.1.1.4 Summary (Participant 1)

As discussed by Participant 3 there has been sporadic literature concerning *in vitro* studies which demonstrate that ELF-EMF may possess a genotoxic potential (Lai and Singh 1997c; Singh and Lai 1998; Svedenstal et al. 1999a/b). However, the energy impact to the genome of livings cells exposed to ELF-EMF had been calculated to be too low to generate DNA damage. Since the mainstream literature contradicted the assumption of genotoxic effects (McCann et al. 1993; McCann et al. 1998; Murphy et al. 1993; Moulder 1998), these sporadic findings were considered more or less meaningless. Opposite to this widely accepted view, the data of the REFLEX study which were systematically investigated and confirmed in 4 laboratories, of which two were not members of the REFLEX consortium, support the view that ELF-EMF causes genotoxic effects in certain, but not all cell systems.

Based on the methodology used and the data obtained in the REFLEX study, the findings of genotoxicity caused by ELF-EMF are hard facts. DNA single and double strand breaks were observed in human fibroblasts exposed to ELF-EMF at a flux density as low as 35 µT, which is far below the presently valid safety limit. Increases in micronuclei and chromosomal aberrations were found at higher flux densities (3.1.1.1). These effects, although striking in fibroblast from normal donors and donors with a known repair deficiency, were not observed consistently in all cell types, e.g. in human lymphocytes. This suggests that the genetically determined defence mechanisms of cells play a decisive role as to whether or not the cells respond to ELF-EMF exposure. The question arises why the genotoxic potential of ELF-EMF was not confirmed many years ago when suitable biochemical methods became available the first time. One explanation may be that most of the experiments were carried out with lymphocytes which seem to be resistant to ELF-EMF, and that in experiments with different cell systems the exposure time and the exposure conditions may have been inadequate.

As already stated, for energetic reasons ELF-EMF can neither denature proteins nor damage cellular macromolecules directly. If the energy impact on the genome of living cells exposed to ELF-EMF is too low for damaging their DNA, the genotoxic alterations observed in the REFLEX project must be produced indirectly through intracellular processes. Participant 3 observed in its most recent experiments that the increase of DNA strand breaks in human fibroblasts after ELF-EMF exposure can partly be inhibited by oxygen radical scavengers. This finding speaks for the assumption that the observed DNA damage may be caused by free oxygen radicals which are released by ELF-EMF. This assumption is further supported by results obtained by Simko et al., who measured an increase of free oxygen radicals in macrophages derived from murine bone marrow after exposure to ELF-EMF at a flux density of 1 mT (Simko et al. 2001) and by Lupke et al. who observed an increase of free oxygen radicals in monocytes derived from umbilical cord blood and in a human monocytic leukaemia cell line also after exposure to ELF-EMF (50 Hz) at a flux density of 1 mT (Lupke et al. 2004). Into the same direction hint the results of Zymslony et al., who assessed the effects of ALF-EMF (50 Hz, 40 µT) on the oxidative deterioration of DNA in rat lymphocytes after *in vitro* irradiation by UVA (Zymslony et al. 2004). The free radical hypothesis is further supported by the studies of Lai and Singh (2004) who found that brain cells of rats after whole body-exposure to ELF-EMF (60 Hz) at very low flux densities (0.01-0.25 mT) for 2-48 hrs showed increases in DNA single and double strand breaks, and that these increases could be blocked by pre-treating the animals with the free radical scavengers melatonin, N-tert-butyl-α-phenylnitrone and Trolox (a vitamin E analogue). The work of Lai and Singh, which must still be reproduced by other independent research groups, deserves special attention, since the DNA damage reported by them was observed in the brain of whole-body exposed animals, not in isolated cells as in the REFLEX study.

Based on the data of the REFLEX project it must be assumed that ELF-EMF is able to damage the genome in certain, but not all cell systems after exposure *in vitro*. The work of Lai and Singh suggests that these effects might also be seen after exposure *in vivo*. The genotoxic effects of ELF-EMF may be best explained by an ELF-EMF induced increase of intracellular free radicals within the exposed cells and by the genetic background of the exposed cells. It is well known that a balanced free radical status is the prerequisite for maintaining health and that an unbalanced free radical status promotes the process of ageing and the development of chronic diseases such as cancer and neurodegenerative disorders. Whether the balance of free oxygen radicals can also be impaired through ELF-EMF *in vivo* as suggested by Lai and Singh (2004) needs to be further clarified.

4.1.2 Cell proliferation and differentiation

4.1.2.1 Human neuroblastoma cells (NB69 cell line) (Participant 5)

ELF-EMF enhanced proliferation and reduces spontaneous apoptosis of NB69 neuroblastoma cells.

The described results indicate that 42- or 63-hour exposure to 50 Hz magnetic fields at 10 or 100 µT can increase proliferation and reduce spontaneous apoptosis in human neuroblastoma cells. Initial evidence obtained through cell counting (Trypan blue exclusion) was subsequently confirmed through PCNA labelling, 5-bromo-2'-deoxyuridine (BrdU) labelling for identification of DNA-synthesizing cells, and flow cytometry. The modest, though statistically significant increase in the total number of cells in response to a 100-µT field estimated by the Trypan blue exclusion is consistent with the observed increase in the number of PCNA positive cells. This is also consistent with the increased numbers of cells in G2-M phase and of BrdU positive cells observed 24 hours before the increase in the number of cells was detected. The present data also indicate that a 50-Hz EMF at 100 µT can induce changes in the activation of the transcriptional factor CREB in a time-dependent manner.

A number of experimental studies investigating proliferative effects of EMF using in vitro or in vivo models (Kavet 1996), have provided limited evidence that ELF-EMF can represent a growth stimulus. Kwee and Raskmark (1995) have reported that a 24-h exposure to 50 Hz MF at 80 µT significantly increases the proliferation of transformed human epithelial amnion cells and K14 skin fibroblast cells. Wei et al. (2000) have reported that 60 Hz MF (30-120 µT, 3-72 h exposure) can induce proliferation in human astrocytoma cells and strongly strengthen the effect of two chemical agonists.

Some studies, however, have reported effects that are in apparent contrast to those described above. For instance, Conti et al. (1983) and Cleary (1993) have reported reduced ^{3}H-thymidine incorporation into lymphocyte DNA after exposure to 2.0 - 7.0 mT, 50-Hz magnetic fields. It has been proposed that several physical and biological variables, including different field parameters, exposure protocols, cell types and physiological conditions (degree of differentiation or activation) may account for the conflicting results reported in the literature (see for instance Schimmelpfeng and Dertinger 1997). In fact, the cellular response to the fields seems to be strongly dependent on biological parameters (Simko et al. 1998a/b; Wei et al. 2000). In addition, there is experimental evidence that specific combinations of AC/DC fields interact with biological systems (Blackman et al. 1994; Trillo et al. 1996) and the key to affecting proliferation of cells in a consistent manner might lie in the simultaneous control of the AC field amplitude and frequency, and the AD/DC field intensity ratio (Yost and Liburdy 1992; Blackman et al. 1985a,b; Trillo et al. 1996; Bauréus Koch et al. 2003).

Also, a recent study by Pirozzoli et al., (2003) has shown that the apoptosis induced by camptothecin in neuroblastoma cells (LAN-5) can be prevented by a 24-h exposure to 50 Hz, 1 mT MF. In addition, the cells respond to the stimulus with an increase in the proliferation index after seven days of continuous exposure to the field. In our cellular model (NB69) and under our experimental conditions, a 63-h exposure to 50 Hz, 100 µT MF significantly reduces the spontaneous rate of apoptosis while increasing proliferation in an extent that is similar to that reported by Pirozzoli et al.

The present data on PCNA, a protein that has been reported to be peak in proliferating cells at late G1 and S phases (Oue et al. 1995), indicate that the normal regulation of the PCNA positive cells is altered by the exposure to 50 Hz, 100 µT MF. In the MF-exposed samples the percent of PCNA-positive cells does not differ significantly from that at day 5 post plating, while in the control groups a significant reduction of PCNA positive cells was observed on day 6. These data are consistent with previous results reported by Cridland et al. (1999) on normal human fibroblasts showing a modest though significant increase in the length of the G1 phase when exposed to 50 Hz, 20 and 200 µT MF

The mechanism of interaction between ELF-EMF and NB69 neuroblastoma cells is not known yet.

Regarding the mechanism of interaction of magnetic fields that could underlay the herein described responses of NB69, the mobilization of cellular Ca^{2+} or some Ca^{2+}-regulatory process have been proposed as pre-eminent targets of the MF stimuli (Tonini et al. 2001). Also, in a recent work Zhou et al. (2002) have reported that ELF MF at 100 µT induced a time-dependent activation of CREB DNA binding in HL-60 cells. The effect was dependent on both the extracellular and intracellular Ca^{2+}, which suggests that ELF-EMF can activate CREB DNA binding through calcium-related signal transduction pathways. Similarly, in the present study, the activation of CREB was found to be influenced by the MF stimulus in

a time-dependent manner. Although additional research is needed to determine whether or not calcium is involved directly in the observed response of NB69 cells to 50-Hz MF, the present results are consistent with such a possibility. Further work is also necessary to determine the gene transcription pattern resulting from the increase of CREB activation after exposure to MF. Such an information would be crucial to identify the mechanism(s) by which MF interact with human neuronal cells in vitro. This hypothesis is not in contradiction with the recent results reported by Ivancsits et al. (2002b) and the studies by this group included in the REFLEX project. Their results showed that 50 Hz MF induced a dose dependent and time dependent DNA-single and double-strand breaks, with responses at a magnetic flux density as low as 35 μT.

4.1.2.2 Mouse embryonic stem cells (Participant 4)

ELF-EMF did not exert any influence on neuronal differentiation of embryonic stem cell.

We could not find evidence that under our experimental conditions, ELF-EMF exposure of ES cell derived neural progenitors affected the neural differentiation process, because we did not observe effects on transcript levels of genes involved in neuronal and glial differentiation (nestin, en-1, nurr1, tyrosine-hydroxylase and GFAP). Immunofluorescence analysis did not show any changes in the intracellular distribution and number of cells expressing neuronal markers (ßIII-tubulin, TH, GFAP).

4.1.2.3 Human lymphocytes and embryonic stem cells (Participant 8)

ELF-EMF did not affect proliferation, cell cycle and activation of lymphocytes.

Since the immune System has a key role in contrasting diseases, possible damages induced by exposure of immune cells, such as lymphocytes, could represent a great risk for human health. Thus, the objectives were to determine if different EMF exposures were able to modify human lymphocytes functionality and gene expression using appropriate in vitro tests. Moreover, since immune system efficiency is modified with ageing, a group of elderly donors was enrolled in order to study possible EMF effects age-related. On the whole, the results obtained show no differences between sham- and ELF-EMF exposed lymphocytes for most of the endpoints studied. Obviously, ELF-EMF is not able to modify proliferation, cell cycle and cell activation, which are fundamental phases of lymphocyte function. Negative results are extremely important for evaluations on human health risk.

ELF-EMF activated the expression of cardiac genes in embryonic stem cells thus enhancing their cardiac differentiation.

ELF - EMF were able to promote the differentiation of mice embryonic stem cells into a specific cardiac cell lineage, selectively promoting the expression of fundamental genes involved in the orchestration of cardiac differentiation. At the end of the differentiation process the expression of typical cardiac genes revealed that a specific direction of differentiation into a cardiac phenotype took place, which was also demonstrated by lack of expression of genes related to other cell lineages (e.g., skeletal muscle cells, neuronal cells, etc.).

4.1.2.4 Summary (Participant 1)

As discussed by Participant 5 (4.1.2.1) the findings reported in the literature about a possible influence of ELF-EMF on the proliferation and differentiation of various cell systems in vitro are controversial. Just recently, Lisi et al. (2004) demonstrated that exposure to ELF-EMF (50 Hz, 1 mT) triggered the differentiation of human pluripotent embryonic stem cells. In the REFLEX project, no data were obtained which suggest a major effect of ELF-EMF on cell proliferation and differentiation in human fibroblasts (4.1.1.1), embryonic stem cells (4.1.2.2), human lymphocytes (4.1.2.3) or neuroblastoma cells (3.1.4.2). On the other hand, some influence of ELF-EMF on proliferation and differentiation in certain cell systems cannot be excluded (4.1.2.1).

Participant 5 (3.1.2.1, 4.1.2.1) observed an inhibition of the spontaneous apoptosis in neuroblastoma cells which was followed by an increase of the proliferation rate, when the cells were exposed to ELF-EMF for 63h at a flux density of 50 or 100 μT. This observation is in line with the results of a recent study by Tokalov et al. (2003) and Tokalov and Gutzeit (2003) who reported that ELF-EMF alone does not have

any effect on the proliferation of HL-60 cells, while it protects heat shock treated HL-60 cells from becoming apoptotic. Quite obviously, ELF-EMF enabled heat shock treated HL-60 cells to escape the cell cycle arrest and to re-enter the normal cell cycle thus allowing the cell to continue the proliferation process. The authors explained this phenomenon by an ELF-EMF induced release of hsp-proteins which are thermo- or cytoprotective.

An answer of what may be the reason for the sporadically observed, but until now not unambiguously confirmed influence of ELF-EMF on cell proliferation and differentiation, may be provided by the REFLEX findings on gene and protein expression. As found by Participant 8 (3.1.4.3, 4.1.2.3), ELF-EMF accelerated the cardiac differentiation of embryonic stem cells through enhanced expression of cardiac genes. Further evidence for the validity of such an assumption comes from Participants 3 and 12 (3.1.4.5), who observed in human fibroblasts a remarkable influence of ELF-EMF on the expression of various genes, among them genes regulating Ca-metabolism, cell cycle, apoptosis, extracellular matrix, and cytoskeleton.

Of course, even if there is a relationship between ELF-EMF exposure and an acceleration of cell proliferation and differentiation in in vitro studies through the proposed mechanisms, it is at present not possible to draw any conclusion for the in vivo effects on man and animal.

4.1.3 Apoptosis

4.1.3.1 Mouse embryonic stem cells (Participant 4)

ELF-EMF altered the expression of bcl-2, bax and GADD45 gene in ES-cell derived neural progenitor cells.

In our experiments with ELF-EMF exposed wild-type mouse ES derived neural progenitors, we showed by Q-RT-PCR analysis significant changes in the transcript levels of the anti-apoptotic bcl-2 gene and the related pro-apoptotic bax gene. The biological significance of this finding and its relevance to the in vivo situation is not yet known. Apoptotic cell death is regulated by members of the bcl-2 family for differentiating mouse embryonic stem cells (Sarkar and Sharma 2002). Apoptosis plays an important role during embryonic development, including the development of the nervous system. Bcl-2 over expression was also reported to eliminate deprivation-induced cell death of brainstem auditory neurons (Mostafapour, 2002). Bcl-2 and bax mRNA transcripts in the hippocampus were significantly but transiently upregulated following the administration of the potent neurotoxin domoic acid (Ananth et al. 2001).

Another gene, whose expression was affected after ELF-EMF exposure, was the 'growth arrest DNA-damage' inducible GADD45 gene. The members of the GADD protein family are considered to play an important role in maintaining genomic stability and to regulate cell cycle activity (Chung et al. 2003).

Our results, which demonstrate changes of bcl-2, bax and GADD45 transcript levels indicate that ELF-electromagnetic signals could be perceived in ES cell-derived neural progenitors as environmental stress signals. These signals may trigger cellular responses for the maintenance of cellular homeostasis via alterations of genes that control cell cycle and apoptotic cell death.

In summary, we may conclude that exposure of ES-derived neural progenitor cells to magnetic fields simulating 50Hz power line ELF-EMF may influence transcript levels of genes encoding proteins of the bcl-2 family involved in apoptosis and the p53 responsive growth arrest and DNA damage inducible GADD45 gene. Since the fundamental processes of programmed cell death and cell cycle regulation are closely related to processes underlying cell transformation, the association of ELF-EMF with early stages of carcinogenesis cannot be excluded yet. Further investigations in vivo using genomics analyses and animal studies after EMF exposure have to be performed.

4.1.3.2 Neuroblastoma cells (NB69 cell line) (Participant 5)

ELF-EMF inhibited spontaneous apoptosis in neuroblastoma cells.

Environmental electromagnetic fields (EMF) such as those from electric power transmission and

distribution lines have been associated with increased risk of childhood leukaemia, cancer of the nervous system and lymphomas (Ahlbom et al. 2001; De Roos et al. 2001). In vitro studies of EMF effects have attempted to find an explanation to the epidemiological data and to determine the possible mechanism for cancer risk. Recent evidence has suggested that a common property shared by a number of known and suspected tumour promoters, is their ability to block the process of apoptosis (Jaattela et al., 1999). Therefore, one possible mechanistic explanation for the apparent effect of weak ELF magnetic fields would be their expression of tumour-promoting activities by interfering with the regulation of apoptosis. We have addressed this hypothesis by testing the effects of a 50 Hz 100 μT MF on apoptosis in the human neuroblastoma cell line.

Our data indicate that the field exposure can significantly inhibit spontaneous apoptosis of NB69 cells as revealed through TUNEL assay. This response was associated with significant increase in the number of cells as well as in BrdU incorporation into ADN. Besides, the immunoreactivity for Bcl-2 protein in exposed samples was also significantly increased at 60 min of exposure with respect to controls (data not shown). Regulation of apoptosis is delicately balanced by signalling pathways between apoptosis-promoting factors such as p53 and caspases, and antiapoptotic factors such as Bcl-2 and MDM2. Several lines of evidences have shown that the functional interaction between these factors play important roles in the control of cell growth and apoptosis.

Previous studies investigating changes in susceptibility to apoptosis after EMF exposure have reported both reduced (Simko et al. 1998b; Fanelli et al. 1999; Ding et al. 2001; Kumlin et al. 2002; Robison et al. 2002) and increased susceptibility (Ismael et al. 1998; Tofani et al. 2001; Mangiacasale et al. 2001; Liu et al. 2003). Other studies concerning DNA repair after EMF exposure have reported no effects (Cossarizza et al. 1989a; Frazier et al. 1990; Cantoni et al. 1996). In the majority of the studies reporting effects on apoptosis, cancer cells were exposed to MF (B>100 μT) after apoptosis induction by radiation or chemical treatments. The cellular susceptibility to such MF-driven apoptosis has been reported to be dependent on the cell type, the presence of genetic abnormalities, cell physiology and the MF exposure time. Cancer cells frequently have decreased cell death as a primary mode of increased cell proliferation. Attention has been focused on the expression of the p53 gene, which induces either a stable arrest of cell growth or apoptosis. The final outcome of the different mechanism of action of p53 is to maintain the genomic stability of the cell. Thus, the absent of this protein or their inactivation contributes to genomic instability, the accumulation of mutations and increased tumorigenesis. In the study by Czyz et al. (2004a, included in the present report) the exposure to 50 Hz EMF at 2.3 mT results in up-regulation of egr-1, c-jun and p-21 transcript levels in p53-deficient, but not in wildtype embryonic stem cells. These data indicate that loss of p53 may also affect the sensitivity of cells to external stress factors, such as EMF.

On the other hand, it has been reported (Tian et al. 2002b) that X-ray irradiation followed by 60 Hz EMF exposures can affect cell cycle distribution and transiently suppress apoptosis in xrs5 cells, which show a defect in rejoining of DNA double-strand breaks. The effect has been proposed to be exerted through EMF-induced decrease in the levels of caspase-3, p21, p53 and phospho-p53 and by increasing Bcl-2 expression. Our present results show that a 50 Hz 100 μT MF induces changes in the cell cycle together with a reduction of spontaneous apoptosis associated with increased Bcl-2 expression in NB69 cells. It is possible that a MF action on p53 and Bcl-2 is responsible for the effects on growth and apoptosis observed in our study. In addition we have investigated possible EMF-induced changes in the activation of the phosphorylated cyclic adenosine monophosphate response-element binding protein (p-CREB). CREB appears to be a primary transcriptional activator of the antiapoptotic gene Bcl-2 (Francois et al. 2000). Inhibition of CREB activity induces apoptosis in sympathetic neurones (Riccio et al. 1997) while CREB overexpression inhibits apoptosis induced by okadaic acid (Walton et al. 1999). Our data show that EMF exposure significantly increases the percent of p-CREB positive cells after 60-minute exposures. These results suggest that CREB may also be involved in the above-described effects of 50 Hz, 100 μT EMF on growth/apoptosis of NB69 cells.

4.1.3.3 Human fibroblasts (Participant 3)

ELF-EMF may not affect the apoptotic process in human fibroblasts after intermittent exposure for 24 hours at a flux density of 1 mT.

No differences in cell count between exposed and sham exposed human fibroblasts after any exposure duration could be detected. Therefore, an elimination of cells by apoptosis and cell death during ELF-EMF exposure can probably be ruled out (3.1.1.1).

4.1.3.4 Summary (Participant 1)

As discussed by Participant 5 (4.1.3.2), data reported in the scientific literature on possible effects of ELF-EMF on the apoptotic process are inconsistent. In many studies available to date, inhibition of apoptosis, enhancement of apoptosis and no effect at all have been reported. Most recently, Lai and Singh (2004) found a significant increase both in apoptosis and in necrosis in brain cells of rats after in vivo exposure to ELF-EMF which they explained by an increase in free radicals. Kim et al. (2004) demonstrated that apoptosis in testicular germ cells of mice can be induced by continuous exposure to ELF-EMF (60 Hz, 0.1 and 0.5 mT). The REFLEX findings did not show a significant effect of ELF-EMF on apoptosis in human fibroblasts (3.1.1.1, 4.1.3.3), embryonic stem cells (3.1.4.1, 4.1.3.1), human lymphocytes (3.1.2.3) and neuroblastoma cells (3.1.2.4).

On the other hand, some influence of ELF-EMF on the apoptotic process cannot be excluded at present. Participant 5 observed an inhibition of the spontaneous apoptosis in neuroblastoma cells which was followed by an increase of the proliferation rate, when the cells were exposed for 63 hours to ELF-EMF at a flux density of 50 or 100 µT (3.1.2.1, 4.1.2.1). A similar phenomenon was also reported by Tokalov and Gutzeit (2003) and Tokalov et al. (2003), who did not observe any direct effect of ELF-EMF on apoptosis in HL-60 cells either, while ELF-EMF protected heat shock treated HL-60 cells from becoming apoptotic, thus enabling cells arrested in the cell cycle to continue the proliferation process.

An answer of what may be the cause for the sporadically observed, but probably not systematically enough studied influence of ELF-EMF on apoptosis may be provided by the REFLEX findings on gene and protein expression. As found by Participant 4 (3.1.4.1, 4.1.3.1), ELF-EMF at a flux density of 2 mT up-regulated in neural progenitor cells the transcript levels of the bcl-2 and the GADD45 gene and down-regulated the transcript levels of the bax gene thus influencing cellular processes, which may result in an enhancement of the anti-apoptotic pathway. Further evidence for the validity of such a hypothesis comes from Participants 3 and 12 (3.1.4.5), who observed a remarkable influence of ELF-EMF on the expression of various genes, including those that regulate cell cycle and apoptosis.

From the physiological point of view, inhibition as well as promotion of apoptosis may be induced by ELF-EMF dependent on the type of cell exposed, its genetic background, its immediate metabolic stage and the pattern of exposure. The mechanisms may follow different routes. It may be possible, that two counteracting mechanisms balance out each other which would result in a zero outcome. Taken together, even if a relationship between ELF-EMF exposure and an inhibition or promotion of apoptosis in in vitro experiments were proven, it would in no way be possible to draw any conclusion for the in vivo situation in man and animal.

4.1.4 Gene and protein expression

4.1.4.1 Mouse embryonic stem cells (Participant 4)

Short-term high intensity exposure to ELF-EMF signals may cause a transient up-regulation of immediate early response and regulatory genes in p53-deficient ES cells.

It was found that a high flux density of 2.3 mT of 50 Hz ELF-EMF signals applied to p53-deficient ES cells at an intermittency scheme of 5 min on/30 min off induced a significant up-regulation of transcript levels of the immediate early growth response gene egr-1. This upregulation was paralleled by a transient upregulation of mRNA levels of the cyclin kinase inhibitor p21 and the AP-1 component c-jun in p53-deficient, but not in wt ES cells. This finding confirms our observation that loss of p53, affects the sensitivity of cells to external stress factors, such as GSM-signals. A correlation between loss of p53

function and external stress-induced expression of egr-1 has also been described by Zhang and Chen (2001), who reported experimental evidence for UV-induced egr-1 expression in p53-deficient mouse cells, whereas the effect was suppressed by functional p53. Our data indicate that a similar egr-1-dependent pathway may be triggered upon ELF-EMF exposure.

The role of c-jun and p21 in these processes has not been clarified so far. Egr-1 cooperates with c-jun in the regulation of DNA synthesis and cell survival in response to ionizing radiation (Hallahan et al. 1995). p21 is implicated in G1 arrest following ionizing radiation-induced DNA damage (Brugarolas et al. 1995). Therefore, one could speculate that the tumor suppressor p53 may be involved in the maintenance of cellular homeostasis of ES cells in response to external stress. However, there are also other data showing that despite abundant quantities of p53 in ES cells, the p53-mediated response is inactive, because of a predominantly cytoplasmic localisation and sequestration of p53 (Aladjem et al. 1998). In spite of this, undifferentiated ES cells are sensitive to DNA damage, because they activate a p53-independent apoptotic response. According to Sabapathy et al. (Sabapathy et al. 1997), the balance between positive and negative regulators of the cell cycle is critical for ES cell differentiation and, if disturbed by exogenous factors, this could lead to the activation of a tumorigenic pathway.

The nature of gene-expression responses to ELF-EMF was short-term only.

In our experiments, cellular responses to ELF-EMF signals were observed only immediately after the end of the 6h exposure and disappeared after an 18h recovery time. Similarly, a 48h exposure to ELF-EMF did not result in gene expression-related responses throughout the differentiation process. These results indicate a short-term nature of cell responses to ELF-EMF and the existence of pathways compensating potential stress-evoked effects of ELF-EMF.

There is some indication that threshold of field flux density exists for ELF-EMF biological effects.

We further investigated the influence of the signal strength and the quality of ELF-EMF exposure on cellular reactions in the ES cell system. Our data indicate the existence of threshold values of field flux density that are needed to evoke biological effects by ELF-EMF. Modifications of transcript levels in p53-deficient cells were observed only upon exposure to ELF-EMF signals applied at a high (2.3 mT) flux density, whereas weaker fields did not cause gene expression-related responses.

ELF-EMF effects in p53-deficient cells were dependent on intermittency cycles (on/off cycle duration).

The exposure protocols of ELF-EMF signals involving either intermittent (on/off cycles) or continuous exposure affected the responses of ES cells. Only an intermittency scheme of 5 min on/30 min off ELF-EMF signals exerted effects on transcript levels, whereas intermittency signals of 5 min on/10 min off exposure or continuous exposure showed no effects on transcript levels of ES cells. These findings demonstrate that a specific intermittency scheme of ELF-EMF exposure may be a critical factor to determine the interference of electromagnetic fields with biological systems (Murphy et al. 2002).

The mechanism of action induced by ELF-EMF exposure of living cells is not yet known.

Several hypothetical models have been proposed to explain the mechanisms of interference of ELF-EMF with biological systems, such as an induction of electric currents by acceleration of ions, resonant interactions involving driving vibrations or orbital transitions in biomolecules (Valberg et al. 1997), biochemical reactions involving free radicals (Brocklehurst and McLauchlan 1996; Eveson et al 2000) or direct interactions of EMF with moving electrons within DNA (Blank 1997). It was also suggested that external oscillating fields cause forced vibrations of free ions of the cellular surface and distort the gating of electro-sensitive channels on the plasma membrane. This would explain, why pulsed electromagnetic fields could have a higher biological activity than continuously applied fields (Panagopoulos et la. 2000, 2002). According to another model (Binhi and Goldman 2000), specific ‘windows’ of the electric-field frequency and amplitude might be predicted. These properties of ELF-EMF could explain the positive results of certain exposure schemes with a specific on/off cycle (in our case, 5 min on/30 min off) and the lack of biological effects at other experimental conditions.

4.1.4.2 Neuroblastoma cells (SY5Y cell line) (Participant 11)

The function of neuronal nicotinic receptors in the brain

Neuronal nicotinic receptors (nAchRs) are a family of ligand-gated cationic channels expressed both in the peripheral and central nervous system where they play a fundamental role in synaptic transmission. At the periphery nAchRs are expressed in post-ganglionic neurons of the autonomic nervous system (Wang et al. 2002 and references therein). In the CNS they seem to be located predominantly at the presynaptic and preterminal parts of the axons where they control the release of a number of different neurotransmitters, such as glutamate, GABA and dopamine (Wonnacott 1997).

nAchRs are composed of different subunits: so far nine ligand binding subunits, alpha 2 - alpha 10, and three structural subunits, beta2 - beta4, have been cloned from different species (Wang et al. 2002). Different combinations of alpha and beta subunits can form different receptor subtypes with their own pharmacological and biophysical characteristics. Neuronal nAchRs are involved in a number of functional processes including cognition, learning and memory (Jones et al. 1999). Alterations in the expression and/or activity of nAchRs have been implicated in different neurological disorders. For instance, mutations in the alpha4 or beta2 subunits produce in humans the autosomal dominant nocturnal frontal lobe epilepsy (Steinlein et al. 1995; De Fusco et al. 2000). Roles for the alpha7 subunit have been suggested in Alzheimer's disease (Dineley et al. 2001) and schizophrenia (Lindstrom 1997; Freedman 1999; Freedman et al. 2000). It has been recently shown that the expression of alpha7 is increased in a well-established mouse model of Alzheimer's disease, whereas the beta-Amyloid (1-42) peptide binds with high affinity to alpha7, suggesting a pathogenetic role for this receptor subtype (Grassi et al. 2003).

Insights into the functional role of nAchRs and their possible involvement in neurological disorders have been obtained by means of knock-out mice (Cordero-Erausquin et al. 2000). By this approach, it has been possible to show that the absence of the beta2 subunit as well as the hyperactivity of the alpha7 subunit are conditions sufficient to promote neurodegeneration. Epidemiological studies have shown that exposure to electromagnetic fields (EMF) might be responsible for neurodegenerative diseases such as Alzheimer's (Sobel et al. 1995, 1996). In light of the role of nAchRs in physiological and pathological conditions, we wondered whether EMF might affect the expression of these molecules. With this aim, we have characterized some neuronal cell lines for their ability to express nAchRs. We have identified some human neuroblastoma cell lines that are currently used to evaluate whether extremely low frequency EMF (ELF-EMF) can interfere with the expression of alpha3, alpha5 and alpha7 nAchR subunits. The expression of these subunits has been studied both at mRNA level by Northern blotting, and at protein level by radioligand assays, upon exposure to different protocol settings.

The function of the cathecolaminergic system in the brain

The cathecolaminergic system is very relevant for many brain functions. Moreover, in the periphery, cathecolamines, in particular norepinephrine, are released by the post-ganglionic neurons of the autonomic nervous system, representing the main neurotransmitters of the ortosympathetic division. In collaboration with Participant 1, we decided to investigate the expression of Dopamine beta-hydroxylase (DßH), the limiting enzyme for the synthesis of norepinephrine, in order to investigate whether ELF-EMF might modify its expression, therefore interfering with autonomic functions as it has been reported in some papers (Kim et al. 2002). With this aim, we carried out Northern blot analyses with RNA extracted from neuroblastoma cells exposed to ELF-EMF.

Finally, we have also been investigating the effects of ELF-EMF on the expression of two transcription factors, Phox2a and Phox2b. These homeodomain proteins are the main regulators of the expression of Dopamine beta-hydroxylase (Yang et al. 1998). In particular, they are responsible for the development of all three divisions of the autonomic nervous system (Lo et al. 1999; Stanke et al. 1999). Indeed Phox2b KO mice fail to develop the whole autonomic nervous system (Pattyn et al. 1999), whereas Phox2a mice show an apparently less severe phenotype, but die the day of birth (Morin et al. 1997). Furthermore, preliminary results from our laboratory have shown that they seem to play a role in the regulation and maintenance of the expression of nAchR alpha3 subunit gene (Flora, personal communication). In order to understand whether ELF-EMF can interfere with the expression of these transcription factors, therefore affecting the formation and function of the autonomic nervous system, we have been carrying out Northern blot experiments to evaluate possible variation in the expression of Phox2a and Phox2b mRNA.

ELF-EMF did not affect the expression of neuronal genes such as nAchRs, DβH, Phox2a and Phox2b, either at mRNA or protein level.

A human neuroblastoma cell line, SY5Y, was used in all the experiments, as it expresses the ganglionic-type nAchR subunits alpha3, alpha5 and alpha7 as well as DßH, Phox2a and Phox2b genes. Cells were

exposed by means of the ELF-EMF generator, setup by Participant 10, under different exposure protocol. The intensity of the electromagnetic field applied was always higher (2 mT and 1 mT) than that of a real life situation, in order to highlight possible, if any, macroscopic effect on gene expression due to ELF-EMF exposure. The duration time of the exposure varied from a relatively short period of time (16h) to a longer period (48h), in order to investigate a time-dependent effect upon exposure to EMF. Finally, the type of exposure, intermittent (5 min on/5 min off) rather than continuous was chosen in order to mimic different kinds of situations that may be encountered during the life-time of an individual. The cells were always collected immediately after the end of the exposure for gene expression analysis, except in one case (1 mT continuous exposure for 48h), when the cells were harvested 48 hours after the end of the exposure, in order to investigate possible indirect effects on the expression of nAchR subunits, DßH, Phox2a and Phox2b, due to the activation of second messenger cascades. We found that exposure of human neuroblastoma cells to continuous (magnetic field intensity of 2 mT and 1 mT) and intermittent (2 mT and 1 mT) low-frequency EMF either for a relatively short period (16h) as well as a longer period (48h) does not seem to influence the expression of neuronal genes for nAchRs, DßH, Phox2a and Phox2b, either at mRNA or protein level. In order to validate these negative results, every exposure condition was tested in at least three to nine independent experiments.

4.1.4.3 Embryonic stem cells of mice during cardiac differentiation (Participant 8)

ELF-EMF up-regulated the expression of cardiac specific genes thus promoting cardiogenesis.

