

# Exposure of Rat Brain to 915 MHz GSM Microwaves Induces Changes in Gene Expression But Not Double Stranded DNA Breaks or Effects on Chromatin Conformation

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We investigated whether exposure of rat brain to microwaves (MWs) of global system for mobile communication (GSM) induces DNA breaks, changes in chromatin conformation and in gene expression. An exposure installation was used based on a test mobile phone employing a GSM signal at 915 MHz, all standard modulations included, output power level in pulses 2 W, specific absorption rate (SAR) 0.4 mW/g. Rats were exposed or sham exposed to MWs during 2 h. After exposure, cell suspensions were prepared from brain samples, as well as from spleen and thymus. For analysis of gene expression patterns, total RNA was extracted from cerebellum. Changes in chromatin conformation, which are indicative of stress response and genotoxic effects, were measured by the method of anomalous viscosity time dependencies (AVTD). DNA double strand breaks (DSBs) were analyzed by pulsed-field gel electrophoresis (PFGE). Effects of MW exposure were observed on neither conformation of chromatin nor DNA DSBs. Gene expression profiles were obtained by Affymetrix U34 GeneChips representing 8800 rat genes and analyzed with the Affymetrix Microarray Suite (MAS) 5.0 software. In cerebellum from all exposed animals, 11 genes were upregulated in a range of 1.34–2.74 fold and one gene was downregulated 0.48-fold ( $P < .0025$ ). The induced genes encode proteins with diverse functions including neurotransmitter regulation, blood–brain barrier (BBB), and melatonin production. The data shows that GSM MWs at 915 MHz did not induce PFGE-detectable DNA double stranded breaks or changes in chromatin conformation, but affected expression of genes in rat brain cells. *Bioelectromagnetics* 27:295–306, 2006. © 2006 Wiley-Liss, Inc.

**Key words:** gene microarrays; mobile phones; blood–brain barrier; neurotransmitter regulation; melatonin

## INTRODUCTION

The growing concern about possible effects on the brain after microwave (MW) exposure from mobile phones, especially in children, have been discussed in many countries because of increasing usage of wireless communication systems. Various effects of MWs from mobile phones on the central nervous system, including permeability of the blood–brain barrier (BBB), neuronal electrical activity, neurotransmitter balance, cognitive function, and sleep have recently been reviewed [Hossmann and Hermann, 2003]. It has been reported that increased incidence of brain tumors and acoustic neurinoma is correlated with exposure to mobile phone MWs depending on duration of mobile phone use [Hardell et al., 2003; Lonn et al., 2004; Hardell et al.,

2005]. Other groups reported no correlation between brain tumors and MWs from mobile phones, but the

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results of these epidemiological studies are not directly comparable because of methodological and other differences, as has recently been reviewed [Kundi et al., 2004; Belyaev, 2005].

There is growing evidence for nonthermal effects of MWs that suggests possible relationship of MW exposure with adverse health effects [Navarro et al., 2003; Santini et al., 2003; Hallberg and Johansson, 2004], immune response in human lymphocytes [Dabrowski et al., 2003], permeability of BBB in rat brains [Persson et al., 1997], increase in calcium ion efflux and ornithine decarboxylase (ODC) activity in brains of exposed rats [Paulraj and Behari, 2002], release of manganese and zinc ions in the serum of the exposed rats [Aksen et al., 2004], decreases in the reproductive capacity of *Drosophila melanogaster* [Panagopoulos et al., 2004], formation of dicentric chromosomes among mobile users [Gadhia et al., 2003], gene expression in human skin fibroblasts [Pacini et al., 2002], and stress response in human epithelial amnion (AMA) cells [Kwee et al., 2001]. Recent data provided evidence for neuronal damage in both the cortex, the hippocampus and the basal ganglia in the brains of exposed rats [Salford et al., 2003; Ilhan et al., 2004]. Although exposure at 0.6 W/kg alone did not affect ultrastructure of neurons in rat brains, exposure of 3-nitropropionic acid (3-NP)—treated animals reduced the effects of 3-NP [Seaman and Phelix, 2005].

Accumulating evidences suggest that nonthermal MW effects do occur, but only under specific parameters of exposure depending on several physical and biological variables [Blackman et al., 1989; Adey, 1999; Pakhomov and Murphy, 2000; Binhi, 2002; Banik et al., 2003; Mileva et al., 2003; Belyaev, 2005]. Such dependences may account for various outcomes from studies employing various physical and biological variables. There is substantial lack of knowledge in biophysical modeling of MW-induced nonthermal biological effects [Adey, 1999; Binhi, 2002]. Such targets as DNA, radicals, proteins and ions in protein cavities may be involved in effects of MWs [Belyaev et al., 1992; Chiabrera et al., 2000; Binhi, 2002; de Pomerai et al., 2003; Ritz et al., 2004].

It has been reported that MWs induced DNA strand breaks in rat brain cells as measured by the single cell electrophoresis technique [Lai and Singh, 1997]. The mechanisms of this effect are not understood and could relate to the induced changes in interaction of DNA with proteins rather than DNA damage [Belyaev et al., 1999; Crumpton and Collins, 2004]. In the present study, we analyzed these possibilities using the pulsed-field gel electrophoresis (PFGE) and anomalous viscosity time dependencies (AVTD). PFGE is a widely

accepted technique to measure DNA double strand breaks and fragmentation in response to genotoxic agents. The AVTD assay measures changes in chromatin conformation, which are indicative of genotoxic effects and stress response [Belyaev et al., 1999, 2001]. In a related project, we found that GSM MWs at 915 MHz induce changes in chromatin conformation and inhibited formation of the DNA-repair 53BP1/ $\gamma$ -H2AX foci in human lymphocytes in vitro [Sarimov et al., 2004; Belyaev et al., 2005; Markova et al., 2005]. In the present in vivo investigation, we used the same conditions of MW exposure and investigated whether GSM MWs induce DNA breaks, change the chromatin conformation, and alter the gene expression in rat brains.