The exposure of EC (P19) cells to ELF-EMF yielded conflicting results and poor reproducibility of the data. On the contrary, the development of a model of in vitro cardiogenesis based on "gene trapping" selection of cardiomyocytes from pluripotent (GTR1) cells provided a potentially homogenous and reproducible approach to assess whether ELF-EMF may afford developmental decisions (i.e. cardiogenesis) in ES cells. In this ES cell model, ELF-EMF afforded a consistent increase in the expression of genes tightly involved in coaxing ES cells to the cardiac lineage. As shown by in vitro run-off analyses, ELF-EMF affected the transcriptional machine of ES cells. These responses led to the expression of cardiac specific genes and ultimately ensued into a high-throughput of cardiogenesis, as shown by the increase in the number of spontaneously beating colonies in ELF_EMF-exposed cells. Failure of ELF-EMF to affect the transcription of a gene promoting skeletal muscle determination and the faint effect on neuronal specification seem to exclude a generalized activation of repressed genes and suggests that coupling of ELF-EMF with GATA-4, Nkx-2.5 and prodynorphin gene expression may represent a mechanism pertaining to ES cell cardiogenesis.

4.1.4.4 rCx46 in oocytes of *Xenopus laevis* (Participant 7)

The influence of ELF-EMF exposure on the expression of rCx46 in single and paired oocytes of *Xenupous laevis* was analysed. Especially the expression level as well as the corresponding regulatory properties of conducting hemi-channels and cell-to-cell channels (Bruzzone et al., 1996) were studied. ELF-EMF exposure neither significantly influenced the expression level of conducting hemi-channels composed of rCx46 (Figures 59 to 61), nor their gating properties by voltage, pH, Ca2+ (Figure 64). A similar result was found for cell-to-cell channels, which could be formed by pairing of oocytes expressing rCx46 (Figure 65). This finding is in contrast to the observation that in general ELF-EMF exposure causes a decrease of cell-to-cell coupling (Hu et al. 2001; Lohmann et al. 2003; Trosko and Ruch 1998; Vander Molen et al. 2000; Yamaguchi et al. 2002; Zeng et al. 2003), but different regulatory mechanisms were suggested. It was proposed that ELF-EMF increases Ca^{2+}-influx which in turn inhibits gap junctional coupling in synovial fibroblasts (Marino et al. 2003). But in osteoblast like cells such an increase of Ca^{2+} was not observed, despite the finding that ELF-EMF induced a decrease of gap junctional coupling (Yamaguchi et al. 2002). In contrast to further reports (Lohmann et al. 2003; Zeng et al. 2003) the authors showed that ELF-EMF does also not effect the distribution of the corresponding membrane protein connexin (Cx43) between the cytoplasmic and the membrane pool. Therefore, a change in the state of Cx-phosphorylation was considered as target of ELF-EMF exposure causing a decrease of cell-to-cell coupling (Yamaguchi et al. 2002, see also Lacy-Hulbert et al. 1998). By ELF-EMF exposure of oocytes expressing rCx46 cytoplasmic free Ca^{2+} and/or signal transduction pathways involved in protein phosphorylation also of rCx46 virtually remain unchanged. This conclusion can be drawn from the unchanged behaviour of the leak-current of the oocytes in the absence and presence of

ELF-EMF (Figure 58). The leak-current includes the sum of all electrogenic transport systems which are known to partially depend on cytoplasmic free Ca^{2+} and protein phosphorylation. At present the origin for the different response of cell systems expressing Cx43 (Hu et al. 2001; Lohmann et al. 2003; Trosko and Ruch 1998; Vander Molen et al. 2000; Yamaguchi et al. 2002; Zeng et al. 2003) and oocytes expressing rCx46 at ELF-EMF exposure remains unsolved.

4.1.4.5 Whole-genome analysis of various cell lines exposed to ELF-EMF (Participant 12)

If we look on the numbers in Table 12, it is obvious that members of some gene families are regulated predominantly. Moreover, repetitions of experiments with the same cell line and the same exposure conditions look more similar than repetitions with different cell lines or different exposure conditions. This might tell us that obviously something is happening on the gene-expression level after ELF-EMF exposure. Otherwise, if we only would see experimental variances (differences in experimental procedures, cell cycle stages, etc.), we would expect about the same numbers in each experiment, or higher similarities between same experiments as between different cell lines.

The results with the different cell lines obviously have not the same quality. For example, the results of profiling 1 and 2 of the fibroblasts (Participant 3) seem to be more similar than between the experiments between cells with differentiating potential. Also genes of different gene families react differently on certain influences. Whereas for example structural proteins like cell adhesion proteins are regulated slowly, certain proto-oncogenes like c-fos, c-fos or actin can be regulated within 10 to 30 min of growth factor addition (Quantin and Breathnach 1988). Moreover, the situation is different here from, for example, a disease situation with a certain defect in a single gene. We deal with environmental influences here, which are complex and variable. Even adaptation to the electromagnetic fields after some hours due to changes in gene expression cannot be excluded.

How the potential molecular changes after ELF-EMF exposure are regulated, remains speculative. However, if we look on the genes extracted by the bio-statistical analysis in more detail, some interesting points become obvious: A remarkable number of members of the actin cytoskeleton and associated proteins are down-regulated (also in RF-EMF experiments). Remarkably, in ELF-EMF treated cells (Participant 3) the actin-associated proteins obviously down-regulated seem to be regulated by Ca, and several Ca regulators were also down-regulated in our experiments. This would mean that the actin cytoskeleton as far as regulated by Ca is down-regulated. In addition to Ca^{2+}-associated proteins, proteins associated with other cations like Fe+, K+, and H+ are down-regulated. More experiments will be necessary for showing if these proteins might be involved in signalling or energy metabolism after ELF-EMF exposure.

4.1.4.6 Summary (Participant 1)

From the REFLEX data, the conclusion must be drawn that ELF-EMF may affect gene and protein expression in various cell systems. Based on the results of the genome analysis of human fibroblasts as carried out by Participant 12 (3.1.4.5), ELF-EMF appears to regulate the expression of a series of genes and proteins such as mitochondrial and ribosomal genes and Ca-, cell cycle-, apoptosis-, extracellular matrix-, and cytoskeleton-related genes. In particular, a number of G proteins and calcium associated proteins involved in signal transduction seem to be strongly regulated by ELF-EMF. Since the variances between the experiments were high, the significance of these findings is limited. Participant 4 observed a transient up-regulation of early response and regulatory genes only in embryonic stem cells deficient for p53 and not in wild type cells after ELF-EMF exposure. This suggests that the genetic background affects the responsiveness of the cells (3.1.4.1, 4.1.4.1). Participant 8 found that ELF-EMF up-regulates the expression of cardiac specific genes in cardiomyocytes derived from embryonic stem cells thus promoting cardiogenesis (3.1.4.3, 4.1.4.3). All these findings were obtained after ELF-EMF exposure at rather high flux densities. It remains, therefore, an open question whether or not these in vitro results are of any significance for the real life exposure of man and animal.

The REFLEX data on gene and protein expression due to ELF-EMF exposure are in line with the results of a series of studies already published in the literature. Goodman et al. (1994) and Lin et al. (2001) reported increased hsp70 transcript concentrations in HL-60 cells after exposure to weak ELF-EMF (60 Hz). Tokalov and Gutzeit (2004) observed an increase in several heat shock proteins in HL-60 cells after

exposure to ELF-EMF (50 Hz, 60 μT, 30 min), which was comparable to that after exposure to heat (41°C, 30 min) or X-ray (200 kV, 5 Gy). Most recently, Zeng et al. (2004a) and Xu et al. (2004) demonstrated that ELF-EMF (50 Hz, 0.4 mT, 24h) altered the signal transduction-related protein expression in human breast cancer cells (MCF-7). Mannerling et al. (2004) who studied the hsp70 expression in several human cell lines reported an increased expression after exposure to ELF-EMF (50 Hz, 0.1 or 0.2 mT, up to 24h). Of course, the expression of genes and proteins induced by ELF-EMF may again be dependent on the type of cell exposed, its genetic background and its immediate metabolic stage and, of course, the pattern of exposure. The available data indicate that the flux density (threshold) at which effects on gene and protein expression are at first found is in the range or not far above the presently valid safety levels of 100 μT for the general public or 400 μT for the workplace.

4.2 Results obtained after RF-EMF exposure

4.2.1 Genotoxic effects

4.2.1.1 Human promyelocytic cell line HL-60 (Participant 2)

Discussion on potential health effects of using mobile telephones has focused on possible cancer-enhancing effects. It seems quite clear that any cancer-related effects of radiofrequency electromagnetic waves cannot be based on direct genotoxic effects, since the energy level is not high enough to damage DNA. Most of RF-field studies concluded that RF-field exposure is not genotoxic or mutagenic. With respect to DNA strand breaks, there is no replicated evidence for DNA and/or repair damage due to RF-field exposure (Lai versus Malayapa, examples see literature cited). On the other hand, some studies have shown that radiofrequency-field/microwave (RF-fields/MW) radiation and extremely low frequency (ELF) fields cause increased DNA strand breakage and chromosome aberrations. This has been shown in cell lines (Phillips et al. 1998), human blood (Verschaeve et al. 1994), animals (Lai and Singh 1995, 1996a/b, 1997a/b/c, 2004) and living human beings (Fucic et al. 1992; Garaj-Vrhovac 1999). The basic strategy in our studies was to test whether RF-EMF are able to alter DNA integrity (MN induction and DNA strand breakage), cell proliferation, cell cycle kinetics and/or apoptosis using the promyelocytic leukaemia cell line HL-60 testing different SAR levels, exposure times and signal modulations. For the experiments a highly standardized exposure system setup was provided by Participant 10. This setup enabled the exposure of suspensions of cells with a highly standardized temperature constancy, an inhomogeneity of SAR of less than 30% and an efficiency of more than 20 W/kg per Watt input power. All experiments were performed blinded, i.e. not knowing, which of the waveguides was exposed to the RF-field and which was the sham control.

RF-EMF exposure for different SAR and different exposure times (1800 MHz, continuous wave) led to the induction of single and double DNA strand breaks.

DNA damage through RF-EMF was evaluated immediately after exposure using the alkaline single cell gel electrophoresis assay (Comet assay). RF-fields at 1800 MHz, continuous wave exposure for different exposure times caused the induction of single and double DNA strand breaks in HL-60 cells. No significant difference was seen between exposed and sham exposed cells at a SAR of 0.2 to 1.0 W/kg. An increase in the steepness of the dose response relation is observed between SAR 1.0 W/kg and 1.3 W/kg. A less expressed increase is observed between 1.6 W/kg and 3.0 W/kg (Figure 73).

Two other laboratories have recorded that RF-field/MW produced significant DNA stands breaks. Verschaeve et al. (1994), who used a GSM cell phone signal to expose human and rat peripheral blood lymphocytes, found significantly increased strand breaks at high, but non-thermal exposure levels. Phillips et al. (1998) exposed Molt-4 T-lymphoblastoid cells with cell phone radiation in the SAR range 0.0024 W/kg to 0.026 W/kg. A 2-hour exposure to these low levels of cell phone radiation significantly increased or decreased the DNA damage. Decreased DNA damage is evidence of increased repair that is, of course, evidence of damage (Meltz 1995). In some other studies the observations of significant increase in DNA single and double strand breaks in brain cells of rats whole body exposed to 2.45 GHz RF-field (Lai and Singh 1995, 1996a/b, 1997a/b) were not confirmed using rodent and human cells

exposed in vitro and in vivo to RF-fields (Malayapa et al. 1997, 1998; Maes et al., 1997; Vijayalaxmi et al., 2000; Li et al., 2001).

RF-EMF exposure for different SAR and different exposure times (1800 MHz, continuous wave) led to an increase in micronuclei.

Micronuclei are easily measured under day light microscopy. They consist of small amounts of DNA that arise in the cytoplasm when chromatid/chromosomal fragments or whole chromosomes are not incorporated into daughter nuclei during mitosis. We have used the conventional cytokinesis-block MN assay to assess induction of cytogenetic damage in HL-60 cells after exposure to RF fields. RF-EMF exposure on HL-60 cells at 1800 MHz, 24h, continuous wave, at the given experimental conditions, caused a significant increase of micronuclei induction in the same SAR-dependent manner as observed for the induction of DNA strand breaks. Whereas at a SAR of 1.0 W/kg no significant difference of micronuclei frequencies was noted compared to sham controls, a clear increase was observed at SAR of 1.3 W/kg and 1.6 W/kg, and, less expressed at a SAR of 2.0 W/kg and 3.0 W/kg (Figure 72).

Induction of both, micronuclei and Comet formation, by RF-EMF was dependent on the time of exposure. A short exposure period of 6 hours caused no increase in MN frequencies compared to longer exposure times of 24 and 72 hours, respectively. Exposure to a 1800 MHz magnetic field at SAR of 1.3 W/kg for 72 hours produces a similar micronucleus frequency in HL-60 cells as that caused by 0.5 Gy ionising radiation (exposure time: 5.2 s), i.e., an average of 22 MN per 1000 BNCs (Figures 75, 76). However, it is not likely that the two entities cause MN induction by similar mechanism and produce the same types of DNA damage. In contrast to these findings, Comet formation already started after short exposure periods of 2 and 6 hours, respectively, with a maximum after 24 hours, and a clear decline occurred towards a longer exposure period of 72 hours. Mechanistically, this finding may be explained by DNA repair phenomena in the case of the DNA strand breakage in contrast to MN induction.

On the other hand, data from several other studies have indicated in primary human lymphocytes an absence of significant differences in the incidence of CA, SCE and MN between RF-EMF-exposed and sham-exposed cells (Vijayalaxmi et al. 1997, 2001a/b; Bisht et al. 2002). The significant increase and a weak effect on sister chromatid exchanges in RF-field exposed human blood lymphocytes reported by Maes et al. (1996, 1997) was not confirmed in their own subsequent investigation (Maes et al. 2001). Some positive findings occurred under conditions in which RF exposure elevated the temperature (Manikowska-Czerska et al. 1985; Sarkar et al. 1994; Varma and Traboulay 1997).

RF-EMF-associated increase of DNA strand breaks and micronuclei (1800 MHz, 1.3 W/kg, 24h) in HL-60 cells was signal-independent.

Interestingly, DNA strand breaks and MN induction were similarly induced by different RF-EMF signal modulations including CW exposure, CW intermittent exposure (5 min on/10 min off), 217 Hz pulse modulation and GSM Talk each at 1800 MHz, SAR 1.3 W/kg for 24h (Figure 77, 78).

RF-EMF induced formation of reactive oxygen species as shown by flow cytometric detection of oxyDNA and rhodamine fluorescence.

ROS, including superoxide anion (O^{-}_{2}), hydrogen peroxide (H_2O_2), hydroxyl free radical ($OH\cdot$) and singlet oxygen ($^1O_2$), continuously generated from the mitochondrial respiratory chain, own a powerfully oxidative potential. ROS are capable of attacking lipids, nuclear acids and proteins, resulting in certain degrees of oxidative damage. The total ROS level in resting HL-60 cells, however, was directly measured in the present study, by flow cytometric detection of Rh123 and the oxidized nucleotide 8-oxoguanosine (Figures 86, 87). Detecting the ROS level by flow cytometry has been a novel approach with the characteristic of fastness, convenience and reproducibility and, to our knowledge, has not been frequently reported before. DHR123, one of common ROS captures, is membrane permeable. It is oxidized intracellularly by ROS to become fluorescent Rh123, which is pumped into mitochondria and remains there. After a period of accumulation it is then detectable by flow cytometry (e.g., Gao et al., 2002) The probe used in the Calbiochem OxyDNA Assay kit is specific for 8-oxoguanine, which, as part of the oxidized nucleotide 8-oxoguanosine, is formed during free radical damage to DNA and is thus a sensitive marker for differences of ROS levels (de Zwart et al. 1999; Kasai 1997; Cooke 1996) in HL-60 cells after exposure to RF-EMF compared to control and sham-exposed cells (Figure 86).

If an involvement of free radicals in the mechanism of RF-EMF induced DNA strand breaks in HL-60 cells could be shown, this would have an important implication on effects to cell integrity due to RF-EMF

exposure. The “free radical hypothesis” stating that EMF increase free radical activity has been proposed by various researchers (Grundler et al. 1992; Reiter 1997; Lai and Singh 2004). Involvement of free radicals in human diseases, such as cancer and atherosclerosis, have been suggested (Beckmann and Ames 1997). Free radicals also play an important role in aging processes (Reiter 1995).

Co-administration of ascorbic acid, a free radical scavenger, inhibited the effects of RF-EMF on HL-60 cells and may, thus, decrease DNA damage without affecting cellular growth.

Two plausible biological mechanisms involving free radicals have to be discussed for the RF-EMF effect. The first involves increased free radical formation and activity and genetic damage as a response to RF-field exposure. The second involves increased free radical activity and genetic damage because of an induced reduction of free radical scavenger, e.g. reduced SOD activity or melatonin (Reiter 1994). Indications were found in our investigations for increased free radicals activities and a correlation with genetic damage (Figures 86 to 89). Cells possess efficient antioxidant defence systems, mainly composed of the enzymes such as superoxide dismutase, glutathione peroxidase, and catalase, which can scavenge the ROS excessive to cellular metabolism, and make ROS level relatively stable under physiological conditions. Under the conditions used in our experiments, endogenous antioxidant enzyme activities of HL-60 cells (SOD and GPx activity) did not show pronounced alterations following RF-field exposure as compared to sham-exposure. Therefore, the first proposed mechanism mentioned above seems to be dominant.

In summary, the findings of an increase of micronuclei induction as well as Comet formation in HL-60 cells after exposure to RF-EMF at the conditions stated above indicate, that RF-EMF might generate genotoxic effects. The results obtained clearly show that RF-EMF under distinct exposure conditions cause DNA damage in human HL-60 cells. Since on the basis of these data RF-EMF have to be regarded as potentially genotoxic, it is pivotal to clarify first the molecular mechanisms involved in these potentially clastogenic effects in forthcoming experiments and secondly the biological consequences of DNA damage induced by RF-EMF, in particular the relevance for inducing mutations and changes in cellular signalling cascades. Responsive to the European Commission´s suggestions, additional independent verification experiments of the results obtained so far have to be conducted in the same cell line and other cell types, which are normal or similar in the metabolic process. Studies on indirect genotoxicity (e.g., reactive oxygen species, oxy-DNA, DNA repair) of RF-EMF on HL-60 cells, have to be extended. Studies on potential changes in gene expression profiles with respect to DNA repair have to be continued in co-operation with other groups.

4.2.1.2 Human fibroblasts and granulosa cells of rats (Participant 3)

RF-EMF generated DNA strand breaks in granulosa cells of rats and DNA strand breaks and chromosomal aberrations in human fibroblasts.

We could demonstrate an induction of DNA single and double strand breaks upon RF-EMF exposure in human diploid fibroblasts and in rat granulosa cells in culture. This induction depended on exposure duration as well as on the applied signal and could be determined in cells of different tissues. Based on the findings which we obtained with ELF-EMF, we also used for the intermittent RF exposure an “on” duration time of 5 minutes and an “off” duration time of 10 minutes. In contrast to ELF-EMF, RF-EMF induced DNA strand breaks also under continuous exposure conditions. However, the effects were more pronounced under intermittent exposure conditions at 5 min on /10 min off cycles.

The identification of the processes which lead to this DNA breakage will help to determine the extent of biological effects induced by RF-EMF exposure. Importantly, cellular effects observed in this study started already at an SAR of 0.3 W/kg which is far below 2 W/kg, the highest level allowed by the European safety limits. This suggests that the currently allowed radiation emission levels for the mobile phones, are clearly not sufficient to protect from biological effects. We have demonstrated, that the effect of ELF-EMF depends on the cell type and the on and off duration times used in research. The negative effects of RF-EMF reported in the literature (McNamee et al. 2002a, b; Tice and Hook 2002; d'Ambrosio and Scarfi 2002a), however, are based on lymphocytes and continuous exposure.

As with Elf-EMF, RF-EMF exposure of human fibroblasts induced also an increase in micronuclei and an even higher incidence of chromosome gaps, chromosome breaks, dicentrics and acentric fragments, which was 10-fold after ELF-EMF exposure as compared to control cells and 100-fold after RF-EMF

exposure. The RF-EMF results regarding chromosomal aberrations are of preliminary nature, but they are in line with the results obtained after ELF-EMF exposure. The evaluation of the micronulei carried out in our laboratory was reproduced blindly with coded slides in two independent laboratories that do not belong to the REFLEX consortium (Universities of Ulm and Kaiserslautern, Germany).

RF exposure revealed a significant decrease in the mitochondrial membrane potential in one experiment, which could not be reproduced. The RF induced formation of DNA strand breaks could not be related to changes in the membrane potential.

4.2.1.3 Mouse embryonic stem (ES) cells (Participant 4)

RF-EMF exposure of ES-derived neural progenitor cells induced a low transient increase of double DNA strand breaks measured by the neutral Comet assay.

Since we observed an up-regulation of GADD45, which is a DNA-damage inducible gene, it was logical to test the induction of primary DNA damages. It has been shown previously that EMF exposition of human HL-60 cells resulted in an increase of DNA breaks, suggesting a direct mutagenic effect (Ivancsits 2002). In addition, a correlation was found between up-regulation of GADD45, of the bcl-2 family member bcl-$X_{L,}$ and an increased amount of early DNA damage measured by the alkaline Comet assay in human preneuronal cells exposed to the amyloid protein (Santiard-Baron 2001). Therefore, we used the alkaline and neutral Comet assay to detect single, and double-strand DNA breaks, resp., in neural progenitors derived from murine pluripotent ES cells after RF-EMF exposure. Under our experimental conditions, 6 hours exposure to GSM signals induced a low transient increase of double-strand DNA breaks, whereas ELF-EMF did not induce a significant DNA damage. Our finding suggests that genotoxic effects of RF-EMF, at least *in vitro*, could not be excluded.

4.2.1.4 Summary (Participant 1)

As discussed by Participant 2 (4.2.1.1) there is sporadic literature about in vitro studies demonstrating that RF-EMF may possess a genotoxic potential (The Royal Society of Canada 1999; Stewart Report 2000). Since the energy impact on the genome of livings cells exposed to RF-EMF was calculated to be too low to cause DNA damage and since the mainstream literature contradicted the assumption of genotoxic effects (Moulder et al. 1999; Meltz 2003), these sporadic findings were considered more or less meaningless. Opposite to this widely accepted view, the data of the REFLEX study which were elaborated in a hitherto unknown systematic approach and confirmed in four laboratories, of which two were not members of the REFLEX consortium, support the view that RF-EMF causes genotoxic effects in certain, if not all cellular systems.

Based on the methodology used and the data obtained in the REFLEX study, the findings on genotoxicity caused by RF-EMF are hard facts. RF-EMF exposure at a SAR value below 2 W/kg induced an increase in DNA single and double strand breaks as well as in micronuclei in HL-60 cells. The DNA damage was dependent on the time of exposure, the field strength of RF-EMF and the type of RF-EMF signals. There is some indication that the effects may be caused via an increase in free oxygen radicals generated by RF-EMF (3.2.1.1, 4.2.1.1). RF-EMF exposure between SAR values from 0.3 to 2.0 W/kg made also DNA single and double strand breaks in human fibroblasts and in granulosa cells of rats dependent on the exposure time and the type of signals. This increase of DNA-strand breaks in human fibroblasts was accompanied by an increase in micronuclei and in chromosomal aberrations thus demonstrating that the DNA repair was not error-free (3.2.1.2, 4.2.1.2. In addition, RF-EMF exposure at a SAR value of 1.5 W/kg caused a slight, but significant increase in DNA double strand breaks in embryonic stem cells of mice (3.2.1.3, 4.2.1.3).

As already stated, for energetic reasons, RF-EMF can neither denature proteins nor damage cellular macromolecules directly. If the energy impact on the genome of living cells exposed to RF-EMF is too low for a DNA damage, the genotoxic alterations observed in the REFLEX project must be produced indirectly through intracellular processes in the course of RF-EMF exposure. In their experiments Participant 2 observed an increase of free radicals in HL-60 cells after RF-EMF exposure. With the oxygen radical scavenger ascorbic acid was it possible to inhibit the generation of DNA strand breaks and of micronuclei during RF-EMF exposure (3.2.1.1, 4.2.1.1). This findings support the assumption that the observed DNA damage may be caused by free oxygen radicals which are released by RF-EMF during

exposure. This possibility is further strengthened by the observation of Lai and Singh (1997a,b), who demonstrated that the increase in single and double DNA strand breaks in brain cells of RF-EMF exposed rats can be blocked with radicals scavengers. A final conclusion whether or not this finding is indisputable is still pending, since an increase in DNA strand breaks at the same model could not be confirmed by another research group (Malyapa et al. 1997, 1998).

Taken together, RF-EMF is able to damage the genome at least in certain cell systems after exposure in vitro. As with ELF-EMF, the genotoxic effects of RF-EMF may be best explained indirectly by an RF-EMF induced intracellular increase in free radicals. It is well known that a balanced free radical status is the prerequisite for maintaining health and that an unbalanced free radical status promotes the process of ageing and the development of chronic diseases such as cancer and neurodegenerative disorders. Whether the balance of free oxygen radicals can also be impaired through RF-EMF in vivo as suggested by the work of Lai and Singh (1997a,b) needs further clarification.

4.2.2 Cell proliferation and differentiation

4.2.2.1 NB69 neuroblastoma cells and neural stem cells (NSC) (Participant 5)

RF-EFM did not affect cell growth of NB69 and neural stem cells.

A short-tem (24h) exposure to the GSM-Basic signal does not modify the cell growth of NB69 cells and NSC. However, as described in 4.2.4.2, this signal induced in both cell a reduction in the proportion of cells expressing FGFR1. Signalling through fibroblast growth factor receptors (FGFRs) is essential for many cellular processes, including proliferation and differentiation (Kovalenko et al., 2003) and nervous system development (Oh et al. 2003). Our results indicate that in the selected exposure conditions, the GSM-basic signal does not induce changes in cell proliferation. Also, the short-term response induced by this GSM-signal on FGFR1 does not seem to be related to changes in cell growth.

4.2.2.2 Human lymphocytes and thymocytes (Participant 8)

RF-EMF may not affect proliferation, cell cycle, apoptosis and activation of human lymphocytes and thymocytes.

The immune system plays a decisive role in health and disease. Therefore, it was important to find out whether or not RF-EMF affect the immune system. Lymphocytes were exposed to RF-EMF at 1800 MHz with three different signals, such as GSM basic, Talk modulated and DTX only (SAR 1.4 - 2 W/kg).The in vitro tests were chosen in order to study the following endpoints: 1) cell proliferation; 2) cell cycle; 3) expression of membrane receptors on T lymphocytes, 4) spontaneous and induced apoptosis; 5) mitochondrial membrane potential (MMP) modifications in induced and spontaneous apoptosis ; 6) cytokine production; 7) Hsp70 levels in induced and spontaneous apoptosis; 8) thymocyte development and apoptosis; 9) T lymphocyte gene expression.

On the whole, the results obtained suggest that no differences exist for the most endpoints studied in RF-EMF exposure. Only some slight differences were observed in PBMCs; in particular, CD8+CD28+ appeared increased in exposed cultures, but the difference (3%) of the order of the calculated standard error did not indicate a relevant effect from a biological point of view. Actually, in a previous work we found that 900 MHz (SAR 76 mW/kg) RF seem to slightly decrease lymphocyte proliferation when these cells are low-stimulated (Capri et al., accepted 2004); thus our results suggest that RF effects on lymphocyte proliferation are frequency-dependent. However, the literature on this field is still scanty. Some groups showed different effect on cytolitic T lymphocyte proliferation (Cleary et al. 1996) and some groups did not found significant effects on mitotic indices between RF-exposed and sham-exposed lymphocytes (Vijayalaxmi et al. 1997).

A more interesting result appears the decrease of CD95 molecules on membrane surface of stimulated CD4 helper T cells, from elderly donors, which was found when cells were exposed to Talk modulated RF in comparison with sham exposed cells. Due to the importance of this receptor in the regulation and homeostasis of immune response, these results deserve further evaluations to confirm this decrease (around 9%) on CD^{4+} helper T lymphocytes from elderly and not from young donors.

An important observation was the observed decrease (around 13%) of IL-1 b production; this effects was found only in low-stimulated PBMCs exposed to DTX RF and suggest that a possible cell target of RF-EMF are monocytes rather than lymphocytes. Also this effect deserves further investigation in order to confirm possible interactions of RF-EMF exposure with human monocytes. Data in the literature are really scanty. A recent study, performed in vivo, demonstrated a transient increase of interferon-g (IFN-g) in mice exposed to GSM-modulated 900 MHz in mice exposed 2 hours/day for 1, 2 and 4 weeks in a TEM cell (Gatta et al. 2003)

Negative results are extremely important for evaluations on human health risk. RF-EMF exposure is obviously not able to interfere with cell cycle, spontaneous or chemically- induced apoptosis, mitochondrial membrane polarisation and cell activation. Negative results were also obtained on thymocyte development. This last result is extremely important, since it was observed in conditions very near to what happens in vivo. Moreover, results from gene expression of quiescent T lymphocyte confirm the absence of significant changes due to RF-EMF exposure.

4.2.2.3 Human promyelocytic cell line HL-60 (Participant 2)

RF-EMF generated genotoxic effects in HL-60 cells within a narrow energy window without affecting cell proliferation, cell progression and apoptosis.

Using the MTT assay, the annexin V assay, the TUNEL assay, cell counting, determination of cellular doubling time and thymidine kinase activity, it could be shown that the RF-field at 1800 MHz, SAR 1.3 W/kg and 24h exposure did not effect cell viability and cell growth, and did not induce apoptosis in HL-60 cells. These findings are in substantial agreement with previous literature reports on effects of RF-EMF in HL-60 cells and other human cells (e.g., Hambrook et al. 2002, Higashikubo et al. 2001). In contrast to the present results, induced cell proliferation and apoptosis have been reported in various other cell types after exposure to EMF (Blumenthal et al. 1997, Philips et al. 1997, Ismael et al. 1998, Kwee and Raskmark 1998, Simko et al. 1998, Velizarov et al. 1999).

4.2.2.4 Mouse embryonic stem (ES) cells (Participant 4)

RF-EMF exerted no influence on ES-derived cardiogenesis and did not affect DMSO-induced cardiac differentiation, proliferation and expression of regulatory genes in P19 EC cells.

Several in vitro studies report negative effects of high frequency EMF on cell cycle, gene expression and differentiation (Fritze et al. 1997; Cain et al. 1997; Goswami et al. 1999; Ivaschuk et al. 1997), DNA and chromatin structure (ICNIRP 1996; Repacholi 1998) and rat embryo development (Klug et al. 1997). In contrast, several reports described positive effects by high-frequency EMF exposure on the length of cell cycle phases, proliferation and gene expression levels in mammalian cells (Cleary et al. 1996; Czerska et al. 1992; Goswami et al. 1999; Lai and Singh 1996a; Sarkar et al. 1994). These studies, however, were performed with different experimental models, carrier frequencies (835 MHz to 2.45 GHz versus 1.71 GHz used in our study) and modulation schemes, and therefore, are not comparable. Moreover, positive RF-EMF effects were often observed at relatively high average SAR values (Cleary et al. 1996; Czerska et al. 1992; Fritze et al. 1997), which suggests that they could arise from RF-EMF-evoked thermal effects. In our studies, GSM signals were applied under conditions of the ICNIRP safety limit using an experimental set-up that enabled precise temperature control (Schönborn et al. 2000), and any temperature increase as a consequence of EMF exposure (Laurence et al. 2000) can be excluded.

For the evaluation of embryotoxic effects of chemical compounds in vitro, the mouse embryonic stem cell test (EST, (Spielmann et al. 1997) using cardiac differentiation of ES cells as endpoint has been established. Therefore, for a further specification of the effects of GSM-217 signals, we analysed EMF exposure during the process of cardiac differentiation. GSM-217 EMF exerted no influence on ES-derived cardiogenesis and did not affect DMSO-induced cardiac differentiation, proliferation and expression of regulatory genes in P19 EC cells. These data present evidence that wild-type ES cells are not sensitive during cardiac differentiation to EMF. However, this finding is in contrast to EMF-induced effects in ES-derived neural progenitors.

The differentiation process in cells is affected by RF-EMF exposure, when applied at the neural progenitor stage.

The intact nervous system might be very sensitive to induced electric fields and currents, due to the high level of spontaneous activity and the greater number of interacting neurons. It has been suggested that induced current densities above 10 mA/m^2 may have effects on some central nervous system functions (Saunders and Jefferys 2002). Because of the special public concern for neurotoxicity due to EMF exposure, we used an experimental protocol successful at selectively differentiating ES cells into the neural lineage (Rolletschek et al. 2001). It provides a tool to investigate in vitro neuropathogenic effects of environmental factors during early development. We exposed the cells to EMF during the differentiation stage when the first neural nestin-positive progenitors appear. This developmental stage is presumably very sensitive to environmental factors. In our experiments, we observed RF-EMF effects on neural differentiation. Among the investigated transcripts (the mRNA levels of the neuronal genes TH, Nurr1 and en-1, and the astrocyte-specific gene GFAP) we observed a statistically significant down-regulation of nurr at 4d+11d and TH at the terminal stage 4d+23d. This might indicate a delayed neural differentiation and would correlate with the up-regulation of the growth arrest gene GADD45 at terminal stage. The significant up-regulation of GADD45 at the terminal stage 4d+23d was also confirmed by quantitative RT-PCR with TaqMan probe. Bcl-2, whose transcript levels were found increased in our study, has also been shown to be involved in neuronal differentiation and axonal regeneration (Daadi et al. 2001). Human teratocarcinoma-derived neurons expressed bcl-2 in 85% of the implanted neurons after transplantation into the rat striatum. In addition, the in vitro induction into the neuronal lineage resulted in an up-regulation of bcl-2 expression. The authors suggested that neuronal differentiation could be mediated at least partially by bcl-2 (Daadi et al. 2001).