Induction of specific genes in cultured cells in response to mobile phone MWs has been reported [Goswami et al., 1999; Pacini et al., 2002]. Induction of the c-fos gene was observed in rat cerebral cortex at the thermal levels of exposure exceeding the specific absorbed rates (SARs), usually observed during mobile telephony [Hossmann and Hermann, 2003].

Screening of gene expression by microarray technology provides new powerful means to search for molecular pathways and to elucidate possible molecular markers of response of brain cells to MWs. However, no data are still available on microarray analysis of brains exposed to MWs from mobile phones in in vivo experiments. In this communication, we present the first pilot data showing that GSM MW exposure induce changes in gene expression as analyzed by microarray technique in rat brains exposed in vivo.

## MATERIALS AND METHODS

### Rats

Eight Fisher 344 male rats, 12 weeks old, weighing 190–260 g, were obtained from A-Lab (Stockholm, Sweden). The rats were maintained by continuous, single-line, brother/sister mating. All rats were kept under the same conditions in a temperature and humidity controlled environment. All animal procedures were in agreement with the Principles of Laboratory Animal Care (NIH publication No. 85-23 revised 1985) and the rules of the Swedish Board of Animal Research. Rats, four animals in each group, were either exposed or sham exposed to MWs for 2 h.

### Microwave Exposure

An installation employing a GSM signal was employed (Fig. 1). This installation was based on a GSM900 test mobile phone. The output of the phone

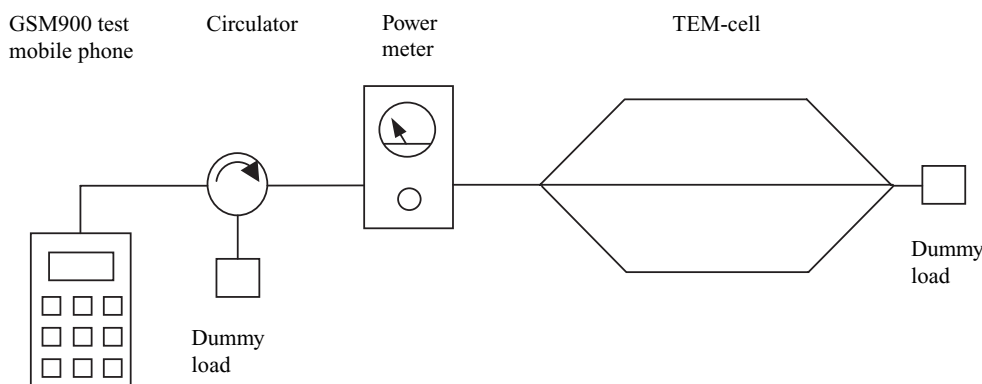


Fig. 1. Block-scheme for the exposure installation employing signal from GSM900 test mobile phone. The output of the phone was connected by coaxial cable to a TEM cell where exposure or sham exposure of rats was performed.

was connected by coaxial cable to a transverse electromagnetic transmission line cell (TEM cell), which has been described previously [Persson et al., 1997; Salford et al., 2003; Sarimov et al., 2004]. In principle, this is a spliced coaxial cable with a central electrode and an outer shield electrode with the unique characteristic of having both linear amplitude and phase response versus frequency. The fields of various waveforms, including continuous waves and pulsed (or modulated) fields, can be accurately generated in the TEM cell without the distortion that is typically introduced when conventional antennas are used to establish impulse test fields [Malmgren, 1998]. The construction of the TEM cell allows relatively homogeneous exposure of samples.

There are 124 different channels/frequencies that are used in GSM900 mobile communication. They differ by 0.2 MHz in the frequency range between 890 and 915 MHz. The test mobile phone was programmed to use channel 124 with the frequency of 915 MHz. The signal included all standard GSM modulations. No voice modulation was applied. A GSM signal is produced as 577  $\mu$ s pulses (time slots), with an interpulse waiting time of 4039  $\mu$ s (seven time slots). The test phone was programmed to regulate output power in the pulses in the range of 0.02–2 W (13–33 dBm). This power was kept constant during exposure at 33 dBm, as monitored online using a power meter (Bird 43, USA).

In our experiments, the average whole-body SAR was 0.4 mW/g and the discontinuous transmission mode (DTX) was off during all exposures. The SAR value was determined both by measurements and by calculations. Both the incident and the reflected power at the input and the transmitted power through the TEM cell were measured using a power meter (Hewlett-Packard 435A, USA) and a coaxial directional coupler (Narda 3001-20, USA). This was done for an input power of 1 W to the TEM cell and for rats of different

sizes. The absorbed power was then calculated and the average whole body SAR-value was determined for each rat size. There were very small variations of average whole body SAR for different rat sizes [Malmgren, 1998]. Numerical calculations were performed using the finite different time domain (FDTD) method [Van Hese et al., 1992; Martens et al., 1993]. It is impossible to provide an exact SAR distribution because the rats were not restrained during exposure inside the TEM cell. The SAR in the brains was found to vary less than four-fold from the average value of 0.4 mW/g in the numerical rat shaped phantom at different positions of the TEM cell. The calculated SAR values were in good agreement with the measured. Sham exposures were performed in the same TEM cell with MW power off. Four independent exposures and four sham exposures were performed during four consecutive days. Two animals were used in each session: one exposed to MWs and the other sham exposed. The order of MW and sham exposure was randomized among sessions. All exposures were performed at room temperature.