Since we observed an up-regulation of GADD45, which is a DNA-damage inducible gene, it was logical to measure the eventual induction of primary DNA damages (4.2.1.3)

4.2.2.5 Summary (Participant 1)

As discussed by Participant 4 (4.2.2.4), the results on possible effects of RF-EMF on cell proliferation and differentiation in vitro which are reported in the literature (The Royal Society of Canada 1999; Stewart Report 2000) are controversial. The REFLEX data do not reveal a significant effect of RF-EMF on proliferation and differentiation of various cell systems such as neuroblastoma cells (NB69) and neuronal stem cells (3.2.2.1, 4.2.2.1), embryonic cancer cells (P19) (3.2.4.1, 4.2.2.4), human lymphocytes and human thymocytes (3.2.2.2, 4.2.2.2) and HL-60 cells (3.2.2.3, 4.2.2.3). In neural progenitor cells only some effect on the differentiation process was observed at a SAR level of 1.5 W/kg (3.2.4.1, 4.2.2.4). Quite obviously, whether or not living cells respond to RF-EMF exposure in vitro may depend on the type of the cell, its genetic background, its metabolic state and, of course, on the exposure conditions.

An answer of what may be the reason for the sporadically observed, but until now not confirmed influence of RF-EMF on cell proliferation and differentiation may be provided by the REFLEX findings on gene and protein expression. As shown by Participant 5, RF-EMF reduced the expression of the receptor FGFR1 of fibroblast growth factor (FGF) in the human neuroblastoma NB69 cell line and in neural stem cells from rat embryonic nucleus striatum (3.2.4.2, 4.2.4.2). Participant 3 (3.2.4.3, 4.2.4.3), Participant 6 (3.2.4.6, 4.2.4.6), and Participant 12 (3.2.4.7, 4.2.4.7) observed, that RF-EMF enhanced the expression of various genes among them ribosomal and mitochondrial genes, ATP related genes and genes encoding calcium-associated proteins and cell cycle proteins.

Of course, the relationship between RF-EMF exposure and the acceleration or inhibition of cell proliferation and differentiation in vitro caused by alteration of gene and protein expression is not proven yet. Should that be shown one day, it is to be found out, whether such cellular events occur also in vivo in RF-EMF exposed man and animal. The most recent data of Weisbrot et al. (2003), who observed an increase in the numbers of off-springs, an elevation of the hsp70 levels, an increase in serum response element (SRE) DNA-binding and an induction of the phosphorylation of the nuclear transcription factor, ELK-1, in Drosophila melanogaster after RF-EMF discontinuous exposure (900/1900 MHz, 1.4 W/kg) during the 10 day developing period, speak in favour of such an assumption.

4.2.3 Apoptosis

4.2.3.1 Brain cells of different origin and human monocytes (Participant 9)

There is no indication that apoptosis is affected in nerve and immune cells after exposure to GSM-like RF-EMF.

Beside the importance of the apoptotic process in cellular homeostasis, only a few papers are available in the literature on the effects of ELF-EMF on apoptosis and almost no data were published on the interaction of RF fields with the apoptotic process. Thus, one of our objectives within the REFLEX programme was to investigate the potential role of environmental electromagnetic fields, specifically GSM-900 radiofrequency radiation (RFR) on the apoptotic process in critical cell types.

Briefly, apoptosis or programmed cell death plays a central role both in development and homeostasis of multicellular organisms (Skulachev 2002). A dual physio-pathological role of cellular apoptosis has been described (Rossi and Gaidano 2003). On the one hand, apoptosis is a major mechanism of protection against genotoxic agents since potential cancer cells are removed by apoptosis. On the other hand, dysregulation in the apoptotic pathways is involved in different pathologies since excessive apoptosis can contribute to diseases such as AIDS or neurodegenerative diseases (Olney 2003) whereas default in apoptosis is involved in cancer or autoimmune diseases (Burns and el-Deiry 2003). Moreover, inducing apoptosis in apoptosis-resistant tumour cells may lead to therapeutic applications (Tolomeo and Simoni 2002) while preventing apoptosis in apoptosis-sensitive cancer cells may be deleterious.

Because the phone is close to the head when in use, brain cells represent a major potential target for RFR emitted by the phones. Furthermore, one of the most critical cell types in the central nervous system is primary neurons. For our studies, we chose rat granule cells. Granule cells of the cerebellum constitute the largest homogeneous neuronal population of mammalian brain. Cerebellar granule cells are a model of election for the study of cellular and molecular correlates of mechanisms of survival/apoptosis and neurodegeneration/neuroprotection (Contestabile 2002). We failed to detect any influence of GSM-900 exposure on apoptosis in this highly critical cell type. All other nerve cell types tested, i.e. SH-SY5Y neuroblastoma cells, human U87 astrocytoma cells and rat C6 glioma cells - a priori less critical than granule cells - were not shown to be sensitive to GSM-900 exposure for up to 24 hours. Hence, no demonstration of an immediate or delayed effect of RFR on apoptosis in nerve cells has been made in rat primary cells and human cell lines. We conclude from our results that nerve cells do not represent a major target, in terms of apoptosis, for RFR emitted by mobile phones.

Because of the role of the immune system for cell homeostasis, cells from the immune system were to be tested. No evidence for an immediate, cumulative or delayed effect of RFR on apoptosis was shown in a human monocytic cell line. Gene expression experiments gave some confirmation that RF-EMF (GSM-900) had no influence on apoptosis in U937 cells as no significant effect was demonstrated on genes involved in apoptosis. We conclude from our results that U937 cells are not sensitive to GSM-900 exposure for up to 48 hours. Taken together, in our experiments, no substantial effect of exposure to RF-EMF (GSM-900) on spontaneous apoptosis of nerve and immune cells was found. No delayed effect could be evidenced either. When tested, interaction between GSM-900 exposure and pro-apoptotic chemicals could not be evidenced.

The results from the REFLEX programme strongly suggest that the apoptotic process may not be a major biological target for GSM mobile telephony-related signals. The REFLEX programme is contributing to most of our current knowledge on the effects of RF fields on cellular apoptosis. Two papers have been very recently published on that topics. Hook et al. (2004) found no evidence of programmed cell death in Molt 4 human lymphoblastoid cells after exposure to 4 different American signals for up to 24 hours at SAR ranging from 0.0024 and 3.2 W/kg. Markkanen et al. (2004) in Finland reported that 900-MHz CW or GSM-modulated RF fields at a SAR of 0.6 W/kg did not induce apoptosis in a control yeast strain and in its temperature-sensitive mutant of cdc48 (apoptosis strain). When yeast strains were pre-exposed to UV, GSM-900 only was able to enhance the UV-induced apoptosis in the mutant yeast strain only. In the REFLEX programme, no significant effect on spontaneous apoptosis was detected in cells from the immune system (human peripheral blood mononuclear cells, human U937 cells) and in EA.hy926 human endothelial cells exposed to RFR-fields (GSM-900 and GSM-1800). No delayed effect (time kinetics) after RFR exposure was demonstrated.

In the present body of work, the status (transformed or non-transformed) of cells used did not influence the effect of RF-fields. The signalling pathways involving bcl-2 was not affected in either $p53^{+/+}$ or $p53^{-/-}$ embryonic stem cells tested after exposure to RFR-fields. The activity of caspase3 was not altered in EA.hy926 cells. In all systems tested, intermittence in the signal did not elicit apoptosis. Data also suggest that for the exposure conditions tested, field effects were not substantially affected by the cell genetics (embryonic stem cells), or the age of the donor (human peripheral blood mononuclear cells).

Hence, no effects of GSM signals (GSM-900 and GSM-1800) have been detected on spontaneous apoptosis of mature and embryonic stem cells in the various groups involved in REFLEX, even in conditions reported to modify other biological endpoints (for instance, an increase of hsp27 expression was detected in EA.hy926 human endothelial cells, 4.2.4.6). However, the expression of the bcl-2 anti-apoptotic gene was shown to increase in murine differentiating embryonic stem cells after exposure to GSM-1800, which correlated with changes in the process of neural differentiation (down-regulation of certain neuronal genes, 4.2.2.4). This needs to be further investigated in order to understand the potential relevance for human health.

We then focused our research on the investigation of some interaction between RF-EMF and known pro-apoptotic drugs. Extension of studies on the expression of apoptosis-related genes was also performed. The data show that cells from the immune and the nervous systems did not exhibit any sensitivity in a concomitant or successive treatment with apoptogenic chemicals and GSM signals, by contrast to cells from the endothelium. However, even in that cell type, GSM-900 was shown to interact with only one chemical (polyHema) over the two chemicals tested. The effect observed was a partial prevention of chemically-induced apoptosis. In this cell line, a weak decrease in pro-apoptogenic genes after exposure to GSM-900 was correlated with the former effect observed. These data on endothelial cells have still to be independently replicated.

Moreover, apoptosis-related genes were shown only weakly affected after exposure to RFR when compared to other gene families such as the ribosomal-related genes (Participant 12). Compared to data of the Juutilainen group, experiments performed within the REFLEX consortium used mammalian cells instead of yeast cells. This, as well as the nature of apoptogenic agent (chemical versus physical), may account for the discrepancy observed in the interaction experiments.

These data suggest that, except for murine differentiating stem cells, low-level RFR are not able to interfere with the spontaneous integrative apoptotic process. If confirmed, interaction with pro-apoptotic chemicals is suggested to be highly dependent of the cell type and the chemical agent used.

4.2.3.2 Human lymphocytes (Participant 8)

RF-EMF may not affect apoptosis in human lymphocytes.

1800 MHz RF (GSM basic, Talk and DTX modulated; SAR 1.3 - 2 W/kg) is not able to modify spontaneous and chemical-induced apoptosis, when human PBMC were exposed 10 min on and 20 min off for 44 hours. This result was also confirmed using cells from old donors (GSM basic, SAR 2 W/kg), since their cells could result differently susceptible to undergo apoptosis (Salvioli et al. 2003). Data were further confirmed by the analysis of mitochondrial membrane potential, which was not affected by RF in all the conditions tested. Negative results were also obtained analysing thymocyte apoptosis during their differentiation. On the basis of these data we can conclude that these types of exposures do not affect apoptotic process, even if this is not established for longer or chronic exposures. (4.2.2.2)

4.2.3.3 Human promyelocytic cell line HL-60 (Participant 2)

Using the annexin V assay and the TUNEL assay, it could be shown that RF-EMF at 1800 MHz, SAR 1.3 W/kg and 24h exposure did not affect cell viability and cell growth, and did not induce apoptosis. (4.2.2.3**)**

4.2.3.4 Mouse embryonic stem (ES) cells (Participant 4)

RF-EMF affected the bcl-2 –mediated anti-apoptotic pathway in differentiating embryonic stem cells.

An up-regulation of bcl-2, bax and GADD45 transcript levels was observed after exposure of ES-derived neural progenitors at specific stages of differentiation to high frequency and extremely low frequency electromagnetic field. We studied the gene expression levels of regulatory genes like hsp70, p21 and apoptosis–related genes of the bcl-2 family (the anti-apoptotic bcl-2 and the pro-apoptotic bax gene). These regulatory genes were pre-selected after our previous experiments with undifferentiated p53-deficient ES cells, where we found significantly increased transcript levels of p21 and hsp70 (Czyz 2004a) after RF-exposure and of p21 after ELF-EMF exposure (50 Hz Powerline) (Czyz 2004b). Therefore, we analysed the effects of EMF on apoptosis-related genes in wild-type (wt) embryonic stem cells at the stage of neural differentiation. Our data demonstrated up-regulation of the transcript levels of bcl-2 in neuronally differentiated ES cells at terminal stages for RF-EMF. However, the biological significance of this finding and its relevance to the situation in vivo has to be clarified.

Apoptotic cell death is executed by caspases and can be regulated by members of the bcl-2 family as reported for differentiating murine embryonic stem cells (Sarkar and Sharma 2002). Apoptosis plays an important role during embryonic development, including the development of the nervous system. Studies applying the model of central axotomy in mouse have shown a degeneration of up to 70% of nigral neurons post transection due to the activation of c-jun, but bcl-2 over-expression leads to a reduced phosphorylation state of c-jun in transected neurons and protection against cell death (Winter et al. 2002). Bcl-2 over-expression was also reported to eliminate deprivation-induced cell death of brainstem auditory neurons (Mostafapour et al. 2002). In other studies, in situ hybridisation revealed a rapid and transient increase in bcl-2 mRNA in neurons following de-afferentation (Wilkinson er al. 2002).

In the RF-EMF experiments, we extended our study by including the analysis of mRNA levels of the growth arrest DNA-damage inducible (GADD45) gene and found a significant up-regulation at the terminal stage of differentiation (at 4+23d). The members of the GADD protein family are considered to play important roles in maintaining genomic stability and in regulating the cell cycle (Chung et al. 2003). The phenotype of GADD45-deficient mice is similar to the phenotype of p53-deficient mice, including genomic instability and sensitivity to radiation induced carcinogenesis (Hollander et al. 1999). GADD45 was found to promote G2/M arrest thus inhibiting entry of cells into S-phase and allowing genomic DNA repair in keratinocytes (Maeda et al. 2002). These findings suggest that GADD45 is a component of the p53 pathway that maintains genomic stability, albeit damage-induced transcription of the GADD45 gene is supposed to be mediated by both p53-dependent (Kastan et al. 1992) and p53–independent mechanisms (Jin et al.2001). Our results, which demonstrate an up-regulation of bcl-2 and GADD45 mRNA levels indicate, that electromagnetic signals are, probably, perceived in embryonic stem cell-derived neural progenitors as environmental stress signals at defined stages of differentiation. Such signals may trigger cellular responses for maintenance of the cellular homeostasis via mobilization of the mechanisms of DNA repair and protection against apoptotic cell death.

4.2.3.5 Human the endothelial cell lines EA.hy926 and EA.hy926v1 (Participant 6)

RF-EMF may affect the hsp27 mediated anti-apoptotic pathway in human endothelial cells.

Stress proteins are known to regulate cell apoptosis (Pandey et al. 2000; Mehlen et al. 1996; Creagh et al. 2000). RF-EMF-induced deregulation of apoptotic process might be a risk factor for tumour development because it could lead to the survival of cells that “should” die. This notion was suggested in the hypothesis presented recently by French et al. (2001). We suggest that the apoptotic pathway regulated by hsp27/p38MAPK might be the target of RF-EMF radiation (6.2.4.6).

4.2.3.6 Summary (Participant 1)

As discussed by Participant 9 (4.2.2.4), knowledge on a possible influence of RF-EMF on the apoptotic process in living cells *in vitro* is rather poor. In the two most recent studies no such effect of RF-EMF was observed (Hook et al. 2004; Markkanen et al. 2004). The REFLEX data did not reveal a significant influence of RF-EMF on apoptosis of various cell types such as brain cells and human monocytes (see 3.2.3.1 and 4.2.31), human lymphocytes (3.2.3.2, 4.2.3.2) and HL-60 cells (3.2.1.1, 4.2.3.3). On the other

hand, an indirect effect on apoptosis via the bcl-2 or hsp27 mediated anti-apoptotic pathway which was detected in differentiating embryonic stem cells (3.2.4.1, 4.2.3.4) and in endothelial cells, respectively (3.2.4.6, 4.2.3.5), cannot be excluded at this time.

Of course, based on the data on gene and protein expression obtained in the REFLEX project, an effect of RF-EMF on the apoptotic pathway, either through inhibition or promotion, seems to be possible. Whether or not cell cultures respond to RF-EMF may depend on the type of cell exposed, their genetic background, their metabolic state and, of course, on the pattern of exposure. But taken together, even if a relationship between RF-EMF exposure and an inhibition or promotion of apoptosis in *in vitro* experiments were proven, it would in no way be possible to draw any conclusion for the *in vivo* situation in man and animal.

4.2.4 Gene and protein expression

4.2.4.1 Mouse embryonic stem (ES) cells (Participant 4)

The genetic constitution of early differentiating embryonic stem cells may play a role on their responsiveness to differently modulated RF-EMF.

In ES cells deficient for the tumour promoter p53, a permanent up-regulation of mRNA levels of the stress response gene hsp70 paralleled by a slight and temporary increase of p21, c-jun and c-myc expression was found in response to GSM-217- but not GSM-Talk-modulated signals characterized by the presence of low frequency components. On the other hand, wt ES and EC cells exposed to GSM-217 signals revealed no effects on gene expression, cardiac differentiation and proliferation. This would indicate, that the genetic background of stem cells may potentially influence the response of early differentiating cells to GSM signals dependent on the modulation schemes, whereas wt cells analysed in this study remained insensitive to GSM-modulated EMF.

p53-/- mice are highly susceptible to the development of spontaneous tumours, in particular, of malignant lymphomas at early age (Attardi and Jacks 1999; Sigal and Rotter 2000). p53 is required for G1 arrest in response to DNA damage and is involved in apoptosis (Attardi and Jacks 1999) via modulating versatile regulatory genes. In our studies using mouse $p53^{-/-}$ ES cells, hsp70 mRNA levels were continuously elevated after 48h EMF exposition. Heat shock proteins act as chaperons whose expression is activated or up-regulated in response to external stress (Beere and Green 2001). Hsp70 has also been defined to regulate homoeostasis in response to external stress during early embryo development (Luft and Dix 1999), while up-regulated hsp70 levels were observed in tumour cells and are correlated with metastases and poor prognosis (Zylicz et al. 2001). A potential involvement of heat shock proteins in cell responses to EMF was reported recently: up-regulated hsp27 protein levels and a transient increase of hsp27 phosphorylation were found in human endothelial cells (Leszczynski et al. 2002).

However, if EMF act as inducers of cellular transformation processes, effects on expression levels of other early response genes should be expected. Indeed, we observed an up-regulation of c-myc, c-jun and p21 mRNA levels in $p53^{-/-}$ cells upon GSM-217 exposure. The same genes were previously shown to be affected by environmental factors, such as UV or X-irradiation in various systems (Amati et al. 1993; Angel et al. 1988; Jean et al. 2001). Because in our model, the shifts in gene expression were low and transient, it is conceivable that EMF signals, while affecting gene expression pattern in $p53^{-/-}$ cells, do not induce permanent cellular transformations in wt cells.

The response of early differentiating cells to RF-EMF is dependent mainly on the carrier frequency of the modulation schemes.

Contrary to GSM-217 signals, which elicited cellular responses in $p53^{-/-}$ ES cells, GSM-Talk modulation exerted no effects on gene expression in our model. This indicates that low frequency components generated by GSM-Talk (2 and 8 Hz) do not promote the action of EMF signals in our cell system. In contrast, time-averaged SAR values (1.5 W/kg for GSM-217 vs. 0.4 W/kg for GSM-Talk) may comprise a factor determining the biological activity of EMF. Furthermore, it cannot be excluded that the modulation scheme (time distribution of high SAR pulses) may also play a role in evoking biological

responses, because slot-averaged SAR values remained similar between the analysed modulation schemes.

The exposure duration may also influence the biological responses to RF-EMF.

In our in vitro studies, up-regulated transcript levels of regulatory genes in $p53^{-/-}$ ES cells were observed after 48 hours exposure to GSM-217 signals, whereas a short-term 6h exposure exerted no effects.

The parameters of genetic constitution, carrier frequency and exposure duration in determining the response of biological systems to RF-EMF have been proposed by in vivo studies using Eμ-Pim1-transgenic mice predisposed to develop spontaneous lymphomas. Repacholi et al. observed an increase in tumour formation after long-term, 18 months exposure of Eμ-Pim1-transgenic mice to 900 MHz EMF (Repacholi et al. 1997), which positive data suggest that mobile phone radiation-induced events may be hazardous to cells deficient in cell repair when occurring frequently over long periods.

It has been shown previously that the exposition of human HL-60 cells resulted in an increase in DNA breaks, suggesting a possible direct mutagenic effect (Ivancsits et al. 2002). In addition, there is a report about a correlation between up-regulation of GADD45, of the member of the Bcl-2 family bcl-$X_{L,}$ and an increased amount of early DNA damage as measured by the alkaline Comet assay in human preneuronal cells exposed to the amyloid protein (Santiard-Baron et al. 2001). Therefore, we used the alkaline and neutral COMET assay to detect single, and double-strand DNA breaks respectively, in the neuronal progenitors derived from murine pluripotent ES cells after ELF or RF-EMF exposure. Under our experimental conditions, however, we could not find a clear evidence of increased single-strand DNA break induction in the alkaline Comet assay. A low but significant increase in double-strand DNA breakage was observed only after a short (6h) RF-EMF exposure in the neutral Comet assay.

In summary, we found that RF-EMF simulating GSM signals caused a transient up-regulation of p21 and c-myc genes and a long-term up-regulation of the stress response gene hsp70 in ES cells deficient for p53 in response to GSM-217- but not GSM-Talk-modulated signals characterized by the presence of low frequency components. Here again we found that the genetic constitution (loss of p53 function) could alter the responsiveness of ES cells and render them sensitive to high frequency EMF, while wild-type cells were irresponsive. However, we did not observe any distinct direct genotoxic effects as measured by the Comet assay.

In ES cell-derived neuronal progenitors we found indication of growth arrest and effects on apoptosis (a significant up-regulation of the growth arrest and DNA damage inducible gene GADD45, the proapoptotic bax and the antiapoptotic gene bcl-2 mRNA levels), which correlated with changes in the process of neural differentiation (down-regulation of the neuronal genes Nurr1 at stage 4d+11d and TH at 4d +23d).

4.2.4.2 NB69 neuroblastoma cells and neural stem cells (NSC) (Participant 5)

RF-EMF reduced the expression of the receptor FGFR1 of fibroblast growth factor (FGF) in the human neuroblastoma NB69 cell line and in neural stem cells from rat embryonic nucleus striatum.

In NB69 cells, this response is similar to that induced by exogenous treatment with 20 ng/ml of bFGF, and by the combined treatment with bFGF plus EMF. In this biological system, an induction of morphological changes (increases in cell size and cell extensions) is also observed after 24 hours of treatment with bFGF. Such morphological changes are accompanied with a reduction in the proportion of cells expressing FGFR1-3 receptors. However, in cultures exposed to the GSM-Basic for 24 hours the effect on FGFR1 was not associated to changes in the cells′ morphology. Evidence exists that treatment of olfactory neuroblastoma cells with bFGF deregulates FGFR1 prior to differentiation (Nibu et al. 2000). It is possible that in our NB69 cells the response induced the GSM-Basic signal on FGFR1 can also be associated to a promotion of differentiation in long-term cultures. Additional work using differentiation markers for neuronal NB69 cells has to be done to verify the above hypothesis.

On the other hand, in neural stem cells, the 24h-exposure also induced a reduced expression of receptor FGFR1, and further significant changes in the cell morphology were observed six days later, in the absence of the GSM-exposure. The oligodendrocytes showed an advanced developmental stage with respect to controls. Similarly, the astrocytes showed longer cell-processes. The morphology of the neuronal progeny of NSC was not significantly changed by the exposure to the GSM-Basic signal.

Evidence exists that treatment of a human astrocytoma cell line, U-87 MG, with 835 MHz electromagnetic radiation induces alterations in F-actin distribution and cell morphology (French et al. 1997). Their astrocytes showed a similar response to that observed in our precursors lineage exposed to the GSM-signal: an increased cell spreading. Taken together, the present data suggest that the reduction of cells expressing FGFR1 induced by signal could be linked to a promotion of the differentiation of non-neuronal populations.

The changes in FGFR1 induced by RF-EMF are dependent mainly on the carrier frequency.

Both, basic and CW signal induced similar changes on FGFR1. This suggests that the cellular response is not dependent on the tested low-frequency modulation, but on the carrier frequency. The signals used in advanced telecommunication systems such as global system for mobile communications (GSM) and universal mobile communications system (UMTS) include extremely low frequency (ELF) amplitude modulation or pulse modulation components. There is a lack of scientific data on the possible health implications of such modulations. A recent study (Huber et al. 2002) has reported that a 30-minute exposure to 900 MHz, 1 W/kg GSM signal can influence the electrical activity of the brain, both before and after sleep onset, in young male test subjects. Both sleep and waking EEG changes were observed only with pulse modulated-EMF. Also, it has been reported a statistically significant micronucleus effect in peripheral blood cultures following 15-minute exposure to phase modulated field (Gaussian minimum shift keying, GMSK), 1.748 GHz, at SAR $\leq$ 5 W/kg (d'Ambrosio et al. 2002b). However, the micronucleus frequency result was not affected by CW exposure. No changes were found either in cell proliferation kinetics after exposure to both CW and phase modulated fields. In our present work we investigated whether fibroblast growth factor receptors (FGFR) could be influenced by the modulation of the GSM-signals. NB69 cells were exposed to GSM- Talk, GSM-Basic and CW signals, 2 W/kg SAR or to DTX-signal, 1W/kg SAR. The exposure to GSM 1800-Basic signal at a 2 W/kg SAR was found to induce a significant decrease in the number of cells expressing the FGFR-1 (15% reduction vs. controls) without affecting significantly the number of cells expressing receptors R2 and R3. The effect on R1 was equivalent to that induced by basic fibroblast growth factor (bFGF) at a 20 ng/ml concentration. The exposure to RF-CW signal (SAR 2 W/kg) induced effects on the expression of FGFR-1 equivalent to those induced by the GSM-Basic signal, whereas the exposure to GSM-Talk signal at the same SAR (2 W/kg) or to DTX-signal (1 W/kg SAR) did not modify significantly the normal expression of the FGF protein receptors R1. Our results indicate that the ELF modulation components resulting from the GSM signals shape (2, 8 and 217 Hz) and higher harmonics are not critical for the EMF-induced changes in FGFR-1 expression. Provided that the Talk mode is a temporal change between GSM Basic (66%) and DTX only (34%) our data together with those from Partner 4 indicate that the exposure duration could also be a critical factor for the herein described response. Future studies may also examine dose-response relationships by varying the exposure time and the specific absorption rate.

4.2.4.3 Human promyelocytic cell line HL-60 (Participant 2)

RF-EMF modulates the gene and protein expression in HL-60 cells.

Applying high resolution two-dimensional polyacrylamide gel electrophoresis to the HL-60 cell system, more than 4000 protein spots can be differentiated on the silver stained protein map. These spots were detected and the master gel image was calibrated. Clear differences in protein expression have been found for RF-field exposed HL-60 cells as compared to control and sham-exposed cells. The quantitative comparison has been completed. Further strategies in the future will include structural and biochemical identification of proteins significantly altered following RF-field exposure, beside mass spectrometry (MALDI-TOF) and mass spectrometric sequencing (ESI-MS/MS) and immunoblotting/functional protein assays, also by comparative studies with reference databases. Clarification of changes in protein expression after exposure to RF-fields will help to understand molecular pathomechanisms.

4.2.4.4 Human lymphocytes (Participant 8)

RF-EMF did not affect gene expression in human lymphocytes.

1800 MHz RF (DTX modulated, SAR 1,4 W/kg) is not able to modify gene expression profile when quiescent T lymphocyte are exposed 10 min on/20 min off for 44 hours. This result, obtained in collaboration with Participant 12 by means of micro-array technique, was expected because lymphocytes

were not stimulated and represents a first step toward a further evaluation in low-stimulated and RF-exposed T lymphocytes; future analyses should clarify the presence of potential gene targets for RF exposure in primary human cells.

4.2.4.5 Brain cells of different origin, human immune cells and human endothelial cell lines (Participant 9)

There is no indication that expression and activity of the inducible Nitric Oxide Synthase (iNOS or NOS_2) is affected in nerve cells after exposure to RF-EMF.

Under pathological conditions, nitric oxide, NO, can act as a neurotoxic agent (Leist and Nicotera 1998; Brown and Bal-Price 2003). A variety of stresses are known to induce neuronal cell death via NOS_2 (or inductible NOS, iNOS) activation and NO production in stimulated astrocytic cells. Hence, activated astrocytes may be involved in the pathogenesis of neurodegenerative diseases. Our goal was to determine whether exposure to a GSM-900 signal could activate C6 glioma cells by increasing the activity of the iNOS enzyme. A potential synergistic effect of such radiofrequency radiation (RFR) on cytokine-induced NO production was also investigated.

A few data are available in the literature on the effect of RF-EMF on nitric oxide, NO, production. Over three identified papers, only one used RF-EMF compatible with mobile telephony. Miura et al. (1993) reported that 10 MHz RFR (10 kHz bursts) caused an increase in NO production in rat cerebellum extracts. Using ultra-wideband pulses, Seaman et al. (2002) recently showed no influence of RFR in the Ultra Wide Band (UWB) range in RAW 264.7 macrophages except when nitrate was added to the culture medium. Paredi et al. (2001) showed a tendency for higher nasal NO levels in humans exposed to GSM-900 for 30 minutes that was due to skin heating experienced by the phone's users. As it has been clearly shown that heating from mobile phone microwaves is negligible, this effect was probably due to the battery's heating. Based on our data, GSM-900 did not appear to be able to alter chemically- induced activation in mammalian astrocytes and thus appeared unlikely to influence tumour cells characteristics and neuronal cells' viability via NO pathways.

There is no indication that expression of heat shock proteins is affected in nerve cells after exposure to RF-EMF.

The first objective was to determine whether exposure to GSM-900 microwaves could influence the expression of hsp70 proteins in neuronal and glial cell lines as reported in an endothelial cell line by Participant 6.

In response to environmental disturbances, cells respond by expressing heat shock proteins. Our study focuses on the 70-kDa family, which is the major form of stress proteins found in the brain (Pavlik et al. 2003) and on hsp27 that is expressed in endothelial cells (Loktionova et al. 1996).

Our data showed that exposure to GSM-900 microwaves were not able to induce hsp70 expression in rat and human nerve cells. These data are not in agreement with results of recent research showing that the expression of heat shock protein (hsp) may be induced in response to radiofrequency radiation exposure at non-thermal levels in different models (de Pomerai et al. 2000, in worms; Kwee et al. 2001, in human amnion cells; Leszczynski et al., 2002 in human EA-hy926 endothelial cells; Weisbrot et al. 2003, in Drosophilae). Thus, a common feature on the effect of GSM-900 at low SAR (about 2 W/kg) on the expression of hsp cannot be drawn. Different cell types could behave differently to exposure to a GSM-900 signal or different members of the hsp family could show a different sensitivity to exposure to GSM signals. Moreover, we can note that, so far, none of the "positive" effects have been independently replicated.

We failed to independently confirm that expression of heat shock proteins is affected in EA-hy926 cells after exposure to GSM-like RF-EMF.

The second objective was to confirm the data of Participant 6 on hsp27 in the EA-hy926 cells. Hsp27 is indeed the major form of stress proteins that is expressed in endothelial cells (Loktionova et al. 1996). Our results obtained by fluorescent image analysis in the two cell lines tested differed from those obtained in one of them by Participant 6 after western blotting experiments. A third method using Elisa test will allow us to quantify precisely if RFR are able to induce changes in hsp27 expression since the sensitivity reported is 1-10 ng for Western Blot and less than 1 ng for ELISA.

Here, with our semi-quantitative method and statistical analysis we were unable to confirm previous data on hsp27 expression in endothelial cell lines. Exposure set-up used in both groups also differed (water bath versus air cooling, homogeneity of SAR distribution at the cells level, …etc), which probably imply different dosimetric features. Whether this could account for the discrepancies observed could be determined (Participant 10). Meanwhile, we cannot conclude yet that RFR induce stress response. Hence, no implication for health hazard can be drawn at the moment based on Hsp expression in mammalian cells after low level RFR exposure.

The possible effect of low-level RFR on the expression of hsp is quite controversial. While some laboratories reported effects in mammalian cells (Leszczynski et al. 2002; Kwee et al. 2001) or simple organisms (de Pomerai et al. 2000; Weisbrot et al. 2003) as stated above, we (present work), as other groups (Cleary et al. 1997; Tian et al. 2002a; Miyakoshi et al. 2003) could not observe any effect in mammalian cells at SAR up to 10 W/kg. To date, no clear and satisfactory explanation can be given. However, we do think that the investigation of hsp expression after RFR exposure in *in vivo* mammalian models will help and enlighten the debate. Some but still sparse data are available, showing that heat shock proteins could not be induced in rat brain below 7 W/kg (Fritze et al., 1997).

There is some indication that gene expression is affected in immune cells after exposure to RF-EMF.

These data were obtained in collaboration with Participant 12 (4.2.4.7). First, these results gave a confirmation that GSM-900 had no influence on apoptosis in U937 cells as no significant effect was demonstrated on genes involved in apoptosis. Then, only a few genes among several thousand tested genes were shown altered after RFR exposure (increase or decrease) in two human immune cell lines. The largest modification in RNA expression corresponded to genes related to signal transduction and energy metabolism. Finally, amine oxidase activity-related genes experienced the largest changes after exposure to mobile phone-like RFR. This later gene is coding for an enzyme which is involved in cell growth and proliferation but also in immune regulation. This gene-profiling analysis showed that RFR can influence some biological processes and gave us trails for further investigations such as looking at energy metabolism in cells exposed to RFR using spectroscopic NMR. Comparing gene profiling obtained in different cell types may provide a "signature" for environmental RF-EMF exposure. It is however still unclear if and how those changes in gene expression can be related to human health.

Reported gene profiling after ethanol stress in yeast (Alexandre et al. 2001) showed that about 6% of the yeast genome were experiencing changes (about 3% upregulated and 3% downregulated). This represents 20 fold more genes than what was found affected after exposure to RF-EMF (0.3%). Genes identified were mainly involved in energetic metabolism, protein destination, ionic homeostasis and stress response with more than 10 hsp members. Stronger criteria were used in Alexandre et al. (2001) for significance of changes in gene expression compared to ours. When compared with the effect of a strong stress agent such as ethanol (in yeast), RF-EMF affected only a few genes in the human immune cell lines we tested. Whereas genes involved in energy metabolism seem to be a common feature for both types of exposure, none of the genes identified in human immune cells after RFR exposure belonged to the stress response family. Based of these comparisons, RF-EMF may be identified as a weak environmental stress, if any.

4.2.4.6 Human endothelial cell lines EA.hy926 and EA.hy926v1 (Participant 6)

RF-EMF induce cellular stress response.

Observed in this study changes in protein phosphorylation and activation of p38MAPK/hsp27 stress response pathway agree with the earlier studies suggesting that mobile phone RF-EMF radiation induces cellular stress response at non-thermal power level. In vitro, Cleary et al. (1997) claimed that RF-EMF exposure has no effect on stress proteins. However, because identification of stress proteins was based solely on their molecular weight in liquid chromatography, the exact identity of proteins claimed to be stress proteins remains unclear. Fritze et al. (1997b), using rat model, have shown increase in expression of stress protein hsp70 in brains of animals exposed for 4 hours to RF-EMF (890-915 MHz) at SAR of 1.5 W/kg. Daniells et al. (1998) and de Pomerai et al. (2000) have shown that overnight in vivo irradiation of nematode worms with RF-EMF (750 MHz) at SAR of 0.001 W/kg cause increase in expression of heat shock protein. Kwee et al. (2001) have shown induction of stress protein hsp70, but not hsp27, in cultures of transformed human epithelial amnion cells exposed for 20 min to RF-EMF (960 MHz) at SAR of 0.0021W/kg.