### Sample Preparations

For preparations of cell suspensions from brain tissues, essentially the same technique as described by Singh [1998] was used. This technique was developed to reduce the amount of DNA breaks in cells and the spin-trap compound N-tert-butyl- $\alpha$ -phenylnitron (PBN), 0.2 mM, was used at all steps of cell suspension preparation. After exposure the rats were anesthetized by i.p. injection of chloral hydrate 5% (0.6 ml/100 g body weight). The rats were placed on ice and the brains were perfused via ascending artery during 3 min, using cold PBS with spin trap PBN.

Afterwards, thymus and spleen were surgically removed; cerebellum was separated from the brains and

frozen in RNAlater™ (Ambion, Austin, TX, USA); and the rest of the brains were dissected into gray and white matter. Briefly, the brains were cut in the midline between the two cerebral hemispheres. Then the cortex with underlying white matter above the lateral ventricles, with the ventricles as cleaving plane was separated. With the two hemispheres cortex and underlying white matter including the corpus callosum, we used a bent thin forceps to lift away and collect the white matter from the inside of the cortex until we reached this. Thereby we could collect an amount of white matter, much less than the volume of the cortex proper, which was collected separately. No white matter was collected from other portions of the brain.

Cell suspensions were prepared from the gray and white matter, as well as from spleen and thymus. Cell suspensions from thymus and spleen were obtained as previously described [Belyaev and Harms-Ringdahl, 1996].

### Gene Microarrays

Total RNA was isolated from cerebellum of the same eight male rats using TRIzol Reagent (Gibco-BRL, Gaithersburg, MD), according to the manufacturer's instructions. Subsequently, the total RNA was purified using an RNeasy kit (Qiagen, Valencia, CA, USA). The OD<sub>260/280</sub> (interval 1.9–2.1) of the RNA was determined by spectrophotometry. The integrity of the RNA was assessed by Agilent 2100 Bioanalyser (Agilent Technologies, Palo Alto, CA, USA), and material without signs of degradation was used. The RNA was processed, labeled and hybridized to the Affymetrix U34A GeneChips in the Affymetrix core facility at NOVUM, Karolinska Institutet, Sweden. cDNA from each sample was synthesized using a T-7 linked oligo-dT primer, and cRNA was then synthesized with biotinylated UTP and CTP. Thereafter, the labeled RNA was fragmented and hybridized to Affymetrix U34 GeneChips representing 8800 genes (Affymetrix) according to Affymetrix's protocols. After hybridization, arrays were washed and stained with streptavidin-phycoerythrin (Molecular Probes, Eugene, OR, USA) in an Affymetrix fluidics station according to the manufacturer's protocol. The probed arrays were then scanned in the Affymetrix scanner. The expression and significance levels for each gene were calculated by using Affymetrix Microarray Suite (MAS) 5.0 software. All probes were scaled to a target intensity value of 100 to allow inter-array comparisons.

### AVTD Measurements

Cells were lysed in polyallomer centrifuge tubes (14 mm, Beckman) by addition of 3.1 ml lysis solution

(0.25 M Na<sub>2</sub>EDTA, 2% w/v sarcosyl, 10 mM Tris-base, pH 7.4) to 0.1 ml of cell suspension. Each tube contained  $3 \times 10^5$  cells. The lysates were prepared in triplicate and kept at 23 °C for 5 h in darkness before AVTD measurements. We also used lysis solution with 4% sarcosyl and results were essentially the same as for 2% lysis solution. Under these conditions of lysis, histones were released from DNA and the resulting DNA-protein complexes represented nuclear matrixes with DNA-loops attached (nucleoids). DNA integrity is preserved and DNA loops can be either relaxed or condensed by appropriate concentrations of ethidium bromide [Belyaev et al., 1999].

The changes in chromatin conformation were measured with the method of anomalous viscosity time dependencies (AVTD) as described previously [Belyaev et al., 1999]. Briefly, this method is based on radial migration of large DNA-protein complexes such as nucleoids in a high-gradient hydrodynamic field of a rotary viscometer [Dill and Shafer, 1976; Kriuchkov et al., 1995]. Radial migration of nucleoids toward the rotating rotor causes anomalous changes of viscosity that can be registered by measuring the rotor rotation period as a function of time. This anomalous viscosity time dependence strongly depends on conformation of nucleoids, molecular weight, and the number of proteins bound to DNA [Dill and Shafer, 1976; Kriuchkov et al., 1995]. Each AVTD is a set of experimental points (period of rotation vs. time of measurement), which are recorded by a personal computer. The AVTDs were measured in the cell lysates at a shear rate of 5.6/s and a shear stress of 0.007 N/m<sup>2</sup> using an AVTD-analyzer (Archer-Aquarius, Ltd., Moscow, Russia). The maximum period of rotation ( $T_{\max}$ ) corresponds to the maximum viscosity and has previously been shown to be the most sensitive AVTD parameter [Kriuchkov et al., 1995]. Each version of the experiment included not less than three AVTD measurements in different tubes. To characterize condensation of chromatin, we used maximal relative viscosity,  $MRV = (T_{\max \text{ ex}})/(T_{\max \text{ sh}})$ , where ( $T_{\max \text{ ex}}$ ) is a mean value in exposed samples and ( $T_{\max \text{ sh}}$ ) is a mean value in sham exposed samples.

### Pulsed-Field Gel Electrophoresis

DNA DSBs were analyzed by PFGE. PFGE grade agarose, *Saccharomyces cerevisiae* and *Saccharomyces pombe* markers were purchased from Bio-Rad (Richmond, CA, USA) and Proteinase K was from Boehringer Mannheim. PFGE was run using a CHEF-DR II apparatus (Bio-Rad) according to the manufacturer's recommendations. Briefly, after two washes with PBS the cells were mixed with 1% low melting point

agarose (Sigma, St. Louis, MO, USA). Agarose blocks were prepared in quadruplicate using 100  $\mu$ l plug moulds. Each plug contained approximately  $5 \times 10^5$  cells. The cells were lysed by incubation of agarose blocks in lysis solution (2% lauryl sarcosyl, 0.5 M EDTA, 10 mM Tris, pH 8.0 adjusted by NaOH) with 0.5 mg/ml proteinase K at 37 °C for 48 h. We also used lysis solution with 4% sarcosyl and the results were essentially the same as for 2% lysis solution. After being washed three times with TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0), blocks were loaded into the wells. PFGE runs were at 14 °C in  $0.5 \times$  TBE buffer in 0.9% agarose gel, run time 72 h, 60 V, and ramped pulse from 3000 to 600 s. On completion, the gels were stained with 0.5  $\mu$ g/ml ethidium bromide (EtBr), destained and images were acquired using a CCD camera (Molecular Dynamics). The plugs were removed from the wells and the amount of DNA released into the gels were analyzed based on EtBr fluorescence as described previously [Rosemann et al., 1993]. The integrated optical density (IOD) of DNA released into lanes was measured using Quantiscan (Biosoft, Cambridge, UK) software.

### Western Blot

The cells were lysed and proteins were separated by 12% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) using Mini-PROTEAN II Electrophoresis Cell according to the manufacturer's instruction manual (Bio-Rad, Richmond, CA). Total proteins were measured using SYPRO Ruby Protein Stain according to the manufacturer (BioRad). Transfer of the proteins onto the membrane was performed using Mini Trans-blot Electrophoretic Transfer Cell (BioRad). Western blot analysis was performed using the RPN 2108 ECL system according to manufacturer (Amersham Life Science, Buckinghamshire, UK) with some modifications. Membranes were incubated with the primary antibodies, 0.1  $\mu$ g/ml mouse monoclonal anti-hsp70 and 5  $\mu$ g/ml mouse monoclonal anti-actin antibodies from Scandinavian Diagnostics Services (Falkenberg, Sweden) and Boehringer Mannheim (Scandinavia AB, Bromma, Sweden), respectively. Secondary anti-mouse Ig antibody NA 931 (Amersham Life Science) was used at the concentration of 1  $\mu$ g/ml. Detection of the labeled antibodies was performed according to the manufacturer's manual for ECL Western blotting detection (Amersham Life Science). The images were obtained from membranes using a charge-coupled device (CCD) camera (FUJIFILM Luminescent Image Analyzer LAS-1000plus). Measurements of IOD were performed on the images using Quantiscan software.

### Statistical Analysis

The data were pooled from four independent exposures and compared with 4 sham exposures using nonparametric Mann–Whitney *U*-test and Wilcoxon matched pairs signed rank test. Results were considered as significantly different at  $P < .05$ .

## RESULTS

### Chromatin Conformation

The changes in chromatin conformation were measured by the AVTD technique (Table 1). The pooled data obtained with the AVTD technique are presented in Figure 2. A slight decrease in the AVTD was observed after MW exposure in splenocytes, but this decrease was not statistically significant (Mann–Whitney *U*-test,  $P > .05$ ). We assume that MW exposure under the chosen conditions of exposure did not affect conformation of chromatin as studied by the AVTD method.

### DNA Fragmentation

DNA DSBs were analyzed using PFGE assay. A representative PFGE image and the pooled data are shown in Figure 3. No MW-induced DNA double strand breaks were detected using PFGE in any of the four types of cells. Slight decrease in fragmented DNA was observed in samples prepared from gray matter of MW-exposed rats, but this decrease was not statistically significant (Mann–Whitney *U*-test,  $P > .05$ ). Thus, MWs induced no PFGE-detectable DNA fragmentation.

### Gene Expression

Affymetrix U34 GeneChips were used to analyze the gene expression in rat brains in response to MWs. RNA from brains of three exposed and three sham exposed animals were selected for microarray analysis based on RNA quality control. One sample from the MW-exposed rat did not pass the quality control and, therefore, was excluded from the analysis along with

**TABLE 1. The Mean Values of Maximum Period of Rotation ( $T_{\max}$ ) as Measured by AVTD in Lysates of Cells Prepared From Exposed and Sham Exposed Rats and the *P*-Values as Analyzed by the Mann–Whitney *U*-test**

| Tissue       | Sham exposure      |      | Exposure           |      | <i>P</i> |
|--------------|--------------------|------|--------------------|------|----------|
|              | $T_{\max}$ sh, sec | SD   | $T_{\max}$ ex, sec | SD   |          |
| Gray matter  | 11.1               | 4.46 | 11.57              | 3.12 | .83      |
| White matter | 11.66              | 2.61 | 11.49              | 2.02 | .89      |
| Spleen       | 24.23              | 3.2  | 19.48              | 3.83 | .15      |
| Thymus       | 31.22              | 4.93 | 31.19              | 2.13 | .97      |