Because of the known broad spectrum of physiological processes that are regulated by stress proteins (Tibbles and Woodgett, 1999), and by hsp27 in particular, it is here hypothesized that mobile phone radiation-induced activation of hsp27/p38MAPK-dependent cellular stress response might: (i) lead to the development of brain cancer due to inhibition of cell apoptosis and (ii) cause increased permeability of blood-brain barrier due to stabilization of endothelial cell stress fibers (Figure 138).

Stress proteins are known to regulate cell apoptosis (Pandey et al. 2000; Mehlen et al. 1996; Creagh et al. 2000). RF-EMF-induced deregulation of apoptotic process might be a risk factor for tumour development because it could lead to the survival of cells that "should" die. This notion was suggested in the hypothesis presented recently by French et al. (2001). We suggest that the apoptotic pathway regulated by hsp27/p38MAPK might be the target of RF-EMF radiation. Hsp27, stress protein shown in this study to be affected by mobile phone radiation exposure, is a member of a family of small heat shock proteins that is ubiquitously expressed in most of cells and tissues under normal conditions in form of large-molecular complexes. In response to stress occurs rapid phosphorylation of hsp27 on serine residues (in human cells Ser-78 and Ser-82) what leads to dissociation of the large-molecular complexes into smaller units (Kato et al. 1994). Various stress factors have been indicated as inducers of changes in expression (accumulation) and/or phosphorylation (activity) of hsp27 (Ito et al. 1995; Deli et al. 1995; Garrido et al. 1997; Huot et al. 1997; Tibbles and Woodgett 1999). Activated (phosphorylated) hsp27 has been shown to inhibit apoptosis by forming complex with the apoptosome (complex of Apaf-1 protein, pro-caspase-9 and cytochrome *c*), or some of its components, and preventing proteolytic activation of pro-caspase-9 into active form of caspase-9 (Pandey et al. 2000; Concannon et al. 2001). This, in turn, prevents activation of pro-caspase-3 which, in order to become active, has to be proteolytically cleaved by caspase-9. Thus, induction of the increased expression and phosphorylation of hsp27 by the RF-EMF exposure might lead to inhibition of the apoptotic pathway that involves apoptosome and caspase-3. This event, when occurring in RF-EMF exposed brain cells that underwent either spontaneous or external factor-induced transformation/damage, could support survival of the transformed/damaged cells what, in favourable circumstances, could help clonal expansion of the transformed/damaged cells - a prerequisite for the tumour development. Furthermore, hsp27 in particular was shown to be responsible for the induction of resistance of tumour cells to death induced by anti-cancer drugs (Huot et al. 1996; Garrido et al. 1997). Thus, it appears possible that RF-EMF induced changes in hsp27 phosphorylation/expression might affect not only tumour development but also its drug-resistance.

Induction of the increase of the permeability of blood-brain barrier by RF-EMF exposure, which has been suggested by some animal and in vitro studies, is one of the controversial health issues that came up in relation to the use of mobile phones. It has been already established that, at thermal levels of exposure, microwave radiation causes increase in the permeability of blood-brain barrier (for review see Jokela et al. 1999; The Royal Society of Canada Report 1999, Stewart Report 2000, Zmirou Report 2001). However, the effect of non-thermal RF-EMF exposure on blood-brain barrier is still unclear. Some studies have suggested that mobile phone radiation, at non-thermal exposure levels, increases permeability of blood-brain barrier *in vivo* (Salford et al. 1994) and *in vitro* (Schirmacher et al. 2000), whereas others suggested lack of such effect (Fritze et al. 1997a; Tsurita et al. 2000). However, the no-effect claimed by Fritze et al. (1997a) is not so straight forward as suggested by the authors because they reported induction of stress response and increased permeability of the blood-brain barrier immediately after the end of irradiation. This effect was short lasting and, because of it, was considered by the authors as insignificant. Also, it remains unclear what would be the blood-brain barrier response to the repeated exposures to mobile phone radiation because the effect of repeated exposures was not examined. The increased blood-brain barrier permeability due to increase of endothelial pinocytosis was suggested by Neubauer et al. (1990) who have demonstrated increase in pinocytosis of cerebral cortex capillaries that were exposed to 2.45 GHz microwave radiation. Finally, the recently reported study by Töre et al. (2001) has shown that 2 hour exposure of rats to RF-EMF (900 MHz) at SAR of 2W/kg (averaged over the brain) causes increase in the permeability of blood-brain barrier. However, the molecular mechanism and the cellular signalling pathways that are involved in the induction of blood-brain barrier permeability are still unknown. We propose that the induction of hsp27 phosphorylation and increased expression by RF-EMF exposure, shown in this study to occur in vitro in human endothelial cells, might be the molecular signalling event that triggers the cascade of events leading to the increase in blood-brain barrier permeability. Phosphorylated hsp27 has been shown to stabilize endothelial cell stress fibers due to the increased actin polymerisation (for review see Landry and Huot 1995). The stabilisation of stress fibres was shown to cause several alterations to endothelial cell physiology: (i) cell shrinkage and opening of spaces between cells (Landry and Huot 1995; Piotrowicz and Levin 1997), (ii) increase in the

permeability of endothelial monolayer (Deli et al. 1995), (iii) increase in pinocytosis (Lavoie et al. 1993), (iv) formation of apoptosis-unrelated blebs on the surface of endothelial cells which may obstruct blood flow through capillary vessels (Becker and Ambrosio, 1987), (v) stronger responsiveness of endothelial cells to estrogen and, when stimulated by this hormone, secretion of larger than normally amounts of basic fibroblast growth factor (bFGF) (Piotrowicz et al. 1997) which could, in endocrine manner, stimulate de-differentiation and proliferation of endothelial cells leading to, the associated with proliferative state - cell shrinkage and unveiling of basal membrane. Occurrence of these events in brain capillary endothelial cells could lead to de-regulation of the mechanisms controlling permeability of blood-brain barrier. Furthermore, in addition to blood-brain barrier effects, the stabilization of stress fibres in endothelial cells may affect apoptotic process - it has been shown that the apoptosis-related cell surface blebbing is prevented by the stabilised stress fibres (Huot et al. 1998).

The proposed hypothetical molecular mechanism for the possible role of mobile phone radiation in development of brain cancer and in increasing permeability of the blood-brain barrier, although a hypothesis, it is reasonably supported by the evidence concerning both effects of microwaves on stress response and effects of hsp27 (increased expression and activity) on cell physiology. Proving or disproving of this hypothesis using in vitro and in vivo models will provide evidence to either support or to discredit the existence of some of the potential health risks that were suggested to be associated with the use of mobile phones.

The recently published hypothesis of French et al. (2001) of the possible effect of chronic/frequent exposure to mobile phone radiation that would induce abnormally high levels of stress proteins in cells still requires experimental confirmation that, indeed, repeating exposures to RF-EMF radiation could cause such an increase. On the other hand, proposed by us hypothetical mechanism of the mobile radiation effect on the brain relies on the single-exposure-induced transient increases in hsp27 phosphorylation and expression. We suggest that the transient effects, induced by repeated exposures, might, by chance of timing coincidence, led to survival of damaged/transformed cells and temporarily increase permeability of the blood-brain barrier. These events, when occurring repeatedly (on daily basis) over the long period of time (years) could become a health hazard because of the possibility of accumulating of brain tissue damage. Furthermore, our hypothesis suggests that other, than RF-EMF, cell-damaging factors might play a co-participating role in the tumour development caused by mobile phone radiation.

Finally, in addition to the p38MAPK/hsp27 stress pathway-induced effects, the extent of the global change of the pattern of protein phosphorylation observed in our study suggests that it is likely that multiple signal transduction pathways might be affected by the RF-EMF exposure. Identification of these pathways will help to determine the extent of biological effects induced by RF-EMF exposure. Importantly, cellular effects observed in this study were induced by RF-EMF irradiation at non-thermal levels, with SAR values set at the highest level that is allowed by the European safety limits. This suggests that the presently allowed radiation emission levels for the mobile phones, although low, might be sufficient to induce biological effects. However, determination of whether these effects might cause any significant health effects requires further studies.

5-step feasibility study of applying proteomics/transcriptomics to mobile phone research.

It has been suggested that high-throughput screening techniques (HTST) of transcriptomics and proteomics could be used to rapidly identify broad variety of potential molecular targets of RF-EMF and generate variety of biological end-points for further analyses (Leszczynski et al. 2004). Combination of data generated by transcriptomics and proteomics in search for biological effects is called the “discovery science”. This term has been coined-in by Aebersold et al. (2000) to define the new approach that will help in revealing biological mechanisms, some of which might be unpredictable using the presently available knowledge. This approach seems to be particularly suited for elucidation RF-EMF health hazard issue because it might reveal effects that are not possible to predict based on the present knowledge about the biological effects of RF-EMF. However, before committing large funds that are needed for HTST studies it is necessary to determine whether indeed this approach will be successful in unravelling physiologically significant biological events induced by RF-EMF. Due to their high sensitivity HTST are able to pick-up very small changes in protein or gene expression which changes might be of insufficient magnitude to alter cell physiology. Thus, although using HTST it might be possible to find biological effects induced by RF-EMF these effects might be of limited or no significance at all, from the physiological stand point. Therefore, to determine the usefulness of HTST

approach to the issue of bio-effects induced by RF-EMF, we have performed a 5-step feasibility study and have shown that HTST might indeed help to identify experimental targets for physiological studies of RF-EMF-induced biological responses. The obtained by us results clearly demonstrate that by using HTST it is possible to identify RF-EMF-induced molecular events that might alter cell physiology. Even though the increase in expression/phosphorylation of the examined hsp27 protein was very modest (ca. 2-3 folds increase) it was possible to determine impact of this event on cell physiology. Whether any impact on organ (e.g., brain) or whole body will be exerted by this change remains to be determined by in vivo studies. Although the use of discovery science-approach employing HTST will not provide direct evidence of health hazard or its absence, it will be essential in unravelling of possibly all biological effects exerted by RF-EMF exposure. Further elucidation of the physiological significance of these biological effects for the health and well-being, in short- and long-term exposure conditions, will allow determination whether any health hazard might be associated with the use of mobile phones at the presently allowed radiation safety levels.

Use of HTST to determine genotype-dependent and modulation-dependent cellular responses.

Our study has shown that proteomics transcriptomics and might be an efficient tool when searching for the proteins and genes responding to a weak stimulus, like the mobile phone radiation. In this pilot study we have found several tens of protein and gene targets of the mobile phone radiation. Functions of the few of the MALDI-MS-identified protein spots suggest possibility of the effects of the mobile phone radiation on such physiological functions as (i) cellular energy production, (ii) protein translation, and (iii) cytoskeleton-dependent processes (e.g. cell size, shape and cell-cell interactions). Potential effects on these processes were supported by the evidence gained with cDNA arrays. Further studies will be needed to determine whether there is any impact of these changes on cell physiology.

The other major finding of the study is the observation that the exposure of cells to the continuous-wave microwaves ("CW-signal" 1800 MHz GSM) does not induce changes in protein expression whereas radiofrequency modulated microwaves ("Talk-signal" 1800 MHz GSM) induces broad changes in protein expression. Analysis of changes in expression of some 1500 proteins using cICAT method combined with liquid chromatography and MS/MS identification of proteins has revealed several tens of affected proteins. Importantly, using other methods such as 2-DE and cDNA arrays the same cytoskeleton-related genes/proteins were detected as being affected by RF-EMF exposure up. It means that with two different proteomics approaches we have observed similar protein changes what strengthens the validity of our observations.

4.2.4.7 Effects of RF-EMF on gene expression in human cells analysed with the cDNA array (Participant 12)

The elevated turnover of ribosomal proteins and proteins involved in energy metabolism allows the hypothesis that the cellular turnover is increased after RF-EMF exposure. To prove this hypothesis for RF-EMF treated HL-60 cells (Participant 2), a very interesting additional comparison was performed: In the 1800 MHz continuous wave experiments (2 expression profiles, 4 hybridisations) we used two controls instead of one: One control in each experiment was a sham-exposed control as usual. Cells from another incubator, neither exposed nor sham-exposed, served as a second control. After both experiments we performed comparisons between both controls as well as between sham-exposed and RF-exposed cells (each comparison with 8 data points per gene, from 2 expression profiling experiments, 4 hybridisations). After going through all investigated gene families listed in Tables 30 and 31, with some gene families we found remarkable differences between the control comparison and the sham-RF comparison (Figure 144). As expected, again ribosomal and mitochondrial genes are much more upregulated in the sham-RF comparison than in the "blinded" comparison (Sham-Ctrl). But there are also other gene families showing the same tendency, as for example ATP related genes, genes encoding calcium-associated proteins and cell cycle proteins.

The increasing ribosomal turnover might lead to cell growth and, in the end, to mitosis and cell proliferation, respectively. This hypothesis has not been confirmed so far by the BrdU-incorporation assay, because no significant increase of DNA synthesis, and therefore, an increase in cell proliferation, could not detected (Participant 2). The same is true for the analysis of protein mass and the MTT assay for the detection of mitochondrial activity (Participant 2).

However, the changes in ribosomal protein synthesis are not so strong that we would expect a very strong increase in cell cycle progression. Ribosomal transcription rates have been found to vary by up to a factor of four (Derenzini et al. 2001; Leary and Huang 2001). It is known from stimulation by growth factors, that an increase of ribosomal activity not necessarily leads to significant changes in cell cycle (Stefanovsky et al. 2001; Bodem et al, 2000). We should investigate the cell cycle distribution after RF-EMF exposure in more detail. Also the analysis of the 45S precursor rRNA by real-time RT PCR or the analysis of the RNA polymerase I and associated proteins would help us to figure out if ribosomal transcription is elevated, which is a pre-requisite for an increase of ribosomal proteins (Stefanovsky et al. 2001; Jacob and Gosh 1999). Compared to the ELF-EMF results (Participant 3) the results with RF-EMF seem to be more uniform. The comparison of HL-60 profiling 2 and 3 for example (Participant 2), shows a much lower number of reproducibly regulated genes than after the analysis of only one experiment (compare Figure 139). If the reason for this is the differentiating potential of the cells (U937 cells of Participant 9, HL-60 cells of Participant 2, T-lymphocytes of participant 8), or the exposure conditions (homogenous ELF-EMF field with or without on/off cycles versus RF-EMF GSM talk signal), or both, remains to be elucidated. More cell lines, each exposed to different fields (ELF, RF), would have to be investigated to draw more reliable conclusions.

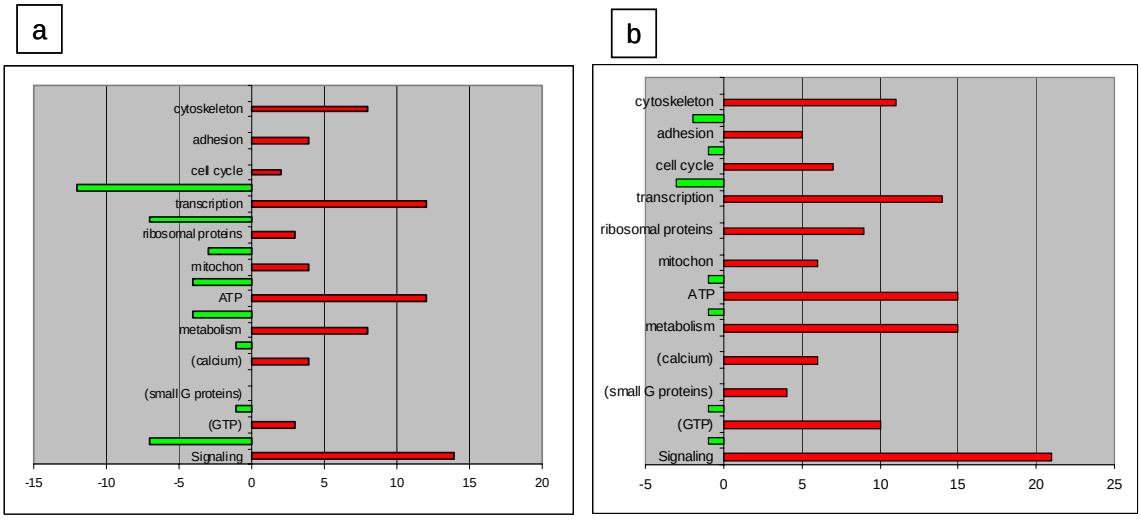


Figure 144. Numbers of regulated genes after RF-EMF exposure sorted according to different gene families. a, comparison between batches of control HL-60 cells (not exposed vs. sham exposed). Red: Genes appearing up-regulated in sham-exposed. Green: Genes appearing down-regulated in sham-exposed. b, comparison between sham-exposed (ctrl) and RF-EMF exposed HL-60 cells. Red: Genes showing up-regulation in RF-EMF. Green: Genes showing down-regulation in RF-EMF.

4.2.4.8 Summary (Participant 1)

Scientific work on gene and protein expression due to RF-EMF exposure using in vitro cell cultures and animal models is still in its early stages and as far as already published difficult to interpret (Independent Expert Group on Mobile Phones 2000). From the REFLEX data the conclusion can be drawn that RF-EMF may affect the gene and protein expression in various cell systems. RF-EMF exposure at a SAR value of 1.5 W/kg caused a transient up-regulation of the p21 and c-myc genes and a long-term up-regulation of the hsp70 gene in p53 deficient embryonic stem cells (3.2.4.1, 4.2.4.1). RF-EMF exposure at a SAR value of 2 W/kg reduced the expression of the receptor FGFR1 of fibroblast growth factor (FGF) in human neuroblastoma cells (NB69) and in neural stem cells of rats obviously without affecting protein transcription (3.2.4.2, 4.2.4.2). RF-EMF exposure up- or down-regulated the expression of various genes and proteins in HL-60 cells (1800 MHz, 1.3 W/kg) and in endothelial cells of human origin (900 MHz, 2.0 W/kg) (3.2.4.3, 3.2.4.6, 3.2.4.7, 4.2.4.3, 4.2.4.6, 4.2.4.7). RF-EMF exposure at a SAR value of 2.0 W/kg activated the p38MAPK/hsp27 stress response pathway and changed the global

pattern of protein phosphorylation in endothelial cells with possible consequences for the signal transduction pathway (3.2.4.6, 4.2.4.6).

Not unexpected, the available literature is controversial (Stewart Report 2000). While Lee et al. (2004) observed an alteration of gene expression in HL-60 cells after exposure to RF-EMF of 2450 MHz and Zeng et al. (2004a) an alteration of protein expression in human breast cancer cells (MCF-57) after exposure to RF-EMF of 1800 MHz, no such effects were found by Miyakoshi et al. (2004), who studied the influence of RF-EMF (1950 MHz) on the expression of hsp27 and hsp70 in human glioma cells (MO54). Opposite to the finding of Participant 6 (3.2.4.6, 4.2.4.6), no significant increase in hsp27 expression in endothelial cells was observed by Participant 9 who used a slightly different method (3.2.4.5, 4.2.4.5). Since the hsp27 expression was significantly increased in one laboratory, while this increase was near to significance in the other laboratory, this discrepancy seems to be neglectable. Furthermore, RF-EMF exposure (1800 MHz) at a SAR value of 1,4 W/kg did not affect gene expression in human lymphocytes (3.2.4.4, 4.2.4.4) and after RF-EMF exposure (900 MHz) at a SAR value of 2 W/kg only a few genes among several thousand tested with the micro-array system were found altered in two human immune cell lines (3.2.4.5, 4.2.4.5). Finally, RF-EMF exposure did not affect the expression and activity of the inducible nitric oxide synthase (iNOS) in nerve cells (3.2.4.5, 4.2.4.5).

The outcome of experiments following the genomics and proteomic approach may essentially depend on the cell system investigated and the RF-EMF signal used. Of course, the question remains as to whether or not these alterations in gene and protein expression are within the normal physiological range and if that is the case, they are without any biological relevance.

5.0 CONCLUSIONS

5.1 Conclusions based on the findings obtained in ELF-EMF research

5.1.1 Human fibroblasts, human lymphocytes, human monocytes, human melanocytes, human muscle cells and granulosa cells of rats (Participant 3)

These are the conclusions that Participant 3 draws from their findings:

1. The data strongly indicate a clastogenic potential of intermittent electromagnetic fields, which may lead to considerable chromosomal damage in dividing cells. However, the induced DNA damage did not persist in form of stable translocations.
2. The induced DNA damage was not based on thermal effects and arouses consideration about environmental safety limits for ELF-EMF exposure.
3. The effects were clearly more pronounced in cells from older donors, which could point to an age-related decrease of DNA repair efficiency of ELF-EMF induced DNA strand breaks.
4. In addition, three responder and three non-responder cell types could be identified, which could in part explain different results in reaction to ELF-EMF reported in the literature so far.
5. Fibroblasts from a donor with the genetically DNA repair defect Ataxia Telangiectasia had a more than two fold increase rate of ELF-EMF induced DNA breaks.
6. Between 3 and 550 Hz the largest DNA breaking effects were seen at 16.66 and 50 Hz, the most commonly used frequencies of alternating current in Europe.
7. Taken together, the results suggest that the observed effects of EMF exposure are caused by indirect mechanisms and are not inflicted due to changes in mitochondrial membrane potential.

5.1.2 Human neuroblastoma cell line NB69 and human hepatocarcinoma cell line HepG2 (Participant 5)

Our present results confirm preliminary observations that a 42- or 63-hour exposure to 50 Hz, sine wave MF at 10 or 100 μT (3 hours on/3 h off exposure cycle) can induce changes in the cell growth of NB69 human neuroblastoma cells. The data indicate that such an effect is exerted through an increase in cell proliferation, as revealed by BrdU-incorporation and flow cytometry.

1. In contrast, a 50-Hz MF at 2000 μT magnetic flux density, 5 min on/30 min off exposure cycle, did not affect significantly cell growth on the NB69 line. Thus, our cells were not responsive to these exposure parameters, which have been reported to be effective on differentiating neural embryonic stem cells (Participant 4). Additional experiments exposing NB69 cells to a 100 μT field in a 5 min on/30 min off cycle showed no significant responses. This indicates that the exposure cycle is crucial to eliciting a detectable cellular response.
2. In the NB69 line, the results on PCNA labelling show that at day 6 post-plating the percent of PCNA-positive cells in samples exposed to a 50-Hz, 100-μT field is significantly increased when compared to controls. Actually, the percent of PCNA positive cells significantly decreases in controls between the days 5 and 6 post-plating, whereas such a decrease did not occur in exposed cells. The results suggest that the MF could impair the normal cell cycle regulation through alterations in the late G1 and S-phases.
3. We have also investigated the response of a different human cancer line, the HepG2 human hepatocarcinoma cell line (data not shown), with a growth pattern different from that of the NB69 line. Fifty-Hertz magnetic fields at 10 or 100 μT elicited similar responses in both cell lines, consisting of significant increase in the number of cells at days 5 postplating. In HepG2, the melatonin, at a 10 nM concentration, inhibited the growth-promoting effect induced by the field (Cid et al., 11th International Congress of IRPA, 2004). In the HepG2 line, the growth effect became even stronger when the exposure was maintained until day 7 post-plating, whereas in the NB69 line, an equivalent extension of the exposure period results in a loss of the effect. The differential responses in both cell lines could be due to the fact that, in control conditions, NB69 cultures become saturated at day 7 post-plating and, consequently, their capability to respond to any stimulus is strongly impaired.

4. A 50-Hz, sine wave MF at 100-μT (3h on/3h off exposure cycle) induces a significant reduction in the spontaneous apoptosis of the human neuroblastoma cell line NB69. This response was associated to an increase in the total number of cells. The data suggest that both responses are a consequence of an effect of the field on cell cycle regulation.
5. In NB69 cells, a 50-Hz, sine wave MF at 100-μT (3h on/3h off exposure cycle) alters the activation of the phosphorylated cyclic adenosine monophosphate response-element binding protein (p-CREB) in a time-dependent manner. The results suggest that the activation of p-CREB is involved in the above described effects of this field on cell growth/apoptosis.

5.1.3 Human lymphocytes (Participant 8)

On the whole, data obtained indicate no response of human PBMCs to ELF-EMF exposure. Thus the conclusions are that ELF-EMF do not affect proliferation and cell activation, two fundamental phases of lymphocyte function. Since previous works indicated that pulsed ELF-EMF may interfere with human lymphocyte functionality (Cossarizza et al. 1989a/b, 1991, 1993), future experiments could be addressed to investigate the role of pulsed signal in biological systems in comparison with the negative results obtained with A.C. 50Hz ELF-EMF.

5.1.4 Mouse embryonic stem cells (Participant 4)

1. ELF-EMF signals at a high flux density are capable to transiently increase transcript levels of the regulatory genes egr-1, p21 and c-jun in ES cells deficient for the tumour suppressor p53.
2. The intermittency scheme of the ELF-EMF signals may play a critical role for changes in transcript levels of some regulatory genes.
3. The genetic constitution of pluripotent embryonic stem cells determined by loss of p53 function can influence ELF-EMF-related cellular responses, whereas wild-type cells are insensitive. It remains to be elucidated, whether ELF-EMF-induced changes of expression levels of regulatory genes may be compensated or normalized, or would result in sustained biological effects in vivo.
4. ELF-EMF exposure of ES-derived neural progenitor cells may influence transcript levels of genes of the bcl-2 family and the p53-responsive growth arrest and DNA damage inducible gene GADD45. This finding is an indication that ELF-EMF may affect, at least transiently, fundamental cellular processes including programmed cell death and cell cycle regulation.
5. Alkaline and neutral Comet assay failed to demonstrate a clear effect on the induction of single- and double-strand DNA breaks after ELF-EMF exposure of ES cell derived neural progenitors.

5.1.5 Experiments with embryonic stem cells of mice during cardiac differentiation (Participant 8)

In the ES cell model (GTR 1), ELF-EMF afforded a consistent increase in the expression of genes tightly involved in coaxing ES cells to the cardiac lineage. As shown by in vitro run-off analyses, ELF-EMF affected the transcriptional machine of ES cells. These responses led to the expression of cardiac specific genes and ultimately ensued into a high-throughput of cardiogenesis, as shown by the increase in the number of spontaneously beating colonies in ELF-EMF-exposed cells. Failure of EMF to affect the transcription of a gene promoting skeletal muscle determination and the faint effect on neuronal specification seem to exclude a generalized activation of repressed genes and suggests that coupling of MF with GATA-4, Nkx-2.5 and prodynorphin gene expression may represent a mechanism pertaining to ES cell cardiogenesis. This work represents, in our opinion, a first step toward an extensive investigation concerning the influence of EMF on the expression of a sequence of genes specifically involved in cell differentiation, and in particular the differentiation into a cardiac phenotype, using genomic and post-genomic techniques.

5.1.6 Experiments with the human neuroblastoma cell line SY5Y (Participant 11)

The results clearly demonstrate that, under the discussed exposure conditions, the expression of major components of the cholinergic and catecholaminergic systems is unresponsive to environmental exposure to ELF-EMF.

5.1.7 *Xenopus laevis* oocytes, human fibroblasts and granulosa cells of rats (GFSHR-17 cell line) (Participant 7)

1. For the applied three exposure protocols (50-Hz powerline, 1.0 mT or 2.3 mT continuously applied for 16 h; 50-Hz powerline, 1.0 mT and 2.3 mT intermittently (on/off: 5 min/10 min) applied for 16h the data indicate that the expression level as well as the voltage dependent gating of rCx46-connexons is not significantly affected. Since we could previously show that protein kinase C dependent phosphorylation processes affect the voltage-dependent gating of rCx46-connexons (Ngezahayo et al. 1998), a significant interaction of ELF-EMF on proteinphosphorylation can be neglected. The formation of cell-to-cell channels composed of two rCx46-hemi-channels, respectively, between a pair of mechanically contacting oocytes indicates an effect of ELF-EMF exposure. Exposure virtually suppresses the formation of cell-to-cell channels, but the effect is not significant on the level of three experiments analysed so far. The known $[Ca^{2+}]_o$-dependent gating property of hemi-channels appears not to be influenced by ELF-EMF exposure.
2. Continuous ELF-EMF exposure at high flux intensity 2.3 mT for 30 min did not significantly influence gap junctional coupling (cell-to-cell channels) of cultured pairs of rat granulosa cells as explored by the double whole cell patch-clamp technique.
3. The presented data indicate that intermittent exposure (5 min on / 10 min off) to ELF-EMF (50 Hz, 1 mT) neither generates a long lasting effect on the time course of $[Ca^{2+}]_i$ in cultured fibroblasts nor granulosa cells. This finding appears to be independent of an exposure for 5 to 18h. The corresponding observation of Ivancsits et al. (2003b), of a time dependent increase/decrease of DNA strand breaks with a maximum at about 15h, therefore seems not to be reflected in a corresponding long lasting change of $[Ca^{2+}]_i$. It is interesting to note that such a long lasting effect is also not found for the mitochondrial potential of fibroblasts. ELF-EMF exposure followed by exposure to further stressors, like 200 µM H_2O_2 or 30 mM KCl, also caused no significant change of $[Ca^{2+}]_i$.
4. Exposure experiments show no significant influence on volume regulatory mechanisms of granulosa cells. Further studies of intracellular signal transduction pathways should allow to understand the unsolved question whether significant effects of ELF-EMF on the genomic level are reflected on the cellular level. At present significant changes of cellular properties could not be derived from the analysed cellular parameters.
5. ELF-EMF exposure of cultured granulosa cells shows a significant time dependent increase of double DNA strand breaks with a maximum at about 18 h as observed by the neutral comet assay. This time dependence was also observed at 8 Hz, 16.66 Hz, 30 Hz, 50 Hz and 300 Hz. Therefore, it appears likely that for the chosen ELF-EMF exposure protocol the observed increase of double DNA strand breaks is not frequency dependent. But, the results of the alkaline comet assay indicate a frequency dependent effect of ELF-EMF exposure on the sum of double and single DNA strand breaks. The data obtained for the granulosa cells, after ELF-EMF exposure at 50 Hz, by the alkaline comet assay are comparable with those obtained by participant 3.

5.1.8 Effects of ELF-EMF on gene expression in human cells analysed with the cDNA array (Participant 12)

The gene expression analyses presented here make it very likely that EMFs – RF-EMF and ELF-EMF – can change gene expression in human cells. Although the in vitro studies do not allow any conclusions concerning health risk, the results are an important pre-requisite for further experiments to elucidate the detailed molecular changes in a cell, caused by EMFs.

The most obvious changes have been detected in the expression of genes involved in ribosomal biogenesis and energy metabolism. If the effects are momentary or lead to more dramatic changes like increase of cell proliferation has to be further investigated by molecular assays. The same is true for first ideas how the signalling after EMF exposure could work, referring to the bio-statistic analysis: The Ca-pathway (PIP3, PKC, ERK MAP and other pathways might be involved, but this is not obvious after our analysis) might be involved in regulation after EMF exposure. The actin cytoskeleton (e.g. stress fibers) and ECM possibly is down-regulated, which might lead to dedifferentiation of cells, again important for growth and proliferation of cells. The actin cytoskeleton behaves different in different cell types (adhesive, non-adhesive cells, cell migration etc.), and therefore also has to be investigated with the help of more specific assays.

5.1.9 Summary (Participant 1)

The ELF-EMF data obtained in the course of the REFLEX project allow the following conclusion:

1. ELF-EMF had genotoxic effects on primary cell cultures of human fibroblasts and on other cell lines. These observations were made in two laboratories within the REFLEX consortium (Participants 3 and 7) and confirmed by two other laboratories from outside the REFLEX project. ELF-EMF generated DNA strand breaks at a significant level at a flux density as low as 35 μT. A strong positive correlation was observed between both the intensity and duration of exposure to ELF-EMF and the increase in single and double strand DNA breaks and micronuclei frequencies. Surprisingly this genotoxic effect was only found when cells were exposed to intermittent ELF-EMF, but not to continuous exposure. Responsiveness of fibroblast to ELF-EMF increased with the age of the donor and in the presence of specific genetic repair defects. The effect also differed among the other types of cells examined. In particular, lymphocytes from adult donors were not responsive. Chromosomal aberrations were also observed after ELF-EMF exposure of human fibroblasts.
2. ELF-EMF at a flux density of 10 and 100 μT increased the proliferation rate of neuroblastoma cells (Participant 5) and at a flux density of 0.8 mT it enhanced the differentiation of mouse stem cells into cardiomyocytes (Participant 8). In contrast to these results, no clear-cut and unequivocal effects of ELF-EMF on DNA synthesis, cell cycle, cell differentiation, cell proliferation and apoptosis were found in the many other cell systems under investigation.
3. Elf-EMF inhibited the spontaneous apoptosis in neuroblastoma cells which was followed by an increase of the proliferation rate, when the cells were exposed for 63 hours to ELF-EMF at a flux density of 50 or 100 μT (Participant 5). In contrst to these results, no clear-cut and unequivoval effects of ELF-EMF on the apoptotic process were found in the many other cell systems under investigation.
4. ELF-EMF at a flux density of about 2 mT up-regulated the expression of early genes, such as p21, c-jun and egr-1, in p53- deficient mouse embryonic stem cells, but not in healthy wild-type cells (Participant 4) and, in addition, may affect the expression of genes and proteins in a variety of other cell systems. The results of the whole genome cDNA micro-array and proteomic analyses indicate that EMF may activate several groups of genes that play a role in cell division, cell proliferation and cell differentiation (Participant 12).

Taken together, the results of the REFLEX project were exclusively obtained in in vitro studies and are, therefore, not suitable for the conclusion that ELF-EMF exposure below the presently valid safety limits causes a risk to the health of people. They move, however, such an assumption nearer into the range of the possible. Furthermore, there exists no justification anymore to claim, that we are not aware of any pathophysiological mechanisms which could be the basis for the development of functional disturbances and any kind of chronic diseases in animal and man.