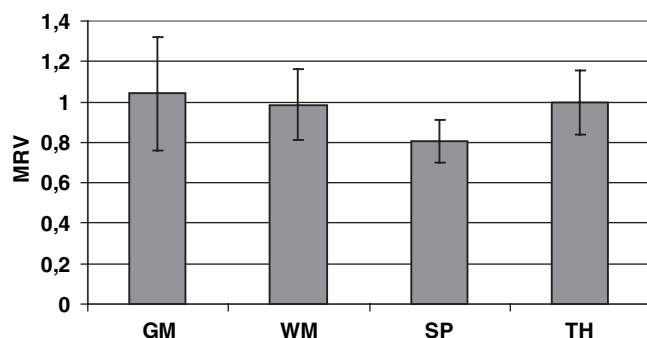


Fig. 2. Responses to GSM (915 MHz) as measured with the AVTD method in cells from gray matter (GM), white matter (WM), spleen (SP), and thymus (TH). Four Fisher rats were exposed to GSM, 2 h, and four rats were sham exposed. The measured parameter was maximum relative viscosity (MRV). The maximum viscosity in the lysates of exposed samples was normalized to the maximum viscosity in the sham exposed samples. Means of four independent exposures, each measured in triplicate, and standard deviations are shown. No statistically significant AVTD changes were seen after exposure to GSM.

the correspondent sham exposed sample. Affymetrix MAS 5.0 software was used for statistical analyses of the chips, including comparison in a three times three matrix. Genes belonging to different functional classes and a number of genes with unknown function were induced. Only those genes that were significantly increased or decreased (Wilcoxon's test,  $P < .0025$ ) in all nine comparisons in the three times three matrix are shown in Table 2. Eleven genes were upregulated in a range of 1.34–2.74 fold and one gene was down-regulated 0.48-fold (Fig. 4). The induced genes encode proteins with diverse functions (Table 2).

### Western Blot

Stress response proteins and particularly hsp70 was activated by MWs of mobile phones in some previous studies as measured by Western blot [Kwee et al., 2001; Weisbrot et al., 2003]. In order to see whether we could miss such activation using gene arrays the cells obtained from the brains were lysed and hsp70 protein was analyzed by Western blot (Fig. 5). No statistically significant changes were observed in hsp70 between

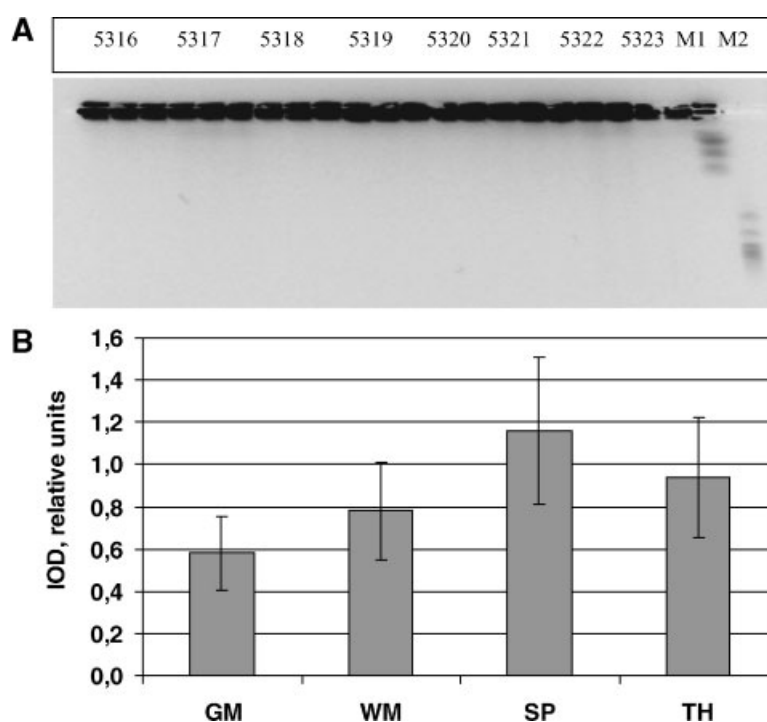


Fig. 3. DNA fragmentation was analyzed by the PFGE. Eight Fisher rats were exposed or sham exposed to GSM MWs, 915 MHz, 2 h. Numbers of exposed (5316, 5319, 5320, 5323) and sham exposed (5317, 5318, 5321, 5322) rats are shown. Cell suspensions were prepared from brain tissues, gray and white matter, as well as from thymus and spleen. **A:** Typical PFGE with brain cells from white matter is shown. Markers are *S. pombe* (M1, 3.5, 4.6, 5.7 Mbp) and *S. cerevisiae* (M2, 225 kbp–2.2 Mbp) chromosomal DNA. **B:** The IOD values of DNA released into lanes were measured using Quantiscan (Biosoft) software. The IOD in exposed samples were normalized to sham exposed samples. Mean values obtained in four independent exposures, each measured two times in duplicate or triplicate, and standard deviations are shown.