5.2 Conclusions based on the findings obtained in RF-EMF research

5.2.1 Human promyelocytic cell line HL-60 (Participant 2)

1. Different SAR levels have been examined with respect to the effect on comet formation and micronuclei induction in HL 60-cells. Comparing RF-EMF exposure (1800 MHz, continuous wave, 24h) at SAR levels ranging from 0.2 W/kg to 3.0 W/kg indicate that both effects appear to be energy dependent. Whereas at SAR of 0.2 W/kg and 1.0 W/kg both, comet formation and micronucleus frequency, were not significantly different from that observed in sham-exposed control cells, comet formation as well as micronucleus frequency were significantly increased at SAR of 1.3 W/kg, 1.6 W/kg and 2.0 W/kg. The maximal effect was observed at a SAR of 1.3 W/kg. At higher SAR levels from 2.0 to 3.0 W/kg micronucleus frequencies and comet formation were less expressed as compared to the effect noted at a SAR of 1.3 W/kg.
2. In order to extend the statistical basis of evaluation average numbers of micronuclei (micronuclei per 1000 BNC) in different experimental groups were calculated comparing cells exposed either at (i) all SAR tested (0.2 W/kg, 1.0 W/kg, 1.3 W/kg, 1.6 W/kg, 2.0 W/kg, 3.0 W/kg, (ii) higher SAR (1.0 W/kg, 1.3 W/kg, 1.6 W/kg, 2.0 W/kg, 3.0 W/kg) or (iii) lower SAR of 0.2 W/kg or 1.0 W/kg. In both groups (i) and (ii) the number of micronuclei was increased at a significant level ($p<0.001$) as

compared to sham-exposed controls, while in group (iii) micronuclei numbers per 1000 BNC were not significantly different from that observed in sham-exposed controls.

3. Likewise, in order to extend the statistical basis of evaluation average values of Olive Tail Moments as a measure of comet formation were calculated in different experimental groups comparing cells exposed either at (i) all SAR tested (0.2 W/kg, 1.0 W/kg, 1.3 W/kg, 1.6 W/kg, 2.0 W/kg, 3.0 W/kg, (ii) higher SAR (1.0 W/kg, 1.3 W/kg, 1.6 W/kg, 2.0 W/kg, 3.0 W/kg) or (iii) lower SAR of 0.2 W/kg or 1.0 W/kg. In both groups (i) and (ii) the comet formation was increased at a significant level (group (i) $p<0.01$; group (ii) $p<0.001$) as compared to sham-exposed controls, while in group (iii) comet formation was not significantly different from that observed in sham-exposed controls.
4. Experiments on the influence of the duration of exposure showed that short exposure period (6h) caused no (MN) or less (Comet) pronounced effects on micronuclei induction and comet formation as compared to longer exposure periods of 24h. While micronucleus frequencies were further increased after exposure for 72h, comet formation after 72h of exposure was less expressed as compared to 24h exposure.
5. Experiments on the influence of RF-signals showed that at a SAR level of 1.3 W/kg all RF-signals tested, i.e. continuous wave (C.W.), C.W. 5 min on/10 min off, GSM-217 Hz, and GSM-Talk exhibited similar effects on micronuclei induction and on comet formation.
6. By applying sequential approaches for the detection of reactive oxygen species (ROS) in HL-60 cells, an increase in the intracellular generation of free radicals accompanying RF-EMF exposure could be clearly demonstrated by flow cytometric detection of the oxidized nucleotide 8-oxoguanosine (oxy-DNA assay) and the fluorescent Rhodamine 123 (DHR 123 assay), respectively.
7. RF-EMF exposure (1800 MHz, 1.3 W/kg, 24h) had no effect on the cellular doubling time and the activity of the enzyme thymidine kinase of HL 60 cells, indicating that RF-EMF exposure does not influence cellular growth rates.
8. RF-EMF exposure (1800 MHz, 1.3 W/kg, 24h) did not induce apoptosis in HL-60 cells.
9. Within the investigated SAR energy ranges RF-EMF under the in-vitro conditions used are genotoxic in HL-60 cells without affecting cell-cycle distribution cell proliferation or cell progression.
10. The partial-body SAR for any 10-gram tissue like for example the head as exposed region to mobile phone electromagnetic fields should not exceed 2 W/kg according to the Radio-Radiation Protection Guidelines. Notably, our findings on genotoxic effects of RF-fields in HL-60 cells have been shown for SAR levels below these acceptable partial-body SAR levels.
11. These results on genotoxicity in the HL-60 cell line cannot be transferred automatically to other cells, especially to primary cells, and definitely not to whole organism.
12. Clear differences in protein expression have been shown for RF-exposed HL-60 cells as compared to control and sham-exposed cells. This indicates that, as also demonstrated by genetic profiling, RF-EMF exposure has an influence on as well the transcriptional as the translational level in these cells. Clarification of changes in protein expression with respect to functional analysis will help to understand molecular pathomechanisms.

5.2.2 Human fibroblasts and granulosa cells of rats (Participant 3)

Our results imply a genotoxic action of RF-EMFs below proposed radiation safety levels.

1. RF-EMFs were able to induce DNA single and double strand breaks in human fibroblasts and SV40 transformed rat granulosa cells. In contrast to ELF-EMF, genotoxic effects were also observed at continuous exposure.
2. In addition, the decline of DNA strand break levels at elongate exposure (16-24 h), which was found in ELF-EMF exposed cells, could not be demonstrated after RF-EMF exposure. These results could point to differences in mechanisms between the genotoxic action of RF and ELF-EMF
3. Differences in genotoxic effects between different cell types after EMF exposure could be found in RF as well as in ELF-EMF exposed cells.
4. RF-EMF exposure of human fibroblasts was able to induce higher incidences of chromosome aberrations than which was found in ELF-EMF exposed cells.
5. No effects of RF-EMF exposure on mitochondrial membrane potential could be observed. These findings are in accordance with the results obtained with ELF-EMF.

5.2.3 Human lymphocytes and thymocytes (Participant 8)

On the whole, the data obtained indicate a very low response of human PBMCs and no response of thymocytes to RF-EMF exposure. Concerning PBMCs, some results suggest a possible effect on the number of CD95 surface molecules in stimulated T lymphocytes from aged donors. Moreover, other results seem to indicate a greater susceptibility to RF of monocytes with respect to lymphocytes, as demonstrated by a decrease of IL-1 b cytokine, specifically produced by monocytes, in RF-exposed cultures. Future work could be addressed to analyse further effects on these type of human cells.

5.2.4 Human neuroblastoma cell line NB69 and neural stem cells (Participant 5)

1. When administered alone, the exposure to the GSM-Basic signal at a 2W/kg SAR induced a decrease in the number of cells expressing the fibroblast growth factor receptor-1 (FGFR-1), both in NB69 cells and NSC, without affecting significantly the number of cells expressing receptors R2 and R3. The magnitude of the effect on R1 was equivalent to that induced by 20 µg/ml bFGF. Since the GSM-Basic treatment did not affect significantly the total cell number or the cell viability, the above data indicate that RF-induced effect in FGFR-1 is not due to a reduction in the number of cells, but to a loss of the cellular expression of receptor-1.
2. The results also indicate that the exposure to GSM 1800-CW signals at a 2 W/kg SAR induced effects on the expression of FGFR-1 equivalent to those described above for the GSM 1800-Basic signal. No significant effects on the expression of FGFR-1 were observed after exposure to GSM 1800-Talk and DTX signals at 2 W/kg SAR and 1 W/kg SAR, respectively. The data obtained with the different GSM-signals suggest that the cellular response is not dependent on the tested low-frequency modulation.
3. The exposure to the GSM-basic signal induces specific, morphological changes in oligodendrocytes and astrocytes derived from neural stem cells, at day 9 post-plating. These results are indicative that GSM-basic radiation at SAR = 2 W/kg can promote differentiation in NSC. The effect would be exerted through short-term changes in the expression of FGF receptor-1. In contrast, the GSM-Basic signal does not influence cytodifferentiation in NB69 cells or in the neuronal progeny of NSC, as revealed with anti-beta-tubulin antibody.

5.2.5 Brain cells of different origin and human monocytes (Participant 9)

1. Our results strongly suggest that the spontaneous apoptotic process is not a biological target for GSM mobile telephony-related signals. This was shown in different primary cells and cell lines from both nerve and immune systems.
2. Based on the expression and activity of inducible nitric oxide synthase (NOS2) in an astrocytic cell line, GSM-like signals did not “activate” the inflammatory process in nerve cells.
3. No evidence was found of effects of GSM-like signals on heat shock proteins in different mammalian nerve cells. Replication of the previously reported increase in hsp27 expression in a human endothelial cell line after exposure failed.
4. Based on the whole data set, our conclusion is that exposure to low-level GSM-900 signal is unlikely to lead to neurodegeneration or to favour tumour development via pathways involving apoptosis, nitric oxide or heat shock proteins.

5.2.6 Mouse embryonic stem cells (Participant 4)

1. Our present data suggest that currently applied GSM radiation levels under certain circumstances might induce biological effects, at least in cells generated from embryonic stem cells in vitro.
2. The genetic constitution of pluripotent embryonic stem cells determined by loss of p53 function influences RF-EMF-related cellular responses at the level of gene expression, whereas wild-type cells are insensitive. It remains to be elucidated, whether RF-EMF-induced changes of mRNA levels of regulatory genes may be compensated or normalized, or would result in sustained biological effects in vivo.
3. RF-EMF exposure of ES-derived neural precursor cells influences the bcl-2 mediated anti-apoptotic pathway, affects the growth arrest and DNA damage inducible gene GADD45 and the neuronal differentiation by inhibition of Nurr1.

4. Short exposure to RF-EMF could induce double-strand DNA breaks in ES-derived neural progenitor cells (as measured by the neutral Comet assay).

5.2.7 Human the endothelial cell lines EA.hy926 and EA.hy926v1 (Participant 6)

1. RF-EMF appears to be recognized by the cells as an external stress factor because it in response to exposure phosphorylation status of several hundreds proteins was altered either up or down; identification of these proteins will be done in due time.
2. RF-EMF appears to be a weak inducer of cellular stress response because it increases expression and phosphorylation of heat shock protein-27 (hsp27) - a known marker of cellular stress response.
3. RF-EMF induced phosphorylation of Hsp27 appears to be regulated by the activation of up-stream stress kinase p38MAPK.
4. RF-EMF-induced hsp27 activation appears to affect down-stream physiological processes in cell - stabilization of F-actin stress fibers what, in turn, alters cell size and shape (causes rounding-up of cells).
5. Using cDNA Expression Arrays and protein separation by 2-dimensional electrophoresis followed by mass spectrometric identification of individual proteins we have determined that the cellular skeleton appears to be a target of RF-EMF exposure as changes in gene/protein expression of some dozen cytoskeletal proteins were induced by RF-EMF exposure.
6. RF-EMF-induced phosphorylation of hsp27 is followed by translocation of hsp27 to cell nucleus where it appears to interfere with the gene expression processes.
7. RF-EMF causes changes in the expression of several tens of genes and proteins as determined by high-throughput screening technologies - cDNA Expression Arrays and protein separation by 2-dimensional electrophoresis followed by mass spectrometric identification of individual proteins.
8. RF-EMF-induced changes in gene and protein expression appear to be dependent on the cell genotype/phenotype what suggests that some cell types might be more and some less responsive to RF-EMF exposure.
9. RF-EMF induced changes in protein expression appear to be modulation dependent since RF-EMF exposure caused changes whereas CW-EMF did not.
10. the ability of RF-EMF to induce cellular stress response indicates that cells recognize this radiation in spite of it low energy but the induction of stress response per se can not be considered as any indicator of potential health risk.
11. We have practically demonstrated that the use of high-throughput screening methods of transcriptomics and proteomics is useful tool in determining the potential targets of RF-EMF exposure in cells.

5.2.8 Effects of RF-EMF on gene expression in human cells analysed with the cDNA array (Participant 12)

The gene expression analyses presented here make it very likely that EMFs – RF-EMF and ELF-EMF – can change gene expression in human cells. Although the in vitro studies do not allow any conclusions concerning health risk, the results are an important pre-requisite for further experiments to elucidate the detailed molecular changes in a cell, caused by EMFs.

The most obvious changes have been detected in the expression of genes involved in ribosomal biogenesis and energy metabolism. If the effects are momentary or lead to more dramatic changes like increase of cell proliferation has to be further investigated by molecular assays. The same is true for first ideas how the signalling after EMF exposure could work, referring to the bio-statistic analysis: The Ca-pathway (PIP3, PKC, ERK MAP and other pathways might be involved, but this is not obvious after our analysis) might be involved in regulation after EMF exposure. The actin cytoskeleton (e.g. stress fibers) and ECM possibly is down-regulated, which might lead to dedifferentiation of cells, again important for growth and proliferation of cells. The actin cytoskeleton behaves different in different cell types (adhesive, non-adhesive cells, cell migration etc.), and therefore also has to be investigated with the help of more specific assays.

5.2.9 Summary (Participant 1)

The RF-EMF data obtained in the course of the REFLEX project allow the following conclusion:

1. RF-EMF produced genotoxic effects in fibroblasts, HL-60 cells, granulosa cells of rats and neural progenitor cells derived from mouse embryonic stem cells (Paticipants 2, 3 and 4). Cells responded to RF-EMF exposure between SAR levels of 0.3 and 2 W/kg with a significant increase in single and double strand DNA breaks and in micronuclei frequency (Participants 2 and 3). Chromosomal aberrations in fibroblasts were also observed after RF-EMF exposure (Participant 3). In HL-60 cells an increase in the intracellular generation of free radicals accompanying RF-EMF exposure could clearly be demonstrated (Participant 2).
2. No clear-cut and unequivocal effects of RF-EMF on DNA synthesis, cell cycle, cell proliferation, cell differentiation and immune cell functionality were found in the cell systems under investigation. (Participants 2, 3, 4, 5, 6, 8). There is some indication that RF-EMF may affect the growth arrest and DNA damage inducible gene GADD45 and the neuronal differentiation by inhibition of Nurr1 in neural progenitor cells (Participant 4).
3. No clear-cut and unequivocal effects of RF-EMF on apoptosis were fond in the cell systems under investigation was observed (Participants 2, 3, 4, 5, 6, 8 and 9). There is some indication that RF-EMF may have some influence on the bcl-2 mediated anti-apoptotic pathway in neural progenotor cells (Participant 4) and on the the p38MAPK/hsp27 stress response pathway in endothelial cells of human origin (Participant 6) which may in turn exert an inhibitory effect on apoptosis.
4. RF-EMF at a SAR of 1.5 W/kg down-regulated the expression of neuronal genes in neuronal precursor cells and up-regulated the expression of early genes in p53-deficient embryonic stem cells, but not in wild-type cells (Participant 4). Proteomic analyses on human endothelial cell lines showed that exposure to RF-EMF changed the expression and phosphorylation of numerous, largely unidentified proteins. Among these proteins is the heat shock protein hsp27, a marker for cellular stress responses (Participant 6). The results of the whole genome cDNA micro-array and proteomic analyses indicated that EMF may activate several groups of genes that play a role in cell division, cell proliferation and cell differentiation (Participants 2, 6 and 12).

Taken together, the results of the REFLEX project were exclusively obtained in in vitro studies and are, therefore, not suitable for the conclusion that RF-EMF exposure below the presently valid safety limits causes a risk to the health of people. They move, however, such an assumption nearer into the range of the possible. Furthermore, there exists no justification anymore to claim, that we are not aware of any pathophysiological mechanisms which could be the basis for the development of functional disturbances and any kind of chronic diseases in animal and man.

6.0 EXPLOITATION AND DISSEMINATION OF RESULTS

6.1 Coordination (Participant 1)

a. Scientific publications, meetings, interviews, and round tables

Adlkofer F et al.: Brochure presenting the REFLEX project, June 2001 (*for distribution*)

Adlkofer F et al.: Oral presentation of results. Bundesamt für Strahlenschutz, Salzgitter/Germany, June 21-22, 2001, p. 18-19

Adlkofer F et al.: Oral presentation of the project. EBEA, Helsinki/Finland, Sep 6-8, 2001. Proceedings, p. 54-56, 269-270

Adlkofer F et al.: Oral presentation of results. EU/Japan/Korea/US workshop on EMF, mobile telephony and health. Brussels/Belgium, Oct 29-30, 2001, Proceedings, p. *(not numbered)*

Adlkofer F et al.: Oral presentation of results. Institut für Zoologie, Technical University of Dresden/ Germany, April 2, 2002

Adlkofer F et al.: Oral presentation of results. COST281/EBEA Forum, Rome/Italy, May 2-5, 2002

Adlkofer F et al.: Oral presentation of the project.. 24th BEMS Meeting, Quebec City/Canada, June 23-27, 2002, Proceedings, p. 91-92, 95, 98-100

Adlkofer F et al.: Oral presentation of the project and of results. PIERS, Cambridge/Mass/USA., July 1-5, 2002. Proceedings, p. 498

Adlkofer F et al.: Oral presentation of results. Cursos de Verano Universidad de Malaga, Ronda/Spain, July 22-26, 2002

Adlkofer F et al.: Oral presentation of the project. Biological Effects of EMF, Rhodes/Greece, Oct 7-11, 2002. Proceedings, p. 514-522

Adlkofer F, Rüdiger HW, Wobus AM: DNA-Doppelstrangbrüche bei intermittierender Exposition. Diskussionsbeitrag. Deutsches Ärzteblatt, Nov 15, 2002, p. 3114-3115

Adlkofer F et al.: Oral presentation of results. Elektromagnetische Felder in der Umwelt, Umwelt-ministerium Nordrhein-Westfalen, Dortmund/Germany, Nov 28, 2002

Adlkofer F et al.: Oral presentation of results. The EMF Biological Research Trust, London/UK, Jan 16, 2003

Adlkofer F et al.: Oral presentation of results. WHO EMF Project, Research Coordination Meeting, Geneva/Switzerland, June 12-13, 2003

Adlkofer F et al.: Oral presentation of the project. 25th BEMS Meeting, Maui/Hawaii, June 22-27, 2003. Proceedings, p. 127, 135 - 136

Adlkofer F: Interview. Television feature on “Elektrosmog”, ARD Germany, Aug 7, 2003

Adlkofer F et al.: Oral presentation of results. 3rd Int. EMF Seminar, Guilin/China, Oct 13-17, 2003. Proceedings, p. 23 - 24

Adlkofer F et al.: Oral presentation of results. O_2 Telecommunication Company, München/Germany, Oct 22, 2003

Adlkofer F et al.: Oral presentation of results. Die Umwelt-Akademie e.V., München/Germany, Dec 5, 2003

Adlkofer F: Interview. Bayer. landwirtschaftliches Wochenblatt, Heft 5, Jan 2004, p. 48

Adlkofer F: Interview. life + sciences, Heft 1, Feb - April 2004, p. 30-31

Adlkofer F et al.: Oral presentation of results. Bündnis 90/Die Grünen, Bavarian State Parliament, München/Germany, April 2, 2004

Adlkofer F: Round Table Discussion. Bayer. Akademie der Wissenschaften, München/Germany,

April 29, 2004

Adlkofer F et al.: Oral presentation of results. EMF-NET, Brussels/Belgium, April 30, 2004

Adlkofer F et al.: Oral presentation of results. BUND, 3. Rheinland-Pfälzisch-Hessisches Mobilfunk-symposium, Mainz, June 12, 2004. Tagungsband, p. 33 - 49

b. Posters

Adlkofer F et al.: Poster presentation. EBEA, Helsinki/Finland, Sep 6-8, 2001

Adlkofer F et al.: Poster presentation. An Environment for Better Health Conference, Arhus/Denmark, May 8-11, 2003

6.2 Experiments with the human promyelocytic cell line HL-60 (Participant 2)

Research performed is basic research with relevance for life science and techniques, respectively. The results obtained by participant 2 have been subsequently actualised and reported in the usual scientific manner. These reports included confidential Annual Reports as progress reports to the European Commission (1st, 2nd, 3rd Annual Report) and public presentations at the following scientific meetings: BEMS 2002 (Radiofrequency EMF and DNA strand breaks), BEMS 2002 (RF-EMF genotoxic effects), PIERS 2002 (1800 MHz radiofrequency exposition of human HL-60 cells induces DNA strand breaks as measured by the alkaline comet assay), BEMS 2003 (Genotoxic effects of RF-EMF on cultured cells in vitro), Deutscher Ärztekongress 2002 (Workshop in German), Deutscher Ärztekongress 2003 (Workshop in German). Peer-reviewed publications have been prepared and will be submitted after the end of the project.

a. Scientific papers

in preparation:

Schlatterer K., Gminski R., Tauber R., Fitzner R. (2004) Radiofrequency (1800 MHz) electromagnetic fields cause DNA strand breaks and micronuclei formation in HL-60 human promyelocytic cells.

b. Scientific meetings

Fitzner R, Gminski R, Schlatterer K (2004) 1800 MHz radiofrequency electromagnetic fields cause energy-dependent genotoxic effects in human promyelocytic HL-60 cells. Session 14: Non thermal biological effects of EM Fields used for mobile communication. Progress In Electromagnetic Research Symposium (PIERS 2004), Pisa, March 28-31, 2004 (oral presentation)

Fitzner R (2004) In-vitro-Untersuchungen an HL-60-Zellen – Einfluss niederfrequenter Magnetfelder (50 Hz; 162/3 Hz). Umweltmedizin – Elektromagnetfelder, Zellen, Gesundheit, 53. Deutscher Ärztekongress, 3.-5. Mai, Berlin 2004 (oral presentation)

Schlatterer-Krauter, K (2004) Einfluss hochfrequenter Elektromagnetfelder des Mobilfunks – Erbgut-veränderungen direkt oder indirekt verursacht. Umweltmedizin – Elektromagnetfelder, Zellen, Gesundheit, 53. Deutscher Ärztekongress, 3.-5. Mai, Berlin 2004 (oral presentation)

c. Posters

Fitzner R, Gminski R, Schlatterer K (2004) Radiofrequency electromagnetic fields (1800 MHz) induce elevated production of reactive oxygen species in human promyelocytic HL-60 cells. Poster presentation. Bioelectromagnetics Society 26th Annual Meeting, 20-24th June, Washington, 2004

6.3 Experiments with human fibroblasts, human lymphocytes, human monocytes, human melanocytes, human muscle cells and granulosa cells of rats (Participant 3)

a. Scientific papers

published:

Ivancsits S, Diem E, Rüdiger HW, Jahn O (2002) Induction of DNA strand breaks by intermittent exposure to extremely-low-frequency electromagnetic fields in human diploid fibroblasts. Mutation Res 519: 1-13

Ivancsits S, Diem E, Jahn O, Rüdiger HW (2003) Intermittent extremely low frequency electromagnetic fields cause DNA damage in a dose dependent way. Int Arch Occup Env Health 76: 431-436

Ivancsits S, Diem E, Jahn O , Rüdiger HW (2003) Age-related effects on induction of DNA strand breaks by intermittent exposure to electromagnetic fields. Mech Age Dev 124: 847-850

submitted:

Diem E, Jahn O, Rüdiger HW. Non-thermal DNA breakage by mobile phone radiation in human fibroblasts and transformed GFSH-R17 (rat granulosa) cells in vitro. Mutation Research

Ivancsits S, Diem E, Jahn O, Rüdiger HW. Chromosomal damage in human diploid fibroblasts by intermittent exposure to extremely low frequency electromagnetic fields. Mutation Research

Ivancsits S, Diem E, Jahn O, Rüdiger HW. Cell type specific genotoxic effects of intermittent extremely low frequency electromagnetic fields. Mutation Research

Ivancsits S, Diem E, Jahn O, Rüdiger HW. Intermittent exposure to extremely low frequency electromagnetic fields increases the genotoxic sensitivity to UV-light or mild thermal stress in cultured human fibroblasts. J Toxicol Envir Health

Pilger A, Ivancsits S, Diem E, Steffens M, Kolb HA, Rüdiger HW. No long-lasting effects of intermittent 50 Hz electromagnetic field on cytoplasmic free calcium and mitochondrial membrane potential in human diploid fibroblasts. Radiat Envir Biophysics

b. Scientific meetings

Wiener Forum Arbeitsmedizin, April 2000 Vienna,"Elektrosmog – Neue Untersuchungen zur gentoxischen Wirkung elektromagnetischer Felder" Oswald Jahn Oral presentation

26th International Conference of Occupational Health (ICOH), 27th August-1st September2000, Singapore, In vitro evaluation of genotoxic potential of low EMF of 50 Hz" Oswald Jahn, Eva Valic, Elisabeth Diem, Hugo W. Rüdiger Oral presentation

Tagung der Österreichischen Gesellschaft für Arbeitsmedizin (ÖGAM), 27-28th September 2002, Vienna. Sabine Ivancsits, Elisabeth Diem, Hugo W. Rüdiger and Oswald Jahn „Biologische Wirkung elektromagnetischer Felder" Oral presentation

Bioelectromagnetics Society 24th Annual Meeting, 23rd-26th June, Quebec, 2002

H.W. Rüdiger, S. Ivancsits, E. Diem, A. Pilger, F. Bersani, O. Jahn. „Genotoxic effects of extremely-low-frequency electromagnetic fields on human cells in vitro" Oral presentation

Gesellschaft für Umwelt und Mutationsforschung 20. Jahrestagung, 17-20th March 2003, Mainz. Sabine Ivancsits, Elisabeth Diem, Oswald Jahn and Hugo W. Rüdiger „Dosisabhängige Induktion von DNA-Strangbrüchen nach niederfrequenter elektromagnetischer Bestrahlung" Oral presentation

Bioelectromagnetics Society 25th Annual Meeting, 22-27th June, Maui, 2003

H.W. Rüdiger, S. Ivancsits, E. Diem, O. Jahn. „Genotoxic effects of extremely-low-frequency electromagnetic fields on human cells in vitro" Oral presentation

Tagung der Österreichischen Gesellschaft für Arbeitsmedizin (ÖGAM), 19-20th September 2003, St. Pölten.. Sabine Ivancsits, Elisabeth Diem, Oswald Jahn and Hugo W. Rüdiger „Induktion von chromosomalen Aberrationen durch niederfrequente elektromagnetische Felder" Oral presentation.

6th Congress of European Bioelectromagnetics Association (EBEA), November 13–15th 2003, Budapest. S. Ivancsits, E. Diem, O. Jahn, H.W. Rüdiger "In vitro genotoxic effects of extremely-low-frequency electromagnetic fields" Oral presentation

c. Poster

Tagung der Österreichischen Gesellschaft für Arbeitsmedizin (ÖGAM), 28-29th September 2001, Salzburg, Sabine Ivancsits, Elisabeth Diem, Hugo W. Rüdiger and Oswald Jahn „Gentoxische Wirkung

von elektromagnetischen Feldern“ Poster presentation

Conference on RF interactions with Humans: Mechanisms, Exposure and MedicalApplications, 27-28th February 2003, London. E. Diem, S. Ivancsits, H.W. Rüdiger “Non-thermal DNA breakage by mobile phone radiation in human fibroblasts and transformed GFSH-R17 (rat granulosa) cells” Poster presentation

Gesellschaft für Umwelt und Mutationsforschung 20. Jahrestagung,17-20th March 2003, Mainz. E. Diem, S. Ivancsits, H.W. Rüdiger “Non-thermal DNA breakage by mobile phone radiation in human fibroblasts and transformed GFSH-R17 (rat granulosa) cells” Poster presentation

4. Gemeinsame Jahrestagung der Österreichischen und Deutschen Gesellschaft für Arbeitsmedizin, April 22-24th 2004, Innsbruck. S. Ivancsits, E. Diem, O. Jahn, H.W. Rüdiger “Induktion von chromosomalen Schäden durch niederfrequente elektromagnetische Felder“ Poster presentation

4. Gemeinsame Jahrestagung der Österreichischen und Deutschen Gesellschaft für Arbeitsmedizin, April 22-24th 2004, Innsbruck. A. Pilger, S. Ivancsits, E. Diem, M. Steffens, H.A. Kolb, H.W. Rüdiger „Intermittierende Belastung mit 50 Hz ELF-EMF bewirkt keine Veränderungen des mitochondriellen Menbranpotentiales und freien Kalziums in humanen Fibroblasten“ Poster Presentation

6.4 Embryonic stem cells (Participant 4)

a. Scientific papers

published:

Jaroslaw Czyz, Kaomei Guan, Qinghua Zeng, Teodora Nikolova, Armin Meister, Frank Schönborn, Jürgen Schuderer, Niels Kuster, and Anna M. Wobus. High frequency electromagnetic fields affect gene expression levels in tumor suppressor p53-deficient embryonic stem cells. Bioelectromagnetics (2004) 25: 296-307.

Jaroslaw Czyz, Teodora Nikolova, Jürgen Schuderer, Niels Kuster, and Anna M. Wobus. Non-thermal effects of power-line magnetic fields (50 Hz) on gene expression levels of embryonic stem cells – the role of tumour suppressor p53. Mutation Research (2004) 557(1): 63-74.

submitted:

Teodora Nikolova, Jaroslaw Czyz, Alexandra Rolletschek, Przemyslaw Blyszczuk, Jürgen Schuderer, Niels Kuster, and Anna M. Wobus. Electromagnetic fields affect the transcript levels of apoptosis-related genes in embryonic stem cell-derived neural progenitor cells. Submitted to Environ Health Persp.

Part of our results were presented by Participant 8 of the REFLEX Project at the International BEMS 2003 Conference in June, 2003 in Maui, Hawaii.

6.5 Experiments with the human neuroblastoma cell line NB69 and neural stem cells (Participant 5)

For our REFLEX studies a specific software for the analysis of immunocytochemical images was developed in collaboration with Escuela Universitaria de Ingeniería Técnica Industrial (Erasmus-Socrates arrangement between Madrid-Belgium).

Part of the results has been presented in different meetings: BEMS, EBEA, and 2nd International Workshop on Biological Effects of EMF.

a. Scientific papers:

published:

Carlos Platero, Kristof Verbiest, Alejandro Úbeda, M-Angeles Trillo, Jaime Gosalvez, and Javier Bartolomé. Platform opened for the processing and management of biomedical images. XXI Jornadas of

Automática 1-7. ISBN: 84-699-3163-6 (2000)

Carlos Platero, M-Angeles Trillo and Alejandro Úbeda. Processing of biomedical images for the study of the potential influence of GSM electromagnetic radiation on neural stem cells. XXIII Jornadas of Automática 1-7. ISBN: 84-699-8916-2 (2002)

in preparation:

M-Angeles Trillo, M-Antonia Cid, M-Antonia Martinez, Vicente-J. Garcia, Alejandro Úbeda and Jocelyne Leal. Influence of 50 Hz magnetic fields on the proliferation and apoptosis of human neuroblastoma cells *in vitro"*.

M.A.Trillo, G. Alegría, M.A. Martínez, D. Reimers, E. Bazán, A. Úbeda, Jürgen Schuderer and J. Leal. Influence of RF fields (GSM signals, 1800 MHz) on the expression of FGFR1 by NB69 human neuroblastoma cell line and neural stem cells from rat embryonic nucleus striatum".

M.A. Trillo, M.A. Martínez, M.A. Cid A. Úbeda and J. Leal. 50 Hz sinus wave magnetic field at 100 μT activates phosphorilated cyclic adenosine monophosphate response-element binding protein (P-CREB) in NB69 human neuroblastoma cell line.

6.6 Human the endothelial cell lines EA.hy926 and EA.hy926v1 (Participant 6)

a. Scientific papers

published:

Leszczynski D, Joenväärä S, Reivinen R, Kuokka R. Non-thermal activation of hsp27/p38MAPK stress pathway by mobile phone radiation in human endothelial cells: Molecular mechanism for cancer- and blood-brain barrier-related effects. Differentiation 70, 2002, 120-129

Leszczynski D, Nylund R, Joenväärä S, Reivinen J. Applicability of Discovery Science-Approach to Determine Biological Effects of Mobile Phone Radiation. Proteomics 4, 2004, 426-431

Nylund R, Leszczynski D. Proteomics analysis of human endothelial cell line EA.hy926 after exposure to GSM 900 radiation. Proteomics, 4, 2004, 1359-1365

submitted:

Leszczynski D. Mobile phone radiation and blood-brain barrier: The available scientific evidence is insufficient to support or dismiss claims of an effect.

in preparation:

Nylund R, Griffin T, Maercker Ch, Schuderer J, Kuster N, Aebersold R, Leszczynski D. Effect of low-energy microwaves on protein expression in human endothelial cell line might be frequency modulation-dependent

Nylund R, Toivo T, Sihvonen AP, Jokela K, Schuderer J, Kuster N, Landry J, Leszczynski D. Mobile phone radiation-induced activation of cellular stress response induces cytophysiological effects.

Nylund R, Reivinen J, Leszczynski D. Cellular response to mobile phone radiation is proteome- and genotype-dependent

Leszczynski D. Induction of Cellular Stress Response by Mobile Phone Radiation: Possible mechanism behind the effects – a molecular biologists perspective. invited review for IEEE Transactions

Nylund R, Toivo T, Sihvonen AP, Schuderer J, Jokela K, Kuster N, Leszczynski D. Mobile phone radiation-induced Hsp27 stress response in human endothelial cell line EA.hy926 is a non-thermal effect.

b. Scientific meetings and reports for the media

invited lectures:

Harvard University, Boston, MA, USA, 15.11.2000, Proteomics: a novel approach to determine health effects of mobile phone radiation.

Centre for Immunology at St. Vincent's Hospital, Sydney, Australia, 11.02.2002, Possible Effects of Mobile Phones on Brain - Should We Be Afraid?

Telstra Laboratories, Melbourne, Australia, 12.02.2002, Mobile Phones and Health Risk: Why Do We Know So Little?

Department of Physics, Sydney University, Sydney, Australia, 15.02.2002, Mobile Phones, Cancer and Blood-Brain Barrier: A Possible Molecular Mechanism.

Zheijang University, School of Public Health, Hangzhou, China, 10.10.2003; Biological effects of mobile phone radiation.

Brooks AFB, San Antonio, TX, USA, 3.12.2003; Application of transcriptomics and proteomics in search for the potential health effects of EMF.

invited presentations at the conferences:

24th Annual Meeting of Bioelectromagnetics Society, Quebec City, Canada, 23-27.06.2002, Effect of mobile phone radiation on gene and protein expression.

27th General Assembly of the International Union of Radio Science (URSI), Maastricht, The Netherlands, 17-24.08.2002, Effect of GSM mobile phone radiation on blood-brain barrier: Use of proteomics approach to define the hypothetical molecular mechanism.