**TABLE 2. Changes in Gene Expression in Response to 915 MHz GSM MWs**

| Affymetrix probeset      | Fold change | Chromosomal location | Gene symbol        | Gene name                                    | Function                                                                                                                               |
|--------------------------|-------------|----------------------|--------------------|----------------------------------------------|----------------------------------------------------------------------------------------------------------------------------------------|
| <b>Metabolic enzymes</b> |             |                      |                    |                                              |                                                                                                                                        |
| Rc_AI014135_g_at         | 2.74 ± 1.13 | 13q13                | Mss4               | Mss4 protein                                 | MSS4 encodes a phosphatidylinositol-4-phosphate 5-kinase that synthesizes phosphatidylinositol (4,5)-bisphosphate.                     |
| J03481mRNA_at            | 1.64 ± 0.37 | 14q21                | Qdpr               | Quinoid dihydropteridine reductase           | Co-factor in the pterin-dependent aromatic amino acid hydroxylating systems. Involved in synthesis of monoaminergic neurotransmitters. |
| U01344_g_at              | 0.48 ± 0.13 | 16p14                | Nat1               | N-acetyltransferase 1                        | Detoxifies carcinogens and xenobiotics. Key role in the circadian rhythm of melatonin synthesis.                                       |
| <b>BBB function</b>      |             |                      |                    |                                              |                                                                                                                                        |
| M96601_at                | 1.56 ± 0.23 | 4q34                 | Slc6a6             | Solute carrier family 6, member 6            | Taurine transporter. Predominantly glial expression.                                                                                   |
| <b>Glia function</b>     |             |                      |                    |                                              |                                                                                                                                        |
| M22357_at                | 1.88 ± 0.51 | 1q21                 | Mag                | Myelin associated glycoprotein               | Involved in myelination and glia-neuron interactions.                                                                                  |
| AF030358_g_at            | 1.61 ± 0.26 | 19p12                | Cx3cl1             | Fractalkine (chemokine3X3C)                  | Activation and chemoattraction of microglia.                                                                                           |
| A03913cds_s_at           | 1.40 ± 0.11 | 9q34                 | GDN_RAT (Serpine2) | Glia-derived nexin-precursor                 | Serine protease inhibitor. Involved in synaptic plasticity.                                                                            |
| <b>Miscellaneous</b>     |             |                      |                    |                                              |                                                                                                                                        |
| AF036548_at              | 1.91 ± 0.67 | 15q12                | Rgc-32             | Rgc32 protein                                | Cell cycle regulatory factor.                                                                                                          |
| L13619_g_at              | 1.81 ± 0.74 | 4q11                 | ISI1_RAT (Insig-1) | Insulin induced growth response protein CL-6 | Key regulator of cholesterol biosynthesis by interaction with HMG-CoA synthetase.                                                      |
| M27207mRNA_s_at          | 1.62 ± 0.22 | 10q31                | Col1a1             | Collagen (I) alpha 1                         | Extracellular matrix structural protein. Its expression is regulated after sciatic nerve injury.                                       |
| rc_AA800851_s_at         | 1.34 ± 0.20 | 11q23                | Gp1bb              | glycoprotein Ib                              | The gene for platelet glycoprotein Ib beta, a critical component of the von Willebrand factor (vWF) receptor. Blood coagulation.       |
| rc_AA799542_at           | 1.58 ± 0.28 | chr12                | Rac3               | Ras-related C3 butolinum toxin substrate 1   | Member of the RAS superfamily of small GTP-binding proteins involved in signal transduction.                                           |

The fold change given is the mean of the log<sub>2</sub>-transformed values of nine pair-wise comparisons with the standard deviation, ±SD. Fold change >1 or <1 represent increase or decrease from baseline, respectively. Only genes that were concordantly induced or suppressed ( $P < .0025$  for each individual pair-wise comparison, Wilcoxon's test) are shown. Functional annotation has been done using OMIM (<http://www.ncbi.nlm.nih.gov>).

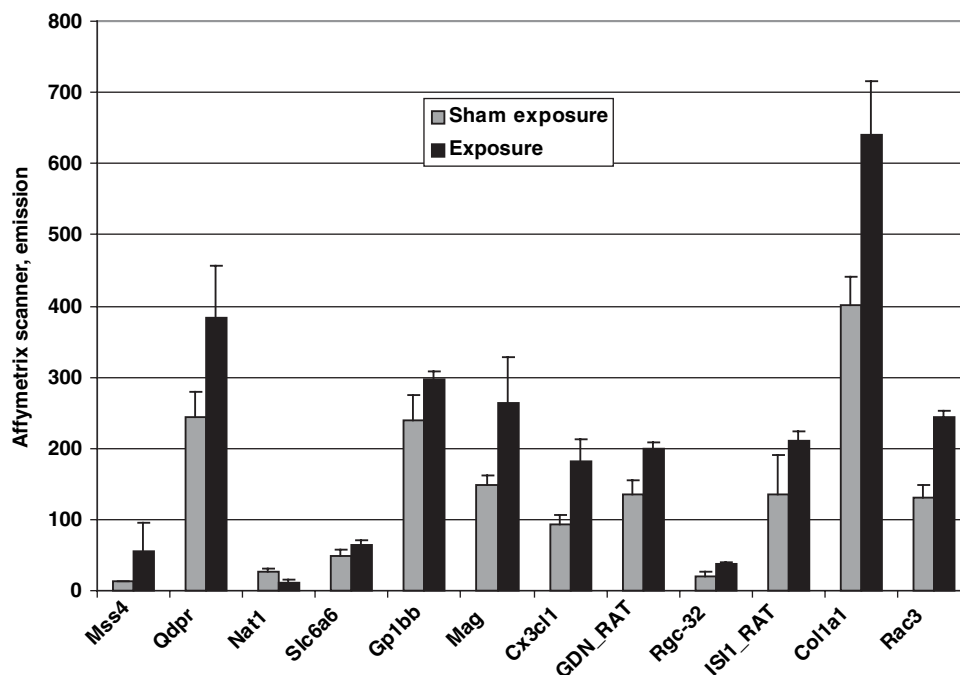


Fig. 4. Affymetrix U34 GeneChips were used to analyze the gene expression in rat brains in response to MWs. The average signals pooled from three exposed and three sham exposed animals and standard deviation are shown for those genes that were concordantly induced or suppressed ( $P < .0025$ , Wilcoxon's test, see also Table 2).

exposed and sham exposed samples (Mann–Whitney *U*-test,  $P > .05$ ). The amount of proteins between lines was equal as controlled by the measurements of total proteins and by probing of actin (data not shown).