COST 281 Seminar "Subtle Temperature Effects of RF-EMF", 12-13.11.2002, London, UK, Indirect evidence of non-thermal biological effects induced by mobile phone radiation in vitro.

FGF & COST 281 Workshop on "Genetic and Cytogenetic Aspects of RF-Field Interaction", 24-27.11.2002, Löwenstein, Germany, Mobile phone radiation-induced gene expression might be cell genotype-dependent.

Proteomica Symposium, University of Madrid, 4-8.02.2003, Cordoba, Spain, Use of discovery science-approach to elucidate bio-effects of electromagnetic fields.

25th Annual Meeting of Bioelectromagnetics Society, Maui, HI, USA, 23-27.06.2003, Use of discovery science-approach to elucidate bio-effects of electromagnetic fields. (Plenary talk)

25th Annual Meeting of Bioelectromagnetics Society, Maui, HI, USA, 23-27.06.2003, Cellular response to mobile phone radiation appears to be cell genotype-dependent.

WHO & ICNIRP & China Health Ministry, 3rd International EMF Seminar in China: Electromagnetic Fields and Biological Effects, 14-17.10.2003, Guilin, China, Discovery science and mobile phone safety: a need for the new research approach. (Keynote talk)

FGF & COST281 Workshop "The Blood-Brain Barrier (BBB) - Can it be influenced by RF-field interactions?", 3-6.11.2003, Reisensburg, Germany, Mobile Phone Radiation and Blood-Brain Barrier: The available scientific evidence is insufficient to dismiss or to support claims of a health risk in humans.

6th Meeting of the European BioElectromagnetics Association, Budapest, Hungary, 12-16.11.2003, New research approach in EMF research - proteomics and transcriptomics (Plenary talk)

IEEE ICES (SCC-28) meeting, San Antonio TX, USA, 4-7.12.2003; Use of high-throughput screening techniques to determine biological effects of mobile phone radiation.

26th Annual Meeting of the Bioelectromagnetics Society, Washington, DC, USA, 20-25.06.2004; Biological effects of EMF: do they exist and what might be their biophysical mechanism - a molecular biologists perspective.

6.7 rCx46 in oocytes of *Xenopus laevis* and human fibroblasts and granulosa cells of rats (Participant 7)

a. Scientific papers

submitted:

Pilger A., Ivancsits S., Diem E., Steffens M., Kolb H-A., Rüdiger H. W. No long-lasting effects of intermittent 50 Hz electromagnetic field on cytoplasmic free calcium and mitochondrial membrane potential in human diploid fibroblasts Radiation and Environmental Biophysics.

in preparation:

Steffens M., Enders O., Behnsen J., Kolb H.-A. Effects of intermittent 50 Hz electromagnetic field on gap junctional coupling of paired *Xenopus laevis* oocytes expressing rCx46.

Steffens M., H.-A. Kolb; Effects of intermittent 50 Hz on conducting connexons of rCx46 expressed in oocytes of *Xenopus laevis*.

Steffens M., Kolb H.-A. Frequency dependent induction of DNA strand breaks by intermittent exposure to extremely low frequency electromagnetic field in various cultered cell lines.

b. Scientific meetings and reports

Steffens M., Kolb, H.-A. Gene expression of rCx46 in *Xenopus* oocytes is not affected by 50 Hz electro-magnetic radiation. Pflügers Arch. 443 (Plenary Lectures, Oral Sessions, Poster Sessions, Symposia) : S280 (2002)

Wobus A.M., Trillo M.A., Ubeda A., Kolb H.-A. Effects of ELF-and RF-EMF on cell proliferation and cell differentiation. 25th Annual Meeting of the BEMS, Maui, USA (June 2003). Proceedings: p. 133

Kolb, H.-A. Die Angst vor dem Strom. Neue Presse. Hannover (September 05, 2003)

Kolb, H.-A. Biologische Wirkungen ELF- und RF- elektromagnetischer Felder (EMF) (Nicht-ionische Wirkungen). 1.Nationaler Kongress Elektrosmog-Betroffener, Biel, Switzerland (22.11.2003)

Kolb, H.-A. Interview with Südwestfunk: Wirkung von EMF auf biologische Systeme. Hannover (July 07, 2004)

c. Poster

M. Steffens, H.-A. Kolb; Gene expression of rCx46 in *Xenopus* oocytes is not affected by 50 Hz electro-magnetic radiation. The Physiological Society Scandinavian Physiological Society, Deutsche Physiologische Gesellschaft (81st Annual Meeting), Tübingen, Germany (15–19 March 2002)

6.8 Experiments with human lymphocytes and thymocytes and with mice embryonic stem cells during cardiac differentiation (Participant 8)

a. Scientific papers

published:

Capri M, Scarcella E, Bianchi E, Fumelli C, Mesircas P, Agostini C, Remondini D, Schuderer J, Kuster N, Franceschi C, Bersani F (2004) 1800 MHz radiofrequency (mobile phones, different Global System for Mobile communication modulations) does not affect apoptosis and heat shock protein 70 level in peripheral blood mononuclear cells from younf and old donors. Int J Radiat Biol, Vol 80, No 6: p. 389 - 397

Ventura C, Maioli M, Asara Y, Santoni D, Mesirca P, Remondini D, Bersani F (2004) Turning on stem cell cardiogenesis with extremely low frequency magnetic fields. The FASEB J, published online Oct 26, 2004.

in preparation:

Magnetic fields and cell fate specification in embryonic stem cells. In preparation for Science

b. Scientific meetings

Effects of ELF-EMF on gene expression of various cell lines. 25th Annual BEMS Meeting 2003, Abstract book, p. 131

6.9 Experiments with brain cells of different origin and human monocytes (Participant 9)

a. Scientific papers

in preparation:

Poulletier de Gannes F. et al., Effects of GSM-900 radiofrequency radiation on apoptosis in brain cells. (Ready to be submitted to Radiation Research)

Lagroye I. et al., GSM-900 signal does not affect iNOS expression in rat C6 glioma cells (in preparation for Radiation Research)

Lagroye I. et al., Apoptosis in U937 after exposure to 217 Hz-modulated GSM-900 radiofrequency radiation. (in preparation for Bioelectromagnetics)

Poulletier de Gannes F. et al., Expression of heat shock proteins in brain cells after exposure to GSM-900 radiofrequency radiation (in preparation for International Journal of Radiation Biology)

b. Scientific meetings

Lagroye I., E. Haro, P.-E. Dulou, B. Billaudel, B. Veyret. Effect of GSM-900 exposure on NOS-II expression in rat C6 glioma cells. 24st Annual Meeting of the BEMS, Quebec, Canada (June 2002)

Lagroye I., E. Haro, P.-E. Dulou, B. Billaudel, B. Veyret. Effect of GSM-900 exposure on NOS-II expression in rat C6 glioma cells. 24st Annual Meeting of the BEMS, Quebec, Canada (June 2002).

Leszczynski, D., Billaudel, B., Czyz, J., Dulou, P-E., Guan, K., Haro, E., Joenväärä, S., Kuokka, R., Lagroye, I. , Meister, A., Reivinen, J., Veyret, B., Wobus, A.M., Zeng. Q. Effects of mobile phone radiation on gene and protein expression in vitro. 24st Annual Meeting of the BEMS, Quebec, Canada (June 2002).

Lagroye I, Bersani F., Billaudel B., Capri M., Czyz J., Dulou P-E., Guan K. Haro E., Joenväärä S., Kuokka R., Kuster N.,., Leszczynski D., Meister A., Reivinen J., Schuderer J., B. Veyret, A.M. Wobus, Q. Zeng. Do ELF or RF fields affect the apoptotic process? Data from the REFLEX programme. 24st Annual Meeting of the BEMS, Québec, Canada (June 2002).

Lagroye I., Poulletier de Gannes F., Haro E., Billaudel B., Dulou P.E., Veyret B. Effect of GSM-900 radiofrequency on apoptosis of immune and nervous cells. 27ème assemblée générale de l'URSI, Maastricht, Pays-Bas, (August 2002).

Lagroye I., Bersani F., Agostini C., Bianchi E.,Billaudel B., Capri M., Dulou P.E., Fumelli C., Haro E., Mesirca P., Poulletier de Gannes F., Scarcella E., Veyret B., Do GSM signals induce apoptosis in mammalian immune and nervous cells? 2nd International Workshop on Biological effects of EMF's, Rhodes, Crète, (October 2002).

Lagroye I., Bersani F., Agostini C., Bianchi E., Billaudel B., Capri M., Dulou P.E., Fumelli C., Haro E., Mesirca P., Poulletier de Gannes F., Scarcella E., Veyret B. Do GSM-900 signals induce apoptosis in mammalian immune and nervous cells? 2nd International Workshop on Biological Effects of Electromagnetic Fields, Rhodes, October 7-11, 2002, page 404-408.

Adlkofer F., R. Tauber, H.W. Rüdiger, A.M. Wobus, A. Trillo, D. Leszczynski, H.-A. Kolb, F. Bersani, I. Lagroye, N. Kuster, F. Clementi, C. Maercker, Risk Evaluation of Potential Environmental Hazards from Low Energy Electromagnetic Field Exposure Using Sensitive in vitro Methods (REFLEX), 2nd International Workshop on Biological effects of EMF's, Rhodes, Crète, (October 2002).

Poulletier de Gannes F., I. Lagroye, E. Haro, P.E. Dulou, B.Billaudel, B. Veyret. Heat shock proteins as sensors of nonthermal effects? Subtil effects of temperature, Cost 281 meeting, London, UK, (november 2002).

Lagroye I., Bersani F., Billaudel B., Capri M., Czyz J., Dulou P-E., Guan K., Haro E., Joenväärä S., Kuokka R., Kuster N., Leszczynski D., Meister A., Poulletier de Gannes F., Reivinen J., Schuderer J., Veyret B., Wobus A.M., Zeng Q. Effects of ELF- and RF-EMF on the apoptotic process. Abstract for the BEMS 25th annual meeting - Maui, Hawaii, June 22-27, 2003, page 134.

Poulletier de Gannes F., I. Lagroye, E. Haro, M. Taxile, P.E. Dulou, B. Billaudel, B. Veyret, Effects of GSM-900 on apoptosis in brain cells. 6th International Congress of the European BioElectromagnetics Association, 2003, Budapest, Hongrie (November 2003).

Lagroye I., Haro E., Billaudel B., Veyret B. The effect of GSM-900 radiofrequency radiation on camptothecin-induced apoptosis in human U937 lymphoblastoma cells. 6th International Congress of the European BioElectromagnetics Association, 2003, Budapest, Hongrie (November 2003).

Poulletier de Gannes, F., Sanchez, S., Lagroye, I., Haro, E., Dulou, P.-E., Billaudel, B., Veyret. B. In vitro and in vivo studies of the effects of GSM-900 microwave exposure on heat shock proteins in the brain and skin. 25th Annual Meeting of the BEMS, Maui, USA (June 2003).

Lagroye I, Bersani F, Billaudel B, Capri M, Czyz J, Dulou P-E., Guan K., Haro E., Joenväärä S., Kuokka R., Kuster N., Leszczynski D., Meister A., Poulletier de Gannes F. Reivinen J., Schuderer J., B. Veyret, A.M. Wobus, Q. Zeng. Effects Of ELF And RF Fields On Apoptosis In Different Cell Lines. . 25th Annual Meeting of the BEMS, Maui, USA (June 2003).

F. Poulletier de Gannes, S. Sanchez, I. Lagroye, E. Haro, B. Billaudel, B. Veyret. Effects of GSM-900 microwave exposure on heat shock proteins: *in vitro* and *in vivo* studies on different models in PIOM laboratory. COST 281bis workshop on "Influence of RF Fields on the Expression of Stress Proteins". April 28-29 2004, STUK Helsinki, Finland.

F. Poulletier de Gannes, I. Lagroye, S. Sanchez, B. Billaudel, B. Veyret. Effect of GSM-900 exposure on hsp27 expression in EA-hy926 endothelial cells: a replication study. 26th Annual Meeting of the BEMS, Washington DC, USA (June 2004).

6.10 Provision of exposure setups and technical quality control (Participant 10)

Exploitation

The success of the exposure setups developed under the umbrella of REFLEX have resulted in additional demands for similar setups being used in further European research programs, e.g. PERFORM B.

Dissemination

a. Scientific papers

published:

J. Schuderer, T. Schmid, G. Urban, N. Kuster, “Novel High Resolution Temperature Probe for RF Dosimetry”, Physics in Medicine and Biology, vol 49, pp. N83-N92, 2004.

J. Schuderer, T. Samaras, W. Oesch, D. Spät, N. Kuster, “High Peak SAR Exposure Unit with Tight Exposure and Environmental Control for In Vitro Experiments at 1800 MHz”, IEEE Transactions on Microwave Theory and Techniques, vol 52, No 8, 2004: 2057-2066

J. Schuderer, D. Spät, T. Samaras, W. Oesch, N. Kuster, “In Vitro Exposure Systems for RF Exposures at 900 MHz”, IEEE Transactions on Microwave Theory and Techniques, vol 52, No8, 2004: 2067-2075

J. Schuderer, W. Oesch, N. Felber, N. Kuster, “In Vitro Exposure Apparatus for ELF Magnetic Fields”, Bioelectromagnetics, in press, 2004.

J. Schuderer, "EMF Risk Assessment: *In Vitro* Research and Sleep Studies", Dissertation, ETH, 2003

J. Schuderer, N. Kuster, “The Effect of the Meniscus at the Solid/Liquid Interface on the SAR Distribution in Petri Dishes and Flasks”, Bioelectromagnetics, vol. 24, pp.103-108, 2003.

submitted:

J. Schuderer, U. Lott, N. Kuster, “UMTS In Vitro Exposure System and Test Signal for Health Risk Research”, Bioelectromagnetics, submitted 2004

in preparation:

W. Oesch, J. Schuderer, N. Kuster, R. Mertens, R. Adey, "Selection of Specific EMF Exposure Conditions for Bioexperiments in the Context of Health Risk Assessments", in preparation, 2004.

b. Scientific meetings

J. Schuderer, T. Samaras, W. Oesch, N. Nikoloski, D. Spät, N. Kuster, "Electromagnetic Field Exposure of Cells at 900 and 1800 MHz: Requirements, Dosimetry and Performance Comparison of Different Setups", FGF & COST281 Workshop on the Influcence of RF Fields on the Expression of Stress Proteins, April, Helsinki, Finland, pp. 21-22, 2004.

J. Schuderer, W. Oesch, U. Lott, N. Kuster, "In Vitro Exposure Setup for Risk Assessment Studies with UMTS Signal Schemes at 1950 Mhz", 25th Annual Meeting of the Bioelectromagnetics Society, June, Maui, USA, p. 68, 2003.

J. Schuderer, W. Oesch, R. Mertens, U. Frauenknecht, N. Kuster, "Exposure Systems, Dosimetry and Quality Control", 25th Annual Meeting of the Bioelectromagnetics Society, June, Maui, USA, pp. 127-128, 2003.

T. Samaras, J. Schuderer, N. Kuster, "Temperature Distributions Inside Cell Cultures Exposed to Electromagnetic Fields In Vitro", Cost 281 Management Committee Meeting, London, GB, Nov. 12-13, 2002.

J. Schuderer, T. Schmid, G. Urban, N. Kuster, "Novel High Resolution Temperature Probe for Micro-dosimetry", 27th General Assembly of the International Union of Radio Science, Maastricht, Netherlands, August, paper No. 2110 (2.p), 2002.

J. Schuderer, W. Oesch, N. Kuster, "In Vitro Exposure Setup for ELF Magnetic Fields Enabling Flexible Signal Schemes and Double Blind Protocols", 24th Annual Meeting of the Bioelectromagnetics Society, June, Quebec, Canada, pp. 105-106, 2002.

J. Schuderer, W. Oesch, R. Mertens, U. Frauenknecht, N. Kuster, "Exposure Systems and Dosimetric Quality Control in the REFLEX Project", 24th Annual Meeting of the Bioelectromagnetics Society, June, Quebec, Canada, pp. 93-94, 2002.

R. Mertens, W. Kainz, N. Kuster, "Simulating Environmental GSM Features for Use in Bioexperiments", 24th Annual Meeting of the Bioelectromagnetics Society, June, Quebec, Canada, p. 105, 2002.
J. Schuderer, R. Mertens, W. Oesch, U. Frauenknecht, N. Kuster, "Flexible and Efficient In Vitro Exposure Setup for Risk Assessment Studies at 1800 MHz Enabling any Modulation Scheme from Sub-Hz up to 15MHz and Double Blind Protocols", 23rd Annual Meeting of the Bioelectromagnetics Society, St. Paul, Minnesota, USA, p. 26, 2001.

N. Kuster, W-R. Adey, "Criteria for Selecting Specific EMF Exposure Conditions for Bioexperiments in the Context of Health Risk Assessments", 23rd Annual Meeting of the Bioelectromagnetics Society, St. Paul, Minnesota, USA, p. 24, 2001.

J. Schuderer, N. Kuster, "The Effect of the Meniscus at the Solid-Liquid Interface on the SAR Distribution in Petri Dishes and Flasks", Millenium Workshop on Biological Effects of Electromagnetic Fields, Heraklion, Greece, pp. 203-207, 2000.

c. Posters

W. Oesch, H-U. Gerber, N. Kuster, "Requirements for Controlling & Monitoring Software of Expoure Systems in (Double-)Blinded Bio Experiments", 24th Annual Meeting of the Bioelectromagnetics Society, June, Quebec, Canada, pp. 152-153, 2002.

d. Reports in the general media

J. Schuderer, "EMF Risk Assessment: In Vitro Research and Sleep Studies", Diss. ETH 15347, 2003.

N. Kuster, "Latest Progress in Experimental Dosimetry for Human Exposure Evaluations and for Characterization and Optimization of Exposure", in "Communication Mobile – Effects Biologique", ed. Claude Legris, CADAS, Académie des Sciences, Paris, France, pp. 63-69, 2001.

6.11 Experiments with the human neuroblastoma cell line SY5Y (Participant 11)

a. Scientific papers

The results described in this final report will be *submitted* for two publications in international scientific journals.

Benfante R., Antonini R.A., Gotti C., Moretti M., Kuster N., Schuderer J., Clementi F, and Fornasari D. – "Extremely low-frequency electromagnetic field (ELF-EMF) does not affect the expression of α3, α5 and α7 nicotinic receptor subunit genes in SY5Y neuroblastoma cell line". Manuscript in preparation

Antonini R.A:, Benfante R., Flora A., Kuster N., Schuderer J., Adlkofer F, Clementi F., and Fornasari D. – "The expression of DβH (dopamine-β-hydroxylase) and noradrenergic phenotype specifying genes Phox2A and Phox2B is unresponsive to exposure to extremely-low-frequency electromagnetic field (ELF-EMF)". Manuscript in preparation

6.12 Effects of EMF on gene expression in human cells analysed with the cDNA array (Participant 12)

a. Scientific publications

submitted:

Schlatterer K, Gminski R, Hermann S, Tauber R, Fitzner R, Maercker C. Gene expression profiling identifies differences in ribosome biogenesis of human promyelocytic leukemia HL-60 cells following exposure to 1800 MHz radiofrequency electromagnetic fields.

in preparation:

Remondini D, Leszczynski D, Nylund R, Ivancsits S, Rudiger HW, Bersani B, Maercker C. The bio-statistical analysis of micro-array data give indications for the induction of calcium-related signaling pathways after exposure of primary fibroblasts and endothelial cells to electromagnetic fields.

Kuokka R, Griffin T, Maercker C, Schuderer J, Reivinen J, Kuster N, Aebersold R, Leszczynski D. Effect of low-energy microwaves on protein expression in human endothelial cell line: Microwave modulation might be the cause of biological response.

b. Oral presentations at scientific meetings and round tables

Maercker C, Czyz J, Ivancsits S, Ruediger HW, Jahn O, Diem E, Pilger A, Rolletschek A, Schuderer J, Kuster N, Guan K, Trillo A, Bazán E, Reimers D, Fornasari D, Clementi F, Schlatterer K, Tauber R, Fitzner R, Adlkofer F, Wobus AM (2002) Gene expression profiling studies on global cDNA arrays show sensitivity of human and mouse cell lines to extremely-low frequency (ELF-EMF) and radiofrequency (RF-EMF) exposure. 24th annual BEMS Meeting, Quebec, Canada.

Maercker C, Wobus AM, Huber W, Poustka A, Ivancsits S, Rüdiger HW, Jahn O, Diem E, Schuderer J, Kuster N, Fornasari D, Clementi F, Schlatterer K, Tauber R, Fitzner R, Reivinen J, Adlkofer F, Leszczynski D (2002) An EU-wide initiative to characterize the biological effects of EMF on human and mouse cell lines by gene expression profiling. 2nd Int. Workshop on Biolocical Effects of Electromagnetic Fields, Rhodes, Greece. Proceedings, pp. 588-594.

Maercker C (2003) In-vitro investigation of molecular effects of electromagnetic fields by high-throughput techniques. Deutscher Ärztekongress, Berlin, Germany.

Maercker C, Schlatterer K, Gminski R, Schuderer J, Kuster N, Adlkofer F, Fitzner R, Tauber R (2003) RF-EMF exposure increases protein synthesis in human promyelocytic cells. 25th annual BEMS Meeting, Hawaii, USA.

Maercker C (2003) Effects of electromagnetic fields on the human genes – expererimental results and assessment of the potential of molecular biology in environmental research. Workshop of the ministry for environment in Nordrhein-Westfalen, Universität Witten-Herdecke, Germany.

Maercker C, Schlatterer K, Gminski R, Schuderer J, Kuster N, Adlkofer F, Fitzner R, Tauber R (2003) In vitro studies on promyelocytic cells with the help of gene expression profiling on cDNA microarrays show an increase of protein synthesis after RF-EMF exposure. EBEA2003 meeting, Budapest, Hungary.

Maercker C (2004) Genomics and proteomics approaches in EMF research. Erice School in Bioelectromagnetics, Erice, Italy.

Maercker C (2004) Invited participant in a round table discussion about research needs in European EMF research. Erice School in Bioelectromagnetics, Erice, Italy.

Maercker C (2004) Do electromagnetic fields induce stress responses? A whole-genome approach helps to identify cellular pathways modulated by RF-EMF and ELF-EMF" COST Workshop "Influence of RF Fields on the Expression of Stress Proteins" Helsinki, Finland.

Maercker C, Schlatterer K, Gminski R, Remondini D, Leszczynski D, Rüdiger H, Bersani F, Fitzner R, Tauber R (2004) The whole-genome gene expression analysis as a powerful method to detect molecular effects of electromagnetic fields. Deutscher Ärztekongress, Berlin, Germany.

Maercker C, Remondini D, Nylund R, Lezczynski D, Schlatterer K, Fitzner R, Tauber R, Ivancsits S, Rudiger H, Bersani F (2004) Analysis of gene expression in EMF research. 26th Annual Meeting of the Bioelectromagnetic Society (BEMS), Washington, USA.

Maercker C (2004) Molecular investigation of the effects of electromagnetic fields. T-Mobile GmbH, Darmstadt, Germany.

c. Posters

Maercker C, Czyz J, Wobus AM, Huber W, Poustka A, Ivancsits S, Ruediger HW, Reivinen J, Leszczynski D, Schlatterer K, Tauber R, Fitzner R, Fornasari D, Clementi F, Schuderer J, Kuster N, Adlkofer F (2002) An EU-wide initiative to characterize the biological effects of electromagnetic fields on different human and mouse cell lines by gene expression profiling. Annual Meeting of the "Nationales Genomforschungsnetz" (NGFN) in Berlin, Germany.

Maercker C, Kuokka R, Reivinen J, Ivancsits S, Ruediger HW, Schuderer J, Kuster N, Fornasari D, Clementi F, Schlatterer K, Tauber R, Fitzner R, Adlkofer F, Leszczynski D (2003) Whole-genome gene expression profiling: a big challenge to find out the molecular answer to EMF exposure. 25th annual BEMS Meeting, Hawaii, USA.

Lupke M, Maercker C, Simkó M (2004) Alteration in gene expression after 50 Hz ELF-MF exposure in human umbellical cord blood-derived monocytes. 3nd Int. Workshop on Biolocical Effects of Electromagnetic Fields, Kos, Greece.

7.0 POLICY RELATED BENEFITS

7.1 Studies on the human promyelocytic cell line HL-60 (Participant 2)

Research performed is basic research with relevance for life science and techniques, respectively. Our results obtained with human promyelocytic HL-60 cells have made a substantial addition to the data base relating to genotoxic and phenotypic effects of RF-EMF in vitro. Its value lies in providing new data that will enable mechanisms of RF-EMF effects to be studied more effectively (e.g. ROS effects) than in the past. Proteomics studies should be extended to identify possible, potential biological and molecular markers. Furthermore, our data provide new information that will be used for risk evaluation by WHO, IARC and ICNIRP.

7.2 Studies on human fibroblasts, human lymphocytes, human monocytes, human melanocytes, human muscle cells and granulosa cells of rats (Participant3)

Based on our findings we propose the suitability of the comet assay, micronucleus test and evaluation of chromosomal aberrations for monitoring and surveillance of EMF exposed subjects. Due to possible cell specific differences in response to EMF the biological material chosen for biomonitoring could be crucial. Our findings arouse concern about environmental threshold limit values and protective measures regarding EMF exposure in particular with respect to older individuals or people suffering from repair syndroms. The observed activation of DNA repair could display beneficial health effects and could be applied for medical treatment.

7.3 Studies on mouse embryonic stem cells (Participant 4)

Our research results confirmed subtle biological effects emanating from both extremely low frequency fields (simulating the magnetic components of 50 Hz power line fields) and high-frequency (RF) electromagnetic fields (EMF) simulating GSM-modulated schemes. The effects were dependent on the genetic constitution of the cells, and especially the transcription of apoptotic/anti-apoptotic related genes was shown to be affected. Neural progenitor cells appeared to perceive EMF at certain stages of differentiation as external stress signals, which may activate at least, a bcl-2 mediated anti-apoptotic pathway.

1. Important for the quality of life and for human health could be the improvement of products emitting EMF. In this context, 'no effect-levels' were observed with regard to flux density, which can be used to determine threshold values more precisely.
2. The analysis of transcript levels affected by EMF should be extended by genomics and proteomics studies in animals, but also in human populations, to identify further potential molecular markers which may serve as "EMF-responsive bio-indicator".
3. Cell biological studies should be continued to elucidate the molecular processes that may be affected by EMF, especially in the context of carcinogenesis.

7.4 Studies on the human neuroblastoma cell line NB69 and neural stem cells (Participant 5)

The described results indicate that a human neuroblastoma cell line can be sensitive to the in vitro exposure to power frequency, sine wave EM fields at magnetic flux densities that are equal to or lower than the exposure threshold (100 μT) recommended by ICNIRP and UE for the general public. The effects, which include changes in the cells' proliferation, apoptosis or the cellular response to growth factors, among others, were found to be dependent on the ELF-EMF density, the exposure time, the cell passage or the cell cycle. The same human cancer cell line, as well as neural stem cells from rat's embryonic nucleus striatum, was found to be sensitive to the in vitro exposure to GSM-1800 signals at a SAR of 2 w/kg, the exposure threshold recommended by ICNIRP and UE for the general public. The effects included changes in the expression of fibroblast growth factor receptor-1, accompanied or not with changes in the cellular morphology linked to a potential promotion of non-neuronal precursors of NSC' progeny. No differential responses were detected when the cells were exposed to GSM signals with

different ELF modulation patterns, suggesting that the observed effects reflect a cellular sensitivity to the RF carrier wave, rather than to the ELF modulation.

1. These data identify cellular mechanisms of response to specific parameters of exposure to ELF and RF electromagnetic fields that are ubiquitous in today's human environment. Such information can significantly contribute to the establishment of adequate strategies for the protection against non-ionising radiation in public, residential or occupational environments.
2. The study of the cellular mechanisms of response to ELF and RF EMF should be extended to properly identify the biophysical phenomena underlying the potential health effects of the exposure to environmental, non-ionising radiation. In this context, two types of studies are of crucial interest. A) Studies aimed to identify potential markers or bio-indicators of EM sensitivity; B) Studies on human cancer cells and the in vitro response to chemicals that could prevent the EMF effects.

7.5 Studies on the human endothelial cell lines EA.hy926 and EA.hy926v1 (Participant 6)

We have found that 900 and 1800 MHz GSM radiation at SAR of 2.0-2.4W/kg causes activation of stress response in human endothelial cell line. The stress response was followed by a physiological response on a single cell level. Cellular stress fibers were stabilized what was followed by the changes in cellular size and shape (cells contraction). Also we have observed effect on other cytoskeletal proteins, in particular on vimentin and formed of it - vimentin filaments. In respect of cell apoptosis we have observed decline in the expression of nearly all proteins involved in Fas/TNFα-dependent apoptosis pathway. This suggests that the mobile phone radiation might have some potential to prevent apoptosis of cells - a possibility that is currently being explored in further research. All-in-all, results of our research suggest that cells recognize mobile phone radiation as an external stress and this in spite of the very low energy of the exposure. Part of the results of our research has been already published in three articles (one in Differentiation 2002; two in Proteomics 2004). These results, although directly can not be used for prediction of any health hazard, they are available for the scientific evaluation of the potential risks associated with the use of mobile phones and for recommendations of further research needs. The remaining experimental data obtained by us within REFLEX is in process of submission for publication (3 manuscripts) and will be available for the scientific community in 2005.

7.6 Studies on rCx46 in oocytes of Xenopus laevis and human fibroblasts and granulosa cells of rats Participant 7)

On the level of DNA we found significant evidence for DNA damage by ELF-EMF for cultured human fibroblasts and granulosa cells of rats. The findings were strongly related to the exposure protocol. At intermittent ELF-EMF exposure maximal effects were observed after 16-18 h of exposure independently on the applied frequency in the range of 8 Hz to 300 Hz.

The effects appeared not to be reflected on the cellular level of free cytoplasmic calcium. Also cellular studies on the expression level of connexin 46 in oocytes of *Xenopus laevis* showed no significant effect on ELF-EMF exposure. Therefore, it is tempting to suggest that significant effects by ELF-EMF exposure on the genomic level appear not to be reflected on the cellular level. But is has to be taken into account that the methods which are applied to study DNA damage are quite different and most probably more sensitive than those for studying cellular parameters.

7.7 Studies on embryonic stem cells during cardiac differentiation and human lymphocytes and thymocytes (Participant 8)

Studies on embryonic stem cells open a totally new perspective: on one side, the possibility to study in a reproducible way the effects of ELF-EMF on cell differentiation and in particular on cardiogenesis; on the other side, the possibility to direct in some way the differentiation processes of stem cells into specific cell phenotypes.

The more or less negative results studies on human lymphocytes and thymocytes are of paramount importance for risk evaluation since they show that the immune system cells are nearly insensitive to ELF and RF EMF exposure.

7.8 Studies on brain cells of different origin and human monocytes (Participant 9)

We have found no evidence of biological effects of GSM-like signals on mammalian immune and nerve cells. The endpoints were apoptosis and the expression of stress- or inflammation induced proteins. Protocols included exposures at SAR levels corresponding to the public limit for local exposure (2 W/kg) and prolonged exposure duration (24 to 48 hours) that represent a "worst-case" exposure condition. These in vitro results will add to the database, on which the next scientific evaluation of RF-EMF health effects will be based. Although the present findings do not suggest a need for a revision of the local exposure limits to RF-EMF (1999/519/CE), more investigations on animal models of neurodegenerative diseases are needed.

7.9 Provision of exposure set-ups and technical quality control (Participant 10)

Our research is basically aimed at guaranteeing appropriate exposure setups and thorough quality control of the engineering aspects of the various experiments. High technical quality control is of special interest, since the variability of experiments within REFLEX is rather broad. Solid risk assessment will contribute to the future development of new communications technologies.

7.10 Studies on the human neuroblastoma cell line SY5Y (Participant 11)

Our results fit in a scientific debate around the contribution of ELF-EMF on brain neurodegenerative diseases, with particular emphasis on Alzheimer's disease (AD). Epidemiological studies showed that workers with likely electromagnetic field exposure may have an elevated risk of AD. On the other hand, experimental studies employing animal models failed to confirm these observations. In our studies we demonstrate that, at molecular level, the cholinergic system, which is one of the most affected neurotransmission system in AD, did not undergo any modification in the expression of relevant nAChR subtypes.

7.11 cDNA array analysis (Participant 12)

The micro-array technique is a state-of-the-art tool to investigate changes in gene expression and therefore molecular defects in human cells. Whereas in the field of medicine this kind of technique is on the way to get a diagnostic standard for certain diseases (e.g. cancer), it is not common so far for the detection of environmental effects. With our study we have shown that the whole-genome analysis is a suitable method to detect potential molecular effects of EMF. Since the different labs participating in the REFLEX project have worked with the same exposure setups (Participant 10) and we have done all hybridisations and data analyses in the same way, we have created a platform which can work with comparable material (RNA) of different cell lines and different experimentators. A quality control of the RNA (test for degradation, concentration) allowed us to make experiments with very different cell lines under comparable conditions and therefore to produce reliable results. This is a big difference to other assays (e.g. microscopical analysis), which strongly depend on people and software. In the near future, whole-genome approaches might support or even replace other measurements of the effects of electromagnetic fields or related environmental influences. Since the technique is very sensitive and specificly applicable for human cells, it also should be applied for in vivo studies in upcoming projects.

7.12 Summary (Participant 1)

The policy related benefits of the REFLEXproject consist in the fact that new knowledge has been generated independent of whether one likes it or not. Biological effects of extremely low-frequency (ELF) and radio-frequency (RF) electromagnetic fields (EMFs) the exposure to which is constantly increasing especially in Europe with its high density of population and industry and with the omnipresence of EMFs in infrastructures and consumer products have become a topic of public concern. This is due to the fear of people that based on the many conflicting research data a risk to their health cannot be excluded with some certainty. Therefore, the overall objective of REFLEX was to find out whether or not the fundamental biological processes at the cellular and molecular level support such an assumption. For this purpose, possible effects of EMFs on cellular events controlling key functions, including those involved

in carcinogenesis and in the pathogenesis of neurodegenerative disorders, were studied through focussed research. Failure to observe the occurrence of such key critical events in living cells after EMF exposure would have suggested that further research efforts in this field could be suspended and financial resources be reallocated to the investigation of more important issues. But as clearly demonstrated, the results of the REFLEX project show the way into the opposite direction.

The REFLEX project has made a substantial contribution to the data base on biological effects of both ELF-EMF and RF-EMF on in vitro cellular systems. The study was designed to investigate whether or not EMF exposure below the energy density reflected by the present safety levels generates in vitro critical cellular events. Gene mutations, deregulated cell proliferation and suppressed or exaggerated programmed cell death (apoptosis) that are caused by or result in an altered gene and protein expression profile are such critical events, the convergence of which is required for the development of chronic diseases. Genotoxic effects and a modified expression of numerous genes and proteins after EMF exposure could be demonstrated with great certainty, while effects on cell proliferation, cell differentiation and apoptosis were much less conclusive. Since all these observations were made in in vitro studies, the results obtained neither preclude nor confirm a health risk due to EMF exposure, but they speak in favour of such a possibility. Because of their fundamental character the findings will be presented to WHO, IARC and ICNIRP. It will be up to these organisations to make use of them for risk evaluation, in combination with findings from animal and epidemiological studies.

A major European added value of REFLEX consists also in the fact that the need for further research and especially how it should look alike have clearly been demonstrated. Furthermore, the outcome of the project should stimulate the research and development departments of the electrical, electronic, and telecommunication industry to make use of the methods developed in order to better adjust the state of technology to the conditions of life, and prompt the European governments to ensure multidisciplinary EMF research in order to take care, that the solution of the presently existing problem of uncertainty about a possible health risk for the people in Europe and beyond due to EMF exposure will not be postponed in the far future.

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ANNEX I - Results submitted after deadline

A) ELF-EMF

1) Re-evaluation of micronucleus frequencies on slides prepared by Participant 3 in two additional laboratories which are not members of the REFLEX consortium

Micronucleus frequencies in fibroblasts which were exposed to ELF-EMF (50 Hz, 1 mT, 15h, 5 min on/10 min off) or sham-exposed in the laboratory of Participant 3 (Vienna, see 2.2 and 3.1.1.1) were re-evaluated under blinded conditions.

a) Prof. Guenter Speit, Medizinische Fakultaet/Humangenetik, University of Ulm, Albert-Einstein-Allee 7, 89081 Ulm, Germany

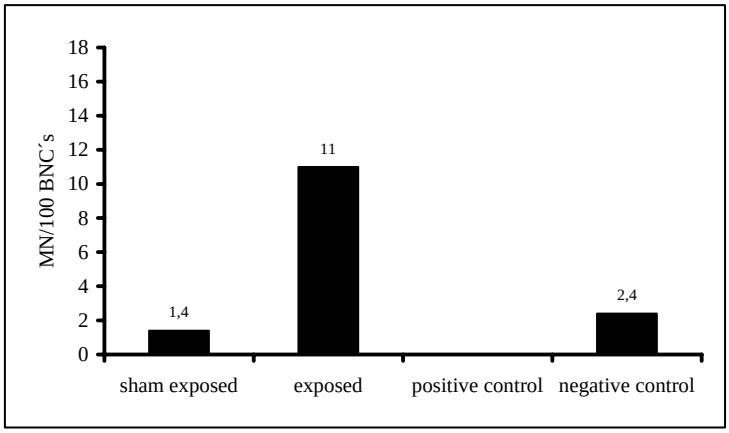

Figure 1: Micronucleus frequencies in ELF-EMF exposed cultured human fibroblasts (50 Hz, 1 mT, 15h, 5 min on/10 min off) and in control cells. Bleomycin treated cells (10µg/ml) were used as a positive control.

b) Prof. Heinrich Zankl, Fachbereich Biologie der Technischen Universitaet Kaiserslautern, Paul-Ehrlich-Strasse 7, 67663 Kaiserslautern, Germany

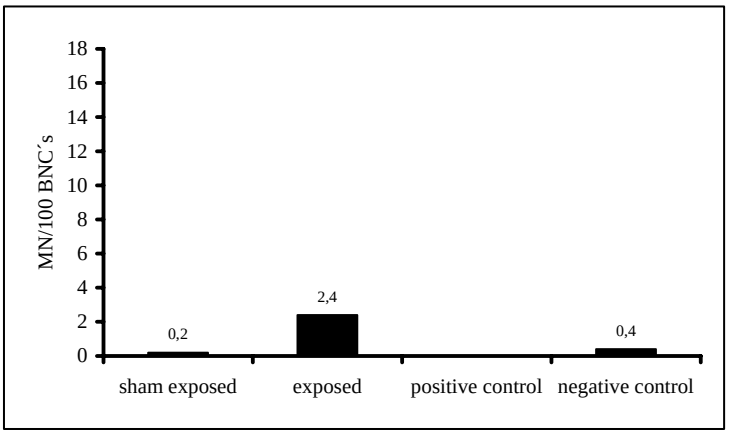

Figure 2: Micronucleus frequencies in ELF-EMF exposed cultured human fibroblasts (50 Hz, 1 mT, 15h, 5 min on/10 min off) and in control cells. Bleomycin treated fibroblasts (10 µg/ml) were used as a positive control.

ELF-EMF exposed cells showed an increase in micronucleus frequencies, which was about 6 to 10-fold as compared to sham exposed cells and negative controls. Although basal levels of micronuclei correlated well with the results obtained in the laboratory of Participant 3, exposed cells showed definitely higher values. The variability in micronuleus frequencies between the laboratories maybe due to the fact that different staining techniques were applied: Vienna and Ulm used a more sensitive fluorescent dye (DAPI), whereas the laboratory in Kaiserslautern stained the slides with GIEMSA. In addition, the laboratories in Ulm and Kaiserslautern scored 500 binucleated cells only instead of 2,000 processed in Vienna.

In conclusion, in three independent laboratories micronucleus frequencies showed a consistent increase in cultured human fibroblasts after ELF-EMF exposure. Differences in micronucleus frequencies between the laboratories can be attributed to different staining techniques and the different a numbers of scored cells.

2) Evaluation of genotoxic effects on human fibroblasts following intermittent exposure to 50 Hz powerline magnetic fields by Participant 8 (Bologna/Naples)

Some experiments related to the evaluation of the induction of genotoxic effects following intermittent (5 min field on/10 min field off) exposures to 50 Hz ELF magnetic fields, 1 mT field intensity, were carried out on human diploid fibroblasts (ES-1). In particular, to evaluate the induction of DNA single strand breaks the alkaline comet assay was applied following 15, 18 and 24 hours exposures, while the induction of micronuclei (MN) was measured following 24 h exposure.

Moreover, positive controls were also provided by treating cells with hydrogen peroxide (Alkaline Comet Assay) or Mitomycin-C (MMC; micronucleus assay) at several concentrations.

The results obtained do not indicate induction of genotoxic effects, neither in terms of comets induction nor in terms of MN frequency increase. On the contrary, positive controls showed an increase in DNA damage, as expected.

Experimental conditions and cytogenetic analysis

Human diploid fibroblasts (ES-1) were cultured in DMEM containing 10% FBS, 20 mM Hepes buffer, 2mM L-glutamine, 100 IU/ml penicillin and 100 µg/ml streptomycin at 37°C in a humidified atmosphere of 5% CO_2. Cells were supplied with fresh culture medium every 48 h and splitted once a week. Cells were received at passage 6 and a master bank was established with cells at passage 8. For experiments, cells from passages 10-16 were used.

Preliminary experiments were devoted to find the dose of chemicals to be used to provide positive controls for both the assays employed. Dose-response curves were set up by treating ES-1 cells with H_2O_2 ranging from 25-100 µM final concentration for 30 minutes (alkaline comet assay) and with MMC ranging form 0,005 – 0,05 µg/ml final concentration for the whole culture period (MN assay). For both chemicals a dose-dependent increase in DNA damage was detected, even for the lowest doses tested. However, the best compromise between induced damage and cell survival was found at 50 µM H_2O_2 and 0,025 µg/ml MMC and these concentrations were used for the experiments.

A second set of preliminary experiments was devoted to define the cell cycle duration of ES-1 cells to block cytokinesis for micronucleus test: it resulted of 28 h (data not shown).

The exposures were carried out at 50 Hz (1 mT field intensity) and the signal used was powerline. Several exposure durations were tested, as reported below.

Alkaline Comet Assay

Ten independent experiments (5 by exposing cells for 24 h, 2 by exposing cells for 18 h, 3 by exposing cells for 15 h) were carried out by setting up 8 cultures each: 4 to be exposed and 4 to be sham exposed.

Moreover, positive controls were provided by treating cultures for 30 min with hydrogen peroxide (H_2O_2, 50 µM). 24 hours before the experiments, 50000 cells/3 ml complete medium were seeded into 35 mm Petri dishes (Corning, cat. 430165). Following the intermittent exposure cultures were processed for the comet assay as described in details in (1). Slides were stained, just before the analysis, with ethidium

bromide (12 μg/ml) and images of 1000 randomly selected cells (250 from each of four replicated cultures) were analyzed by a computerized image analysis system (Delta Sistemi, Rome, Italy) fitted with a Leica DM BL fluorescence microscope at 250 X magnification. This system acquires images, computes the integrated intensity profile for each cell, estimates the comet cell components, head and tail, and evaluates a range of derived parameters. DNA damage was evaluated by calculating the tail factor, as reported in (1). Moreover, tail moment, comet moment and percentage of tail DNA were also measured.

Cytokinesis-block Micronucleus Assay

4 independent experiments were carried out by exposing cells for 24 h. For each experiment 4 cultures were set up: 2 to be exposed and 2 to be sham exposed. Positive controls were provided by adding MMC (0,025 μg/ml) 24 h after the seeding.

50000 cells/5 ml complete medium were seeded in slide flasks (NUNCLON, cod. 170920, 9 cm^2 growth area) and recovered for 24 h. To block cytokinesis, 4 hours before the end of the first cycle (48 h after seeding), Cytochalasin-B (3 μg/ml final concentration) was added and at the end of the second replication cycle (80 h after seeding), cells were incubated for 30 min at 37°C with hypotonic solution (KCl 0,075 M) and fixed for 10 min (methanol 80% in distilled water). Air dried slides were stained for 8 min (10% Giemsa in phosphate buffer pH 6.8). The intermittent MF exposure was carried out during the first 24 hours following the recovery.

MN were scored in binucleated cytokinesis-blocked (CB) cells with well preserved cytoplasm by using a light microscope, and for each experiment their frequency was evaluated in 2000 cells (1000 cells for each duplicate slide). The results were expressed as micronucleated binucleated (MNBN) cells per thousand binucleated cells.

The morphological criteria for MN scoring in binucleated cells were similar to those reported by Fenech for human lymphocytes (2). By classifying 500 cells according to the number of nuclei, the binucleate cell index (BCI) and the cytokinesis-block proliferation index (CBPI) were evaluated for each culture, as reported in (3) and (4), respectively.

Statistical analysis

Differences between treated and untreated samples (sham exposed vs. exposed; sham exposed vs. positive controls) were tested by using the two tailed paired Student's t test. P values lower than 0.05 were considered as statistically significant.

RESULTS

Comet assay

Following 24 h intermittent exposure (5 min on/10 min off) to 50 Hz powerline MF, human diploid fibroblasts did not show statistically significant differences in all the parameters investigated when sham-exposed samples were compared to exposed ones. The results obtained are reported in table 1 as mean ± standard error of 5 independent experiments. Same results have been obtained when cultures exposed for 18 h (2 experiments) and 15 h (3 experiments) were compared to their own sham exposed cultures, as shown in table 2 and 3, respectively.

On the contrary, when sham exposed cultures were compared to H_2O_2 -treated samples, a significant increase in all the parameters investigated was detected following treatments of 30 minutes ($p<0.05$). The results obtained are reported in figure 1 as mean ± standard error of all the parameters investigated.

Micronucleus assay

Following 24 h exposure no genotoxic effects were detected by comparing sham-exposed with exposed samples. The proliferation index (CBPI) also resulted not affected by the exposure. The results are reported in table 4 (4 independent experiments), where data related to MMC treatments are also shown. By comparing sham-exposed with MMC treated cultures a statistically significant increase in MN frequency was detected, together with a decrease of cell proliferation and of percentage of binucleated cells ($p<0.05$ in all cases).

DISCUSSION

The data here reported do not support the hypothesis that intermittent exposures to 50 Hz MF (powerline signal) induce genotoxic effects in human diploid fibroblasts.

Concerning the alkaline comet assay, our finding is also supported by the results obtained by treating cells with hydrogen peroxide as positive control, where an increase in all the comet parameters investigated has been detected. Moreover, the cells investigated showed an high sensitivity to 30 min treatment already at 25 µM final concentration (data not shown), while most of the data reported in literature on several cell types indicate that 30 min treatments at concentrations between 50 and 100 µM are needed to induce a statistically significant effect.

Concerning the data on the induction of micronuclei (MN), also in this case we have not found genotoxic effects induced by the field. On the contrary, MMC treatments at doses of 0,025 µg/ml resulted cytotoxic (CBPI: 1,18 vs. 1,45 in treated and untreated cultures, respectively) while a dose of 0,033 µg/ml is necessary to induce MN increase in human peripheral blood lymphocytes without affecting cell proliferation.

The data here reported refer only to the exposures performed with the powerline signal: experiments devoted to test the sinusoidal signal are in progress.

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Table 1 – mean values ± standard error of the parameters investigated following alkaline comet assay in ES-1 human fibroblasts exposed for 24 h to 50 Hz powerline magnetic field. Results of 5 independent experiments (1000 cells/treatment investigated)

Parameters	Sham-exposed	MF-exposed
Tail Factor (%)	5.65 ± 0.98	6.36 ± 0.93
Comet Moment	1.86 ± 0.43	2.01 ± 0.49
% DNA	3.8 ± 0.97	4.12 ± 1.12
Tail Moment	1.10 ± 0.32	1.29 ± 0.36

Table 2 – mean values ± standard error of the parameters investigated following alkaline comet assay in ES-1 human fibroblasts exposed for 18 h to to 50 Hz powerline magnetic field. Results of 2 independent experiments (1000 cells/treatment investigated)

Parameters	Sham-exposed	MF-exposed
Tail Factor (%)	6.24 ± 0.25	6.11 ± 0.26
Comet Moment	2.32 ± 0.04	2.25 ± 0.04
% DNA	4.37 ± 0.22	4.20 ± 0.22
Tail Moment	1.56 ± 0.05	1.54 ± 0.11

Table 3 – mean values ± standard error of the parameters investigated following alkaline comet assay in ES-1 human fibroblasts exposed for 15 h to to 50 Hz powerline magnetic field. Results of 3 independent experiments (1000 cells/treatment investigated)

Parameters	Sham-exposed	MF-exposed
Tail Factor (%)	5.23 ± 0.26	5.20 ± 0.63
Comet Moment	1.76 ± 0.08	1.68 ± 0.18
% DNA	3.07 ± 0.29	3.08 ± 0.68
Tail Moment	0.89 ± 0.03	0.88 ± 0.15

Table 4 – MN frequency, proliferation index (CBPI) and binucleate cell index (BCI) in cultures sham-exposed, exposed for 24 h and positive controls. Data are reported as mean ± SD of 4 independent experiments. * Sham-exposed vs. MMC treated cultures: $p<0.05$.

Parameters	Sham-exposed	MF-exposed	MMC (0.025 µg/ml)
MN/1000 CB cells	0.45±0.06	0.43±0.07	2.35±0.35*
CBPI	1.45±0.02	1.43±0.02	1.15±0.01*
% BCI	45.2±2.45	43.3±2.01	15.0±1.41*

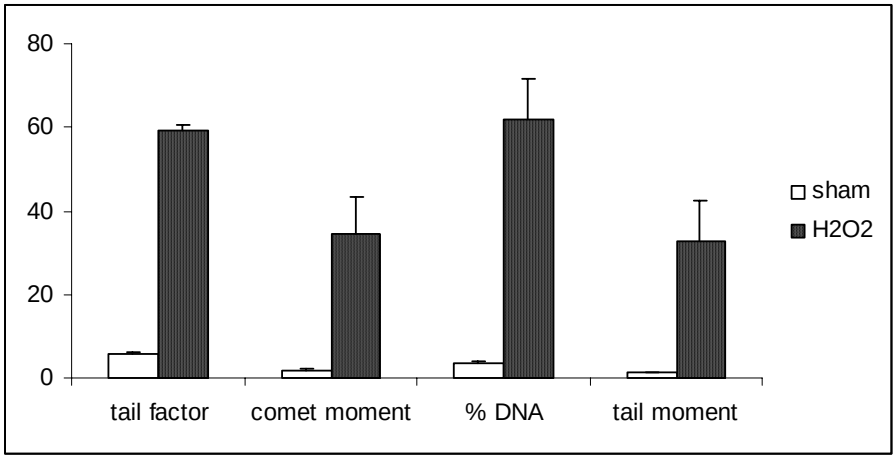

Figure 1 – mean values ± standard error of the parameters investigated following alkaline comet assay in ES-1 human fibroblasts treated for 30 min with 50µM hydrogen peroxide compared to sham-exposed samples.

B) RF-EMF

1) Re-evaluation of micronucleus frequencies on slides prepared by Participant 3 in two additional laboratories which are not members of the REFLEX consortium

Micronucleus frequencies in fibroblasts which were exposed to RF-EMF (GSM basic 1950 MHz, 15h, 2 W/kg) or sham-exposed in the laboratory of Participant 3 (Vienna, see 2.2 and 3.2.1.2) were re-evaluated under blinded conditions.

a) Prof. Guenter Speit, Medizinische Fakultaet/Humangenetik, University of Ulm, Albert-Einstein-Allee7, 89081 Ulm, Germany

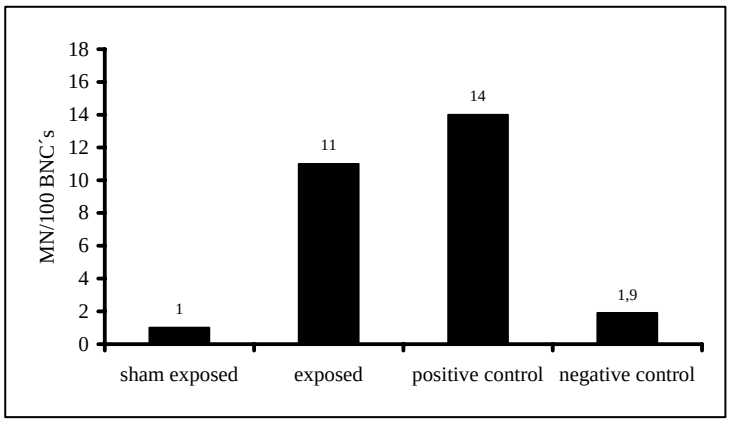

Figure 1: Micronucleus frequencies of RF-EMF exposed (GSM basic 1950 MHz, 15h, 2 W/kg) cultured human fibroblasts and control cells. Bleomycin-treated cells were used as a positive control.

b) Prof. Heinrich Zankl, Fachbereich Biologie der Technischen Universitaet Kaiserslautern, Paul-Ehrlich-Strasse 7, 67663 Kaiserslautern, Germany

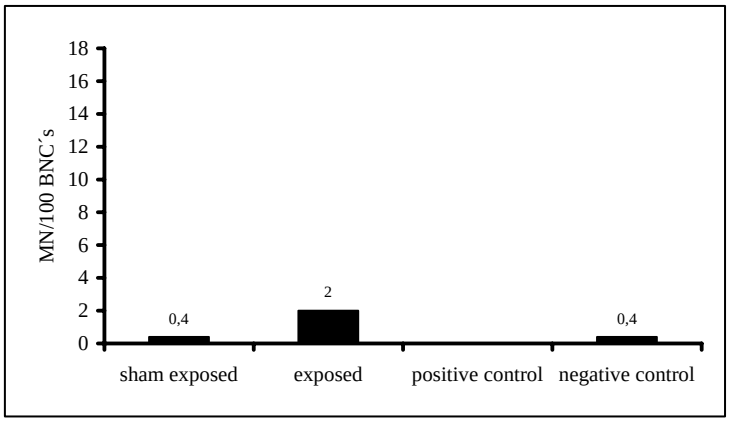

Figure 2: Micronucleus frequencies of RF-EMF exposed (GSM basic 1950 MHz, 15h, 2 W/kg) cultured human fibroblasts and control cells. Bleomycin-treated cells were as a positive control.

RF-EMF exposed cells showed an increase in micronucleus frequencies, which was about 5-fold compared to sham exposed cells and negative controls. Although, basal levels of micronuclei correlated well with the results obtained in the laboratory of Participant 3, exposed cells showed definitely lower levels. These variability could be attributed to different staining techniques. Laboratories in Vienna and Ulm used a more sensitive fluorescent dye (DAPI), whereas the laboratory in Kaiserslautern stained the slides with GIEMSA. Positive controls could not be evaluated, due to a too low number of assessable cells.

In conclusion, in three independent laboratories micronucleus frequencies showed a consistent increase in cultured human fibroblasts after RF-EMF exposure. Differences in micronucleus frequencies between the laboratories can be attributed to different staining techniques and a various number of scored cells.

IT'IS FOUNDATION
Foundation for Research on Information Technologies in Society

Prof. Dr. Niels Kuster
Director Foundation IT'IS
Professor of Swiss Federal Institute of Technology – ETHZ
Phone +41 1 245 9690 · kuster@itis.ethz.ch

ANNEX II

Technical support (Participant 10)

OBJECTIVES

To be able to compare the results of investigations carried out in the different laboratories and to ensure the conclusiveness of the data obtained in the studies, it is of the utmost importance that the conditions of exposure to EMFs be strictly controlled. Therefore, the objective are:

- Evaluation of existing setups
- Development of an optimised ELF setup
- Development of an optimised RF setup (900 MHz)
- Development of an optimised RF setup (1800 MHz)
- Comprehensive dosimetry ELF setup
- Comprehensive dosimetry RF setups
- Technical quality control during the entire period of exposure

Detailed information about the applied exposure systems and their dosimetry are given in

- [Schuderer et al., 2004a] for the ELF setup
- [Schuderer et al., 2004b] for the RF setup (GSM)
- [Schuderer et al., 2003] for the RF setup (UMTS)
- [Schönborn et al., 2000] for the waveguide setup of Participant 4
- [Laval et al., 2000] and [Schuderer et al., 2004c] for the Wire-patch cell of Participant 9
- [Toivo et al., 2001] and [Schuderer et al., 2004c] for the resonator setup of Participant 6
- [Kuster et al., 2002] for the choice of the exposure signals

REQUIREMENTS

The requirements for the exposure systems are formulated in Kuster and Schönborn (2001). In particular, the following parameters need to be fulfilled:

ELF setup:

- large loading volume with uniform exposure
- high dynamic (µT - mT) and frequency ranges (subHz - kHz)
- enabling complex signals and intermittent exposure
- good isolation between exposure and sham
- identical atmospheric parameters for exposed and sham cells (preferably placed in the same incubator)
- blinded exposure protocols by a computer-controlled random decision maker
- continuous monitoring of all environmental and technical parameters in order to detect any malfunctions
- evaluation of possible artefacts such as parasitic E-fields, temperature loads, vibrations, etc.

RF setup:

- peak SAR exposure: >100 W/kg
- maximum time averaged SAR exposure for thermal load < 0.1°C: > 2 W/kg
- deviations from uniformity of exposure: < 30%
- variability of exposure: < 10%
- loading volume with uniform exposure: min. 50 cm^2
- flexible signal unit with high dynamic range enabling complex modulation such as:
  - continuous wave (CW)
  - pulse or sinusoidal modulation at any frequency and repetition rate
  - GSM DTX (discontinuous transmission mode)
  - GSM non-DTX
  - GSM talk (temporal changes between non-DTX and DTX)
  - UMTS signal schemes
- flexible intermittent exposure protocols: seconds to hours
- good isolation between exposure and sham: > 30 dB
- identical atmospheric parameters for exposure and sham: μT < 0.1°C
- blinded exposure protocols
- continuous monitoring of exposure and environmental parameters
- detailed numerical modelling including meniscus
- evaluation of SAR distribution and experimental verification by dosimetric measurements
- uncertainty and variability analysis for SAR
- evaluation of the temperature load
- reliability, user-friendliness and self-detection of malfunctions
- minimal setup cost

MATERIAL AND METHODS

Overview

High-end exposure setups for ELF, RF-GSM and RF-UMTS were developed and fully characterized with respect to exposure parameters, design and possible artifacts.

Since the budget did not allow to equip every laboratory with these setups, existing setups were evaluated with respect to their suitability.

Evaluation of Existing Setups

In order to assess the performance of the already existing setups, the exposure systems in the laboratories of Participants 2 - 9 were evaluated with respect to dosimetric performance and characterization. Possible modifications to improve the performance were also evaluated.

In addition to the newly developed exposure systems, it was decided to use five existing setups (Participants 4, 5, 6, 8, 9) and to modify and improve the two RF setups of Particioants 4 and 9. Similar methods for dosimetry and characterization as described for the newly developed RF setups were used.

ELF setups:

- *Helmholtz coils (Participant 5)*: A pair of Helmholtz coils is placed inside a μ-metal shield; exposure and sham are kept in different incubators; and sinusoidal B-fields (50 Hz) up to 0.1 mT can be applied.
- *4-coil system (Participant 8)*: Two unshielded 4-coil systems are arranged in the same incubator; B-fields up to 1 mT (50 Hz) can be applied.

Both setups provide acceptably uniform B-fields, and no further modification and optimisation was applied.

RF setups:

- *Waveguide setup (Participant 4)*: The waveguide setup of Participant 4 is operated at 1710 MHz and allows the exposure of eight 60 mm diameter Petri dishes. The original system [Schönborn et al., 2000] was enhanced with
  - a new signal unit, allowing complex GSM modulation
  - field sensors to monitor the exposure
  - temperature sensors to monitor the incubator environment
  - an optimised air flow system to reduce temperature differences between both chambers
- *Wire-patch cell (Participant 9)*: The Wire-patch cell is an open radiating setup operated at 900 MHz. The setup is based on a 150 mm x 150 mm parallel plate configuration (distance 29 mm), short-circuited at the edges by four plots [Laval et al., 2000]. Eight 35 mm Petri dishes (placed inside a 60 mm dish with distilled water) are arranged symmetrically around the central coaxial feed. Since the WP cell is an open setup, exposure and sham groups need to be placed in two different incubators. For the purpose of REFLEX, the original system was enhanced by
  - distance keepers for the Petri dishes,
  - optimised Petri dish loading (to reduce the thermal load, distilled water instead of cell medium is used for the 60 mm dish),
  - E-field sensors for monitoring and regulation
  - A computer-controlled signal unit, allowing complex GSM modulation
- *Resonator setup (Participant 6)*: The resonator setup consists of a short-circuited waveguide chamber at 900 MHz [Toivo et al., 2001]. Four 60 mm diameter Petri dishes are exposed in a standing wave E-field maximum in E-polarization. The dishes are placed on a glass plate which is water-cooled from below. In this way a temperature stability of ± 0.3°C over the range from 0 – 10 W/kg average SAR is achieved. No modification of the resonator setup was performed.

Development of ELF Exposure Setup

An ELF setup was developed and four copies were installed in the laboratories of Participants 3, 4, 7 and 11. The following methods have been applied to achieve an optimised design for the ELF exposure system:

- Two coil chambers are placed inside the same incubator to guarantee identical environmental parameters for exposure and sham groups. A fan system serves for enhanced atmospheric exchange between coil chambers and incubator.
- µ-metal shielding is applied for the coils in order to provide sufficient sham isolation.
- E-field shielding of the exposure area is applied to remove parasitic E-fields, generated by the voltage drop over the inductive coils.
- Elastically damped dish holders are used to minimize the coupling of the mechanical vibrations to the Petri dishes.
- Numerical field calculation was used to optimise the 4-coil system within the µ-metal shielded exposure chamber. Optimisation parameters were size of the coils, number of windings and distance between the windings. B-field uniformity was used as an optimisation target.
- Exposure control is realized by monitoring and feedback regulation of the coil currents.
- Complex ELF signals can be applied by using an arbitrary function generator together with a custom-made current source to generate any signal with a point length of 16000 points, a point resolution of 12 bit and frequencies up to 1.5 kHz.
- A power-line signal was defined as the maximum accepted distortion for low- to medium-voltage power systems by the IEC (spectral components up to 1250 Hz are present).
- Environmental monitoring is applied with temperature sensors inside the chambers and by controlling the fan system (current measurement).
- Computer control allows blind protocols and easy handling of the system.

Development of RF Exposure Setup (GSM)

An RF setup (GSM) was developed and four copies were installed in the laboratories of Participants 2, 5, 6 and 8. The following methods have been applied to achieve an optimised design for the RF exposure system:

- The study from Schönborn et al. (2001) showed that the most suited setup with respect to highly uniform cell monolayer exposures should be based on waveguides.
- A waveguide setup was numerically analysed by using the FDTD method. The length of the waveguides was optimised, so that the system is operated at a fundamental resonator mode at 1800 MHz. In this way, superior power efficiency can be achieved.
- The required uniformity and maximum SAR is achieved for a fan-cooled cell monolayer exposure in E-polarization, which provides minimum temperature load.
- Exposure control is realized by field sensors.
- Environmental control is realized by placing the waveguides inside an incubator and using fans for atmospheric exchange. A common air inlet of the fan system was realized in order to reduce temperature differences between both waveguides.
- Environmental monitoring is applied with air temperature sensors and fan monitoring.
- Low variability is realized with a field sensor feedback regulation and by using dish holders together with distance keepers to provide defined positions of the Petri dishes with respect to the incident fields.
- Flexible signal schemes and blinded protocols are realized with a computer-controlled signal unit. AM modulation of the RF generator is applied via an arbitrary function generator and additionally via software commands. Temporal changes between different modulation modes like GSM DTX and non-DTX is realized with a GSM frame unit, blanking the output of the RF amplifier.

Development of RF Exposure Setup (UMTS)

An RF setup (UMTS) was developed and installed in the laboratory of Participant 3. Similar methods as for the RF setup (GSM) have been applied to achieve an optimised design for the UMTS exposure system:

- Similar to the GSM setup, two waveguides are used, equipped with field and temperature sensors and an optimized fan cooling system.
- Due to the different carrier frequency at 1950 MHz, new positions for the Petri dishes inside the waveguide are necessary. Because of the 5 MHz bandwidth of the UMTS signal, a broadband coax-to-waveguide adapter was required.
- The signal unit was updated by a UMTS signal generator. The fast power control of the signal is realized by AM modulation of the RF generator with a fading function stored on the arbitrary function generator.
- A UMTS test signal was defined, which represents worst-case exposure with respect to ELF spectral content. The signal is based on closed loop power controlled fades and additionally covers compressed mode and an open loop power controlled sequence for the physical random access channel.

Dosimetry ELF Exposure Setup

The following methods for dosimetry of the ELF setup were applied:

- *Numerical B-field characterization*: Mathematica V4.1 was used for analytical calculation of the B-field distribution as resulting from the 4-coil configuration.
- *Experimental B-field characterization*: A 3-axis Gaussmeter (FH49, Magnet-Physik, Germany) was used to measure the B-field distribution inside the exposure chamber.
- *Uncertainty and variability*: Uncertainty of the dosimetric assessment and exposure variability were analyzed for the applied numerical and experimental methods.
- *Induced E-field characterization*: The distribution of the induced E-fields within the cell medium was assessed by calculation.
- *Artefact characterization*:
  - *Parasitic E-fields*: A Wandel and Goltermann EFA-3 sensor system was used to determine the electric fields inside the exposure chamber as produced by the setup.
  - *Temperature*: Temperature was measured inside the cell medium with a SPEAG T1V3 probe as well

as for several positions inside the exposure chamber with Thermometrics Pt100 temperature sensors.

- *Vibrations*: To assess the acceleration resulting from coil vibrations, a Wilcoxon Research accelerometer Model 728T equipped with an amplifier unit P704T was applied.

Dosimetry RF Exposure Setup (GSM, UMTS, Wire-Patch Cell)

The following methods for dosimetry of the RF setups were used:

- *Numerical field simulation*: The FDTD simulation platform SEMCAD was applied for a full 3D electromagnetic field analysis.
- *Numerical modelling*: High resolution numerical models including menisci at the solid/liquid interfaces have been used to achieve realistic modelling. SAR extrapolation to the monolayer was applied to compensate discretization error in the strong SAR gradients.
- *Experimental verification*: Simulation was experimentally verified using the DASY3 near-field scanner equipped with 3-axis free space E- and H-field probes (SPEAG EF3DV2, H3DV6) and a dosimetric E-field probe with a diameter of only 1 mm [Pokovic et al., 2000].
- *Uncertainty and variability*: The uncertainty of the SAR assessment was evaluated with respect to the applied numerical and experimental methods. Possible variability of SAR values was additionally evaluated.
- *Thermal load*: The thermal load for the exposed group was assessed by measurement and simulation: A SPEAG T1V3 temperature probe was used for a single-point measurement of the temperature response of the medium (probe was fixed in the temperature maximum). Additionally, a coupled electro-thermal FDTD simulation was used for the 3D assessment of the temperature distribution as a function of exposure duration.

Quality Control and Maintenance

Quality control is ensured by the analysis of the exposure data as recorded from the monitoring unit and stored within an encoded file. All experimental settings and software commands are saved together with the sensor data for field exposure and environment. The monitoring sampling rate is 0.1 Hz. Decoding of the data files can only be provided by a dedicated software and is done by the quality assurance group after biological evaluation. A detailed report of all exposure parameters is then provided.

Furthermore the controlling and monitoring software is able to self-detect malfunctions and responds with warnings or abortions if required (tracing and handling of 60 errors).

For the RF setups, the ambient ELF-fields in the different laboratories were determined for several positions within the incubator and laboratory using a Wandel & Golterman EFA-3 sensor system.