## DISCUSSION

The biological effects of nonthermal MWs are dependent on such physical parameters as frequency, intensity, polarization, and modulation [Blackman et al., 1989; Adey, 1999; Pakhomov and Murphy, 2000; Banik et al., 2003; Grigoriev et al., 2003; Mileva et al., 2003; Belyaev, 2005; Lai, 2005]. Such data provide the rationale to test the effects of modulated GSM MWs produced by mobile phones in operation.

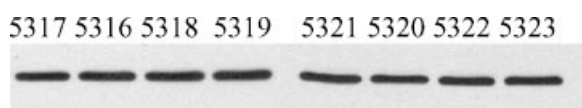


Fig. 5. Hsp70 protein as measured by Western blot in cells from the brains of four Fisher rats (5316, 5319, 5320, 5323) that were exposed to GSM MWs (915 MHz, 2 h), and four rats (5317, 5318, 5321, 5322) that were sham exposed. No statistically significant hsp70 changes were seen after exposure to GSM.

Using AVTD technique, it has been shown that MWs affected conformation of chromatin in cells of different types exposed in vitro under specific conditions [Belyaev, 2005]. In human lymphocytes, the AVTD usually decreased after exposure to GSM MWs at 915 MHz in contrary to the increase in AVTD that was observed immediately after genotoxic impacts such as ionizing radiation [Belyaev et al., 2001, 2005; Sarimov et al., 2004; Markova et al., 2005]. Here, we analyzed effects of MWs on chromatin conformation in brain cells from white and gray matter, as well as in lymphocytes from thymus and spleen. While a weak trend toward decrease in AVTD, indicative of chromatin condensation, was seen in cells from thymus, no statistically significant change in chromatin conformation was detected. There might be several possible reasons for the observed discrepancies between these data and results obtained in vitro with human lymphocytes [Sarimov et al., 2004; Belyaev et al., 2005; Markova et al., 2005]. First, the cells in vitro might be more sensitive to stress response than cells in vivo that are under control of tissues. Second, cell type might be of importance. Third, in studies with human lymphocytes, the individual data were normalized to own individual controls and these normalized data were compared between groups. Therefore, possible

individual variability between cells did not affect the comparison. In the present study, absolute data from exposed and sham exposed animals were compared. Thus, individual variability might mask the MW effects.

Using PFGE technique, we were not able to detect any DNA fragmentation induced by MW exposure. The employed PFGE technique was sensitive to detect the DNA fragmentation immediately after irradiation of cells with 1 Gy of  $\gamma$ -rays [Torudd et al., 2005]. PFGE has lower sensitivity as compared to the comet assay that has shown DNA breaks under specific conditions of MW exposure [Lai and Singh, 1996, 1997; Diem et al., 2005]. It should be noted that the neutral comet assay is sensitive to both DSBs and DNA-loop relaxation due to single-strand breaks (SSBs) [Belyaev et al., 1999; Crumpton and Collins, 2004]. Both alkaline and neutral comet assays may also be sensitive to physiologically derived DNA strand discontinuities occurring in transcription and replication and some other processes of DNA-protein interactions with formation of intermediate physiological DNA strand discontinuities such as those mediated by topoisomerase I and II [Torudd et al., 2005]. Therefore, induction of several processes such as transcription, replication and interaction of DNA with topoisomerases can be detected by alkaline and neutral comet assays and can be misinterpreted as induction of DNA breaks.

Limitations in sensitivity of PFGE to detect MW-induced DNA damage was recently described [Aitken et al., 2005]. Aitken et al. observed no DSBs as measured with PFGE in the bulk of DNA of mouse spermatozoa after MW exposure at 900 MHz but they found statistically significant DNA damage as measured by quantitative PCR in the mitochondrial genome and the nuclear  $\beta$ -globin locus. It should be noted that despite its lower sensitivity, PFGE is the only technique that provides a possibility directly to measure DSBs, which are the most important DNA damage. Other techniques, including AVTD, comet assay and quantitative PCR measure DSBs and also SSBs, which are less important and repaired more efficiently.

The AVTD technique is more sensitive to DNA damage as compared to PFGE. DNA breaks after irradiation at doses of 5–10 cGy, around 50–100 SSB/genome, can be detected in human lymphocytes and cultured mammalian cells by AVTD [Belyaev and Harms-Ringdahl, 1996; Torudd et al., 2003]. The sensitivity of the AVTD technique is comparable with the sensitivity of the comet assay as used by Singh in human lymphocytes [Singh et al., 1994]. It should be mentioned, however, that sensitivities of these two assays might be different for other cell types, for example, for transformed cells that have higher background levels. Therefore, comparison of the AVTD data

with the results of comet assay in brain cells should be made with caution.