RESULTS

Dosimetry ELF Exposure Setup

The performance of the ELF setup can be summarized by:

- Dynamic range for B-field amplitude (50 Hz): 0.02 – 3.6 mT_{rms}
- Dynamic range for B-field frequency: mHz –1500 Hz
- Nonuniformity of B-field: < 1%
- Uncertainty for B-field assessment: 4.3%
- Variability of exposure: 1.6%
- Loading volume: 3500 cm^3
- Parasitic E-fields (50 Hz): < 1 V/m
- Vibrations:
  - < 0.1 m/s^2 for elastically damped holder
  - < 1 m/s^2 for non-damped holder

- Signal schemes:
  - Sinusoidal 3 –1000 Hz
  - 50 Hz power-line signal (components up to 1250 Hz)
  - Arbitrary intermittency
- Exposure control and monitoring: provided by current measurements (sampling rate 0.1 Hz)
- Environmental control: provided by incubator and fan system (air temperature difference between exposure and sham: < 0.1°C)
- Environmental monitoring: provided by temperature probes and fan current sensing (sampling rate 0.1 Hz)

Dosimetry RF Exposure Setup (GSM)

The performance of the RF setup (GSM) can be summarized by:
- Dynamic range for peak SAR: 0.01 W/kg to > 100 W/kg
- Nonuniformity of SAR: < 30%
- Thermal load: < 0.03 °C / (W/kg)
- Uncertainty of SAR assessment: 20%
- Variability of exposure: 5.1%
- Loading surface for cell monolayers: 60 cm^2
- Signal schemes:
  - Continuous wave
  - 217 Hz pulse modulation
  - GSM non-DTX
  - GSM DTX
  - GSM Talk
  - Arbitrary intermittency
- Exposure control and monitoring: provided by field sensor (sampling rate 0.1 Hz)
- Environmental control: provided by incubator and fan system (air temperature difference between exposure and sham: < 0.1°C)
- Environmental monitoring: provided by temperature probes and fan current sensing (sampling rate 0.1 Hz)

Dosimetry RF Exposure Setup (UMTS)

The performance of the RF setup (UMTS) can be summarized by:
- Dynamic range for peak SAR: 0.01 W/kg to > 200 W/kg
- Nonuniformity of SAR: < 26%
- Thermal load: < 0.03 °C / (W/kg)
- Uncertainty of SAR assessment: 18%
- Variability of exposure: 1.9%
- Loading surface for cell monolayers: 60 cm^2
- Signal schemes:
  - Continuous wave
  - UMTS test signal (maximized ELF spectral content)
  - 217 Hz pulse modulation
  - GSM DTX
  - GSM non-DTX
  - GSM Talk
  - Arbitrary intermittency
- Exposure control and monitoring: provided by field sensor (sampling rate 0.1 Hz)

- Environmental control: provided by incubator and fan system (air temperature difference between exposure and sham: < 0.1°C)
- Environmental monitoring: provided by temperature probes and fan current sensing (sampling rate 0.1 Hz)

Quality Control and Maintenance

Quality Control:

- In the course of the REFLEX project, approximately 1800 *in vitro* experiments have been performed. Each of these experiments is documented with a dosimetric evaluation report covering the time courses and statistics for the field values, air temperatures and fan currents as well as all experimental settings.
- Average ambient ELF B-fields in the incubators of laboratories 2, 3, 4, and 8 are:
  - Participant 2: $B = 0.3 \pm 0.2\ \mu T_{rms}$
  - Participant 4: $B = 3.2 \pm 2.0\ \mu T_{rms}$
  - Participant 5: $B = 3.5 \pm 2.2\ \mu T_{rms}$
  - Participant 8: $B = 2.8 \pm 1.9\ \mu T_{rms}$

Maintenance:

Maintenance and assistance was provided in the course of the project for:

- installation of the setups
- handling of the setups
- exchange of several Pt100 temperature probes
- exchange of some RF dish holders
- exchange of one malfunctioning ELF current source
- exchange of one malfunctioning RF generator
- provision of software updates
- evaluation of data files

DISCUSSION

All tasks except development of an optimised RF setup at 900 MHz have been fully solved. More setups than initially planned needed to be developed, since the quality of the setups available in the laboratories were not sufficient to meet the requirements of the project. In order to stay within the budget, the consortium decided to develop a new RF setup only for 1800 MHz and use already available setups for 900 MHz (setups of Participants 6 and 9). Furthermore, the setup of Participant 9 was updated to allow complex GSM modulation. In addition to the required deliverables, a novel UMTS exposure system was developed.

CONCLUSIONS

High-end exposure systems for conducting *in vitro* laboratory studies in several European research institutes were realized and characterized. These systems have already become standard exposure setups for bioexperiments around the world.

An ELF exposure system that allows flexible signal and intermittent exposure schemes has been developed and characterized. It is easy to handle due to automated software control. Coil currents, chamber temperatures and fan speed are continuously monitored and allow the experimental history to be traced with 10 s resolution. B-field and E-field distributions were characterized. The B-field shielding of the 4-coil configuration considerably enhances the uniformity of the field distribution, and a highly efficient E-field shielding inhibits strong parasitic electric fields generated by the coils. Temperature differences between exposed and sham-exposed cells are kept below 0.1°C. The vibration load on the

exposed Petri dishes is sensitive to mechanical resonance; however, a mechanically isolated and elastically damped dish holder limits this to less than $0.1 m/s^2$, which is no more than twice the background vibration of the sham setup.

The waveguide-based, computer-controlled RF (GSM) setup enables the exposure of cell monolayers with excellent efficiency > 20 W/kg /W. The flexible signal unit allows the generation and control of complex modulated signals, e.g. temporal changes between different GSM operation modes (DTX/non-DTX). Exposure field strength and environmental parameters (air temperature, fan system) are continuously monitored. Due to the field regulation, exposure variability is kept below 10%. A coupled electro-thermal FDTD analysis was performed and resulted in a nonuniformity of SAR of < 30%. The temperature load was assessed by measurement and simulation, and a maximum temperature increase of less than 0.03°C was found. No localized temperature hot "spots" are generated within the cell medium. All simulations were verified by dosimetric measurements.

An exposure setup allowing the blinded exposure of cell monolayers to UMTS signal schemes was developed and dosimetrically analysed. Cells can be exposed to up to 17 W/kg/W with less than 26% nonuniformity of SAR. The temperature load for the exposed cells is less than 0.03°C /(W/kg). The UMTS specifications have been analysed in order to identify ELF spectral components in the signal. These mainly result from inner loop power control; however, pulsed signal structures due to compressed mode and PRACH/PCPCH procedures also contribute to the ELF components. A test signal is proposed which is compliant to the 3GPP FDD modulation specifications and is optimised for maximized ELF spectral power (1 Hz harmonics).

Quality control for the entire duration of the project is ensured due to automatically generated data files. Exposure field strength, temperature, fan currents and all settings and computer commands are stored in the data files with a sampling rate of 0.1 Hz. Evaluation reports are available for every experiment performed in the REFLEX consortium.

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Attachment 14

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THE GEEK SYNDROME

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Autism – and its milder cousin Asperger's syndrome – is surging among the children of Silicon Valley. Are math-and-tech genes to blame?

Nick is building a universe on his computer. He's already mapped out his first planet: an anvil-shaped world called Denthaim that is home to gnomes and gods, along with a three-gendered race known as kiman. As he tells me about his universe, Nick looks up at the ceiling, humming fragments of a melody over and over. "I'm thinking of making magic a form of quantum physics, but I haven't decided yet, actually," he explains. The music of his speech is pitched high, alternately poetic and pedantic – as if the soul of an Oxford don has been awkwardly reincarnated in the body of a chubby, rosy-cheeked boy from Silicon Valley. Nick is 11 years old.

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Nick's father is a software engineer, and his mother is a computer programmer. They've known that Nick was an unusual child for a long time. He's infatuated with fantasy novels, but he has a hard time reading people. Clearly bright and imaginative, he has no friends his own age. His inability to pick up on hidden agendas makes him easy prey to certain cruelties, as when some kids paid him a few dollars to wear a ridiculous outfit to school.

One therapist suggested that Nick was suffering from an anxiety disorder. Another said he had a speech impediment. Then his mother read a book called Asperger's Syndrome: A Guide for Parents and Professionals. In it, psychologist Tony Attwood describes children who lack basic social and motor skills, seem unable to decode body language and sense the feelings of others, avoid eye contact, and frequently launch into monologues about narrowly defined – and often highly technical – interests. Even when very young, these children become obsessed with order, arranging their toys in a regimented fashion on the floor and flying into tantrums when their routines are disturbed. As teenagers, they're prone to getting into trouble with teachers and other figures of authority, partly because the subtle cues that define societal hierarchies are invisible to them.

"I thought, 'That's Nick,'" his mother recalls.

Asperger's syndrome is one of the disorders on the autistic spectrum – a milder form of the condition that afflicted Raymond Babbitt, the character played by Dustin Hoffman in Rain Man. In the taxonomy of autism, those with Asperger's syndrome have average – or even very high – IQs, while 70 percent of those with other autistic disorders suffer from mild to severe mental retardation. One of the estimated 450,000 people in the US living with autism, Nick is more fortunate than most.

He can read, write, and speak. He'll be able to live and work on his own. Once he gets out of junior high hell, it's not hard to imagine Nick creating a niche for himself in all his exuberant strangeness. At the less fortunate end of the spectrum are what diagnosticians call "profoundly affected" children. If not forcibly engaged, these children spend their waking hours in trancelike states, staring at lights, rocking, making high-pitched squeaks, and flapping their hands, repetitively stimulating ("stimming") their miswired nervous systems.

In one of the uncanny synchronicities of science, autism was first recognized on two continents nearly simultaneously. In 1943, a child psychiatrist named Leo Kanner published a monograph outlining a curious set of behaviors he noticed in 11 children at the Johns Hopkins Hospital in Baltimore. A year later, a pediatrician in Vienna named Hans Asperger, who had never seen Kanner's work, published a paper describing four children who shared many of the same traits. Both Kanner and Asperger gave the condition the same name: autism – from the Greek word for self, autòs – because the children in their care seemed to withdraw into iron-walled universes of their own.

Kanner went on to launch the field of child psychiatry in the US, while Asperger's clinic was destroyed by a shower of Allied bombs. Over the next 40 years, Kanner became widely known as the author of the canonical textbook in his field, in which he classified autism as a subset of childhood schizophrenia. Asperger was virtually ignored outside of Europe and died in 1980. The term Asperger syndrome wasn't coined until a year later, by UK psychologist Lorna Wing, and Asperger's original paper wasn't even translated into English until 1991. Wing built upon Asperger's intuition that even certain gifted children might also be autistic. She described the disorder as a continuum that "ranges from the most profoundly physically and mentally retarded person ... to the most able, highly intelligent person with social impairment in its subtlest form as his only disability.

It overlaps with learning disabilities and shades into eccentric normality."

Asperger's notion of a continuum that embraces both smart, geeky kids like Nick and those with so-called classic or profound autism has been accepted by the medical establishment only in the last decade. Like most distinctions in the world of childhood developmental disorders, the line between classic autism and Asperger's syndrome is hazy, shifting with the state of diagnostic opinion. Autism was added to the American Psychiatric Association's Diagnostic and Statistical Manual of Mental Disorders in 1980, but Asperger's syndrome wasn't included as a separate disorder until the fourth edition in 1994. The taxonomy is further complicated by the fact that few if any people who have Asperger's syndrome will exhibit all of the behaviors listed in the DSM-IV. (The syn in syndrome derives from the same root as the syn in synchronicity -- the word means that certain symptoms tend to cluster together, but all need not be present to make the diagnosis.) Though Asperger's syndrome is less disabling than "low-functioning" forms of autism, kids who have it suffer difficulties in the same areas as classically autistic children do: social interactions, motor skills, sensory processing, and a tendency toward repetitive behavior.

In the last 20 years, significant advances have been made in developing methods of behavioral training that help autistic children find ways to communicate. These techniques, however, require prodigious amounts of persistence, time, money, and love. Though more than half a century has passed since Kanner and Asperger first gave a name to autism, there is still no known cause, no miracle drug, and no cure.

And now, something dark and unsettling is happening in Silicon Valley.

In the past decade, there has been a significant surge in the number of kids diagnosed with autism throughout California. In August 1993, there were 4,911 cases of so-called level-one autism

logged in the state's Department of Developmental Services client-management system. This figure doesn't include kids with Asperger's syndrome, like Nick, but only those who have received a diagnosis of classic autism. In the mid-'90s, this caseload started spiraling up. In 1999, the number of clients was more than double what it had been six years earlier. Then the curve started spiking. By July 2001, there were 15,441 clients in the DDS database. Now there are more than seven new cases of level-one autism – 85 percent of them children – entering the system every day.

Through the '90s, cases tripled in California. "Anyone who says this is due to better diagnostics has his head in the sand."

California is not alone. Rates of both classic autism and Asperger's syndrome are going up all over the world, which is certainly cause for alarm and for the urgent mobilization of research. Autism was once considered a very rare disorder, occurring in one out of every 10,000 births. Now it's understood to be much more common – perhaps 20 times more. But according to local authorities, the picture in California is particularly bleak in Santa Clara County. Here in Silicon Valley, family support services provided by the DDS are brokered by the San Andreas Regional Center, one of 21 such centers in the state. SARC dispenses desperately needed resources (such as in-home behavioral training, educational aides, and respite care) to families in four counties. While the autistic caseload is rising in all four, the percentage of cases of classic autism among the total client population in Santa Clara County is higher enough to be worrisome, says SARC's director, Santi Rogers.

"There's a significant difference, and no signs that it's abating," says Rogers. "We've been watching these numbers for years. We

feared that something like this was coming. But this is a burst that has staggered us in our steps."

It's not easy to arrive at a clear picture of whether there actually is a startling rise in the incidence of autism in California, as opposed to just an increase in diagnoses. One problem, says Linda Lotspeich, director of the Stanford Pervasive Developmental Disorders Clinic, is that "the rules in the DSM-IV don't work." The diagnostic criteria are subjective, like "Marked impairment in the use of nonverbal behaviors such as eye-to-eye gaze, facial expression, body posture, and gestures to regulate social interaction."

"How much 'eye-to-eye gaze' do you have to have to be normal?" asks Lotspeich. "How do you define what 'marked' is? In shades of gray, when does black become white?"

Some children will receive a diagnosis of classic autism, and another diagnosis of Asperger's syndrome, from two different clinicians. Tony Attwood's advice to parents is strictly practical: "Use the diagnosis that provides the services."

While diagnostic fuzziness may be contributing to a pervasive sense that autism is on the rise, Ron Huff, the consulting psychologist for the DDS who uncovered the statistical trend, does not believe that all we're seeing now is an increase in children who would have previously been tagged with some other disability, such as mental retardation – or overlooked as perfectly healthy, if quirky, kids.

"While we certainly need to do more research," says Huff, "I don't think the change in diagnostic criteria will account for all of this rise by any means."

The department is making its data available to the MIND Institute at the University of California at Davis, to tease out what's behind the numbers. The results of that research will be published next year. But the effects of a surging influx are already rippling through the local schools. Carol Zepecki, director of student services and special education for the Palo Alto Unified School District, is disturbed by what she's seeing. "To be honest with you, as I look back on the special-ed students I've worked with for 20 years, it's clear to me that these kids would not have been placed in another category. The numbers are definitely higher." Elizabeth Rochin, a special-ed teacher at Cupertino High, says local educators are scrambling to create new resources. "We know it's happening, because they're coming through our schools. Our director saw the iceberg approaching and said, 'We've got to build something for them.'"

The people scrambling hardest are parents. In-home therapy alone can cost $60,000 or more a year, and requires so much dedication that parents (particularly mothers) are often forced to quit their jobs and make managing a team of specialists their new 80-hour-a-week career. Before their children become eligible for state funding, parents must obtain a diagnosis from a qualified clinician, which requires hours of testing and observation. Local facilities, such as the Stanford Pervasive Development Disorders Clinic and its counterpart at UC San Francisco, are swamped. The Stanford clinic is able to perform only two or three diagnoses a week. It currently has a two- to six-month waiting list.

For Rick Rollens, former secretary of the California Senate and cofounder of the MIND Institute, the notion that there is a frightening increase in autism worldwide is no longer in question. "Anyone who says this epidemic is due to better diagnostics," he says, "has his head in the sand."

Autism's insidious style of onset is particularly cruel to parents, because for the first two years of life, nothing seems to be wrong.

Their child is engaged with the world, progressing normally, taking first steps into language. Then, suddenly, some unknown cascade of neurological events washes it all away.

One father of an autistic child, Jonathan Shestack, describes what happened to his son, Dov, as "watching our sweet, beautiful boy disappear in front of our eyes." At two, Dov's first words – Mom, Dad, flower, park – abruptly retreated into silence. Over the next six months, Dov ceased to recognize his own name and the faces of his parents. It took Dov a year of intensive behavioral therapy to learn how to point. At age 9, after the most effective interventions available (such as the step-by-step behavioral training methods developed by Ivar Lovaas at UCLA), Dov can speak 20 words.

Even children who make significant progress require levels of day-to-day attention from their families that can best be described as heroic. Marnin Kligfeld is the founder of a software mergers-and-acquisitions firm. His wife, Margo Estrin, a doctor of internal medicine, is the daughter of Gerald Estrin, who was a mentor to many of the original architects of the Internet (see "Meet the Bellbusters," Wired 9.11, page 164). When their daughter, Leah, was 3, a pediatrician at Oakland Children's Hospital looked at her on the examining table and declared, "There is very little difference between your daughter and an animal. We have no idea what she will be able to do in the future." After eight years of interventions – behavioral training, occupational therapy, speech therapy – Leah is a happy, upbeat 11-year-old who downloads her favorite songs by the hundreds. And she is still deeply autistic.

Leah's first visit to the dentist required weeks of preparation, because autistic people are made deeply anxious by any change

in routine. "We took pictures of the dentist's office and the staff, and drove Leah past the office several times," Kligfeld recalls. "Our dentist scheduled us for the end of the day, when there were no other patients, and set goals with us. The goal of the first session was to have Leah sit in the chair. The second session was so Leah could rehearse the steps involved in treatment without actually doing them. The dentist gave all of his equipment special names for her. Throughout this process, we used a large mirror so Leah could see exactly what was being done, to ensure that there were no surprises."

Daily ordeals like this, common in the autistic community, underline the folly of the hypothesis that prevailed among psychologists 20 years ago, who were convinced that autism was caused by a lack of parental affection. The influential psychiatrist Bruno Bettelheim aggressively promoted a theory that has come to be known as the "refrigerator mother" hypothesis. He declared in his best-selling book, The Empty Fortress, "The precipitating factor in infantile autism is the parent's wish that his child should not exist. ... To this the child responds with massive withdrawal." He prescribed "parentectomy" – removal of the child from the parents – and years of family therapy. His hypothesis added the burden of guilt to the grief of having an autistic child, and made autism a source of shame and secrecy, which hampered efforts to obtain clinical data. The hypothesis has been thoroughly discredited. Richard Pollak's The Creation of Dr. B exposed Bettelheim as a brilliant liar who concocted case histories and exaggerated both his experience with autistic children and the success of his treatments.

One thing nearly everyone in the field agrees on: genetic predisposition. Identical twins share the disorder 9 times out of 10.

But the debates about the causes of autism are certainly not over. Controversies rage about whether environmental factors – such as mercury and

other chemicals in universally administered vaccines, industrial pollutants in air and water, and even certain foods – act as catalysts that trigger the disorder. Bernard Rimland, the first psychologist to oppose Bettelheim and promote the idea that autism was organic in origin, has become a leading advocate for intensified investigation in this area. The father of an autistic son, Rimland has been instrumental in marshaling medical expertise and family data to create better assessment protocols.

The one thing that almost all researchers in the field agree on is that genetic predisposition plays a crucial role in laying the neurological foundations of autism in most cases. Studies have shown that if one identical twin is autistic, there's a 90 percent chance that the other twin will also have the disorder. If parents have had one autistic child, the risk of their second child being autistic rises from 1 in 500 to 1 in 20. After two children with the disorder, the sobering odds are 1 in 3. (So many parents refrain from having more offspring after one autistic child, geneticists even have a term for it: stoppage.) The chances that the siblings of an autistic child will display one or more of the other developmental disorders with a known genetic basis – such as dyslexia or Tourette's syndrome – are also significantly higher than normal.

The bad news from Santa Clara County raises an inescapable question. Unless the genetic hypothesis is proven false, which is unlikely, regions with a higher than normal distribution of people on the autistic spectrum are something no researcher could ask for: living laboratories for the study of genetic expression. When the rain that fell on the Rain Man falls harder on certain communities than others, what becomes of the children?

The answer may be raining all over Silicon Valley. And one of the best hopes of finding a cure may be locked in the DNA sequences

It's a familiar joke in the industry that many of the hardcore programmers in IT strongholds like Intel, Adobe, and Silicon Graphics – coming to work early, leaving late, sucking down Big Gulps in their cubicles while they code for hours – are residing somewhere in Asperger's domain. Kathryn Stewart, director of the Orion Academy, a high school for high-functioning kids in Moraga, California, calls Asperger's syndrome "the engineers' disorder." Bill Gates is regularly diagnosed in the press: His single-minded focus on technical minutiae, rocking motions, and flat tone of voice are all suggestive of an adult with some trace of the disorder. Dov's father told me that his friends in the Valley say many of their coworkers "could be diagnosed with ODD – they're odd." In Microserfs, novelist Douglas Coupland observes, "I think all tech people are slightly autistic."

Though no one has tried to convince the Valley's best and brightest to sign up for batteries of tests, the culture of the area has subtly evolved to meet the social needs of adults in high-functioning regions of the spectrum. In the geek warrens of engineering and R&D, social graces are beside the point. You can be as off-the-wall as you want to be, but if your code is bulletproof, no one's going to point out that you've been wearing the same shirt for two weeks. Autistic people have a hard time multitasking – particularly when one of the channels is face-to-face communication. Replacing the hubbub of the traditional office with a screen and an email address inserts a controllable interface between a programmer and the chaos of everyday life. Flattened workplace hierarchies are more comfortable for those who find it hard to read social cues. A WYSIWYG world, where

respect and rewards are based strictly on merit, is an Asperger's dream.

Obviously, this kind of accommodation is not unique to the Valley. The halls of academe have long been a forgiving environment for absentminded professors. Temple Grandin – the inspiring and accomplished autistic woman profiled in Oliver Sacks' An Anthropologist on Mars – calls NASA the largest sheltered workshop in the world.

A recurring theme in case histories of autism, going all the way back to Kanner's and Asperger's original monographs, is an attraction to highly organized systems and complex machines. There's even a perennial cast of hackers: early adopters with a subversive streak. In 1944, Asperger wrote of a boy "chemist [who] uses all his money for experiments which often horrify his family and even steals to fund them." Another boy proved a mathematical error in Isaac Newton's calculations while he was still a freshman in college. A third escaped neighborhood bullies by taking lessons from an old watchmaker. And a fourth, wrote Asperger, "came to be preoccupied with fantastic inventions, such as spaceships and the like." Here he added, "one observes how remote from reality autistic interests really are" – a comment he qualified years later, when spaceships were no longer remote or fantastic, by joking that the inventors of spaceships might themselves be autistic.

Clumsy and easily overwhelmed in the physical world, autistic minds soar in the virtual realms of mathematics, symbols, and code. Asperger compared the children in his clinic to calculating machines: "intelligent automata" – a metaphor employed by many autistic people themselves to describe their own rule-based, image-driven thought processes. In her autobiography, Thinking in Pictures, Grandin compares her mind to a VCR. When she hears the word dog, she mentally replays what she calls "videotapes" of various dogs that she's seen, to arrive at something close to the average person's abstract notion of the

category that includes all dogs. This visual concreteness has been a boon to her work as a designer of more humane machinery for handling livestock. Grandin sees the machines in her head and sets them running, debugging as she goes. When the design in her mind does everything it's supposed to, she draws a blueprint of what she sees.

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These days, the autistic fascinations with technology, ordered systems, visual modes of thinking, and subversive creativity have plenty of outlets. There's even a cheeky Asperger's term for the rest of us – NTs, "neurotypicals." Many children on the spectrum become obsessed with VCRs, Pokémon, and computer games, working the joysticks until blisters appear on their fingers. (In the diagnostic lexicon, this kind of relentless behavior is called "perseveration.") Even when playing alongside someone their own age, however, autistic kids tend to play separately. Echoing Asperger, the director of the clinic in San Jose where I met Nick, Michelle Garcia Winner, suggests that "Pokémon must have been invented by a team of Japanese engineers with Asperger." Attwood writes that computers "are an ideal interest for a person with Asperger's syndrome ... they are logical, consistent, and not prone to moods."

This affinity for computers gives teachers and parents leverage they can use to build on the natural strengths of autistic children. Many teenagers who lack the motor skills to write by hand find it easier to use a keyboard. At Orion Academy, every student is required to buy an iBook fitted with an AirPort card. Class notes are written on electronic whiteboards that port the instructional materials to the school server for retrieval. (At lunch, the iBooks

are shut off, and if the kids want to play a two-person game, they're directed to a chess board.) The next generation of assistive technology is being designed by Neil Scott's Archimedes Project at Stanford. Scott's team is currently developing the equivalent of a PDA for autistic kids, able to parse subtle movements of an eyebrow or fingertip into streams of text, voice, or images. The devices will incorporate video cameras, head and eye tracking, intelligent agents, and speech recognition to suit the needs of the individual child.

The Valley is a self-selecting community where passionately bright people migrate from all over the world to make smart machines work smarter. The nuts-and-bolts practicality of hard labor among the bits appeals to the predilections of the high-functioning autistic mind. The hidden cost of building enclaves like this, however, may be lurking in the findings of nearly every major genetic study of autism in the last 10 years. Over and over again, researchers have concluded that the DNA scripts for autism are probably passed down not only by relatives who are classically autistic, but by those who display only a few typically autistic behaviors. (Geneticists call those who don't fit into the diagnostic pigeonholes "broad autistic phenotypes.")

The chilling possibility is that what's happening now is the first proof that the genes responsible for bestowing certain special gifts on slightly autistic adults – the very abilities that have made them dreamers and architects of our technological future – are capable of bringing a plague down on the best minds of the next generation. For parents employed in prominent IT firms here, the news of increased diagnoses of autism in their ranks is a confirmation of rumors that have quietly circulated for months. Every day, more and more of their coworkers are running into one another in the waiting rooms of local clinics, taking the first uncertain steps on a journey with their children that lasts for the rest of their lives.

In previous eras, even those who recognized early that autism might have a genetic underpinning considered it a disorder that only moved diagonally down branches of a family tree. Direct inheritance was almost out of the question, because autistic people rarely had children. The profoundly affected spent their lives in institutions, and those with Asperger's syndrome tended to be loners. They were the strange uncle who droned on in a tuneless voice, tending his private logs of baseball statistics or military arcana; the cousin who never married, celibate by choice, fussy about the arrangement of her things, who spoke in a lexicon mined reading dictionaries cover to cover.

The old line "insanity is hereditary, you get it from your kids" has a twist in the autistic world. It has become commonplace for parents to diagnose themselves as having Asperger's syndrome, or to pinpoint other relatives living on the spectrum, only after their own children have been diagnosed.

High tech hot spots like the Valley, and Route 128 outside of Boston, are a curious oxymoron: They're fraternal associations of loners. In these places, if you're a geek living in the high-functioning regions of the spectrum, your chances of meeting someone who shares your perseverating obsession (think Linux or Star Trek) are greatly expanded. As more women enter the IT workplace, guys who might never have had a prayer of finding a kindred spirit suddenly discover that she's hacking Perl scripts in the next cubicle.

One provocative hypothesis that might account for the rise of spectrum disorders in technically adept communities like Silicon Valley, some geneticists speculate, is an increase in assortative mating. Superficially, assortative mating is the blond gentleman who prefers blondes; the hyperverbal intellectual who meets her soul mate in the therapist's waiting room. There are additional

pressures and incentives for autistic people to find companionship – if they wish to do so – with someone who is also on the spectrum. Grandin writes, "Marriages work out best when two people with autism marry or when a person marries a handicapped or eccentric spouse.... They are attracted because their intellects work on a similar wavelength."

That's not to say that geeks, even autistic ones, are attracted only to other geeks. Compensatory unions of opposites also thrive along the continuum, and in the last 10 years, geekitude has become sexy and associated with financial success. The lone-wolf programmer may be the research director of a major company, managing the back end of an IT empire at a comfortable remove from the actual clients. Says Bryna Siegel, author of The World of the Autistic Child and director of the PDD clinic at UCSF, "In another historical time, these men would have become monks, developing new ink for early printing presses. Suddenly they're making $150,000 a year with stock options. They're reproducing at a much higher rate."

Genetic hypotheses like these don't rule out environmental factors playing a role in the rising numbers. Autism is almost certainly not caused by the action of a single gene, but by some orchestration of multiple genes that may make the developing child more susceptible to a trigger in the environment. One consequence of increased reproduction among people carrying some of these genes might be to boost "genetic loading" in successive generations – leaving them more vulnerable to threats posed by toxins in vaccines, candida, or any number of agents lurking in the industrialized world.

At clinics and schools in the Valley, the observation that most parents of autistic kids are engineers and programmers who themselves display autistic behavior is not news. And it may not be news to other communities either. Last January, Microsoft became the first major US corporation to offer its employees insurance benefits to cover the cost of behavioral training for

their autistic children. One Bay Area mother told me that when she was planning a move to Minnesota with her son, who has Asperger's syndrome, she asked the school district there if they could meet her son's needs. "They told me that the northwest quadrant of Rochester, where the IBMers congregate, has a large number of Asperger kids," she recalls. "It was recommended I move to that part of town."

For Dov's parents, Jonathan Shestack and Portia Iversen, Silicon Valley is the only place on Earth with enough critical mass of supercomputing resources, bio-informatics expertise, genomics savvy, pharmaceutical muscle, and VC dollars to boost autism research to the next phase. For six years, the organization they founded, Cure Autism Now, has led a focused assault on the iron-walled fortress of the medical establishment, including the creation of its own bank of DNA samples, available to any scientist in the field on a Web site called the Autism Genetic Resources Exchange (see "The Citizen Scientists," Wired 9.09, page 144).

At least a third of CAN's funding comes from donors in the Valley. Now Shestack and Iversen want to deliver the ultimate return on that investment: better treatments, smarter assistive technology – and, eventually, a cure.

"We have the human data," says Shestack. "Now we need the brute-force processing power. We need high-density SNP mapping and microarray analysis so we can design pharmaceutical interventions. We need Big Pharma to wake up to the fact that while 450,000 people in America may not be as large a market as for cholesterol drugs, we're talking about a demand for new products that will be needed from age 2 to age 70. We need new technology that measures modes of perception,

and tools for neural retraining. And we need a Web site where families with a newly diagnosed kid can plug into a network of therapists in their town who have been rated by buyers – just like eBay."

The ultimate hack for a team of Valley programmers may turn out to be cracking the genetic code that makes them so good at what they do. Taking on that challenge will require extensive use of technology invented by two people who think in pictures: Bill Dreyer, who invented the first protein sequencer, and Carver Mead, the father of very large scale integrated circuits. As Dreyer explains, "I think in three-dimensional Technicolor." Neither Mead nor Dreyer is autistic, but there is a word for the way they think – dyslexic. Like autism, dyslexia seems to move down genetic pathways. Dreyer has three daughters who think in Technicolor.

One of the things that Dan Geschwind, director of the neurogenetics lab at UCLA, finds fascinating about dyslexia and autism is what they suggest about human intelligence: that certain kinds of excellence might require not just various modes of thinking, but different kinds of brains.

"Autism gets to fundamental issues of how we view talents and disabilities," he says. "The flip side of dyslexia is enhanced abilities in math and architecture. There may be an aspect of this going on with autism and assortative mating in places like Silicon Valley. In the parents, who carry a few of the genes, they're a good thing. In the kids, who carry too many, it's very bad."

Issues like this were at the crux of arguments that Bryna Siegel had with Bruno Bettelheim in a Stanford graduate seminar in the early '80s, published in Bettelheim's The Art of the Obvious. (Siegel's name was changed to Dan Berenson.) The text makes poignant reading, as two paradigms of scientific humanism clash in the night. Siegel told "Dr. B" that she wanted to do a large study of children with various developmental disorders to search

for a shared biochemical defect. Bettelheim shot back that if such a marker were to be uncovered it would dehumanize autistic children, by making them essentially different from ourselves.

Still an iconoclast, Siegel questions whether a "cure" for autism could ever be found. "The genetics of autism may turn out to be no simpler to unravel than the genetics of personality. I think what we'll end up with is something more like, 'Mrs. Smith, here are the results of your amnio. There's a 1 in 10 chance that you'll have an autistic child, or the next Bill Gates. Would you like to have an abortion?'"

For UCSF neurologist Kirk Wilhelmsen – who describes himself and his son as being "somewhere on that grand spectrum" – such statements cut to the heart of the most difficult issue that autism raises for society. It may be that autistic people are essentially different from "normal" people, he says, and that it is precisely those differences that make them invaluable to the ongoing evolution of the human race.

"If we could eliminate the genes for things like autism, I think it would be disastrous," says Wilhelmsen. "The healthiest state for a gene pool is maximum diversity of things that might be good."

One of the first people to intuit the significance of this was Asperger himself – weaving his continuum like a protective blanket over the young patients in his clinic as the Nazis shipped so-called mental defectives to the camps. "It seems that for success in science and art," he wrote, "a dash of autism is essential."

For all we know, the first tools on earth might have been developed by a loner sitting at the back of the cave, chipping at thousands of rocks to find the one that made the sharpest spear, while the neurotypicals chattered away in the firelight. Perhaps certain arcane systems of logic, mathematics, music, and stories – particularly remote and fantastic ones – have been passed down from phenotype to phenotype, in parallel with the DNA

that helped shape minds which would know exactly what to do with these strange and elegant creations.

Hanging on the wall of Bryna Siegel's clinic in San Francisco is a painting of a Victorian house at night, by Jessy Park, an autistic woman whose mother, Clara Claiborne Park, wrote one of the first accounts of raising a child with autism, The Siege. Now 40, Jessy still lives at home. In her recent book, Exiting Nirvana, Clara writes of having come to a profound sense of peace with all the ways that Jessy is.

Jessy sent Siegel a letter with her painting, in flowing handwriting and words that are – there is no other way to say it – marvelously autistic. "The lunar eclipse with 92% cover is below Cassiopeia. In the upper right-hand corner is Aurora Borealis. There are three sets of six-color pastel rainbow on the shingles, seven-color bright rainbow on the clapboards next to the drain pipe, six-color paler pastel rainbow around the circular window, six-color darker pastel rainbow on the rosette ..."

But the words aren't the thing. Jessy's painting is the thing. Our world, but not our world. A house under the night sky shining in all the colors of the spectrum.

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