Effects of MWs have been shown to depend on several physical parameters such as frequency, modulation, and flux density during exposure [Adey, 1999; Pakhomov and Murphy, 2000; Banik et al., 2003; Grigoriev et al., 2003; Mileva et al., 2003; Balmori, 2005; Belyaev, 2005]. The role of biological variables such as genotype and physiological conditions is also important for manifestation of the MW effects, as has recently been reviewed [Belyaev, 2005]. Therefore, the data obtained here should be compared with care with the experimental data of others showing DNA damage under other conditions of exposure [Lai and Singh, 1997; Diem et al., 2005]. The absence of DNA breaks under specific parameters of exposure used in this study does not support a conclusion of absence of genotoxic effects of MWs in general and one-sided interpretations of the obtained results should be avoided. Based on the reported beneficial effects of MWs, it should not be excluded that exposure to MWs may enhance repair at specific parameters of exposure [Pakhomov and Murphy, 2000]. Thus, no significant effect, induction of damage or decrease in breaks may be observed, depending on the parameters of MW exposure.

A novel result of this pilot study is that exposing rats to GSM MWs for 2 h altered the gene expression profile in the brain. The genes that were statistically significantly changed in expression by MW exposure appear unrelated and they do not show any pattern of activation or in distribution on the chromosomes (Table 2). One possible explanation is that the samples of cerebellum that were analyzed are composed of different types of cells. Hypothetically, specific gene patterns may be induced in each of these types and this induction may be masked when analyzing the whole cerebellum. Another possible explanation is that a number of genes belonging to different functional classes and in addition a number of genes with unknown functions was induced, but that this induction was not statistically significant. The statistical power of this study was limited by the number of gene arrays and also by the fact that the brain normally shows small changes in gene expression. This probably led to that not all induced genes were revealed.

We discuss further only the genes that were statistically significantly induced. The induced genes cover such functions as activation and chemoattraction of microglia into the infarcted tissue (fractalkine), cell cycle regulation (Rgc32 protein) and enzymatic hydroxylation (quinoid dihydropteridine reductase) (Table 2). Of special interest is that the expression of Slc6a6 and Nat1 genes was changed (Table 2). The Slc6a6 gene is involved in regulation of neurotransmitter transport and

neurotransmitter regulation and is thereby involved in BBB function [Mori et al., 1999; Kang et al., 2002], which has been shown to be affected by GSM MWs [Persson et al., 1997]. The *Nat1* gene encodes acetyltransferases (ATs) that can acetylate the endogenous arylalkylamines tryptamine, 5-hydroxytryptamine (serotonin), and 5-methoxytryptamine, the immediate precursor of melatonin. Melatonin response to electromagnetic fields has been discussed in the literature for a long time and some studies have indicated a potential of melatonin to suppress MW effects on brain, kidney, and skin of rats [Lai and Singh, 1997; Ayata et al., 2004; Oktem et al., 2005]. Recent evidence indicated a possible decrease in melatonin production among cellular telephone users [Burch et al., 2002].

Compared to studies of tumorigenic or inflammatory processes that often induce large induction of responsive genes, for example, cytokines, growth factors, etc., the observed gene expression differences are relatively modest in the present study. This is usually seen in the analysis of brain tissue when mild stimuli are applied or different rat strains are evaluated [Rimondini et al., 2002; Sommer et al., 2005]. In addition, we deal with a highly heterogeneous tissue and all the nonaffected cells will mask any effect on a distinct cell population. To detect such effects, the experimental protocol should be refined and more fine dissection of brains should be used in further investigations. The cortex, hippocampus and basal ganglia would be interesting to investigate based on recent data showing MW-induced neuronal cell death in these locations [Salford et al., 2003; Ilhan et al., 2004].

Although stress response proteins, particularly hsp70, were activated by MWs of mobile phones in some previous studies, as measured by Western blot [Kwee et al., 2001; Weisbrot et al., 2003], our study did not reveal such activation. These previous studies should be compared with our study with caution because of significant differences in experimental design. First, the previous studies were performed either in vitro with transformed cells in culture [Kwee et al., 2001] or with *Drosophila melanogaster* [Weisbrot et al., 2003]. In our study, rats were exposed and analysis of expression in the brains was performed. Contrary to transformed cells in vitro and fruit flies, the changes in mammalian tissues, especially in the brains, are the subject of more complicated compensatory mechanisms. Therefore, the effects may be masked by these compensatory mechanisms. Second, the changes in gene expression are usually observed within specific time windows that might be dependent on various variables. For example, Lin et al. [1996] reported that a negative feedback mechanism resulted in lowering of c-myc response in HL60 cells to almost zero after 1 h of

continuous exposure. We have chosen 2 h duration of exposure based on previous experiments of our research groups showing that this exposure affects the mammalian brain [Salford et al., 2003] and human primary lymphocytes [Belyaev et al., 2005]. However, whether 2 h is an optimal time for detection of MW-activated gene expression in the exposed brains, we do not know. Third, the parameters of field (intensity, frequency, modulation, polarization) were different between our and previous studies. Bearing in mind the importance of several physical and biological variables for the MW effects, only results of studies with identical parameters of exposure should be compared in order to avoid misleading interpretations and conclusions.

## CONCLUSION

The data showed that GSM MWs at 915 MHz from a mobile phone did not induce PFGE-detectable DNA breaks and changes in chromatin conformation, but affected expression of genes in rat brain. Because the nonthermal effects of MWs appear to be dependent on many variables, both physical and biological, the results of this study should be compared with care with other studies where these variables are different.

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