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Brain proteome response following whole body exposure of mice to mobile phone or wireless DECT base radiation

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The objective of this study was to investigate the effects of two sources of electromagnetic fields (EMFs) on the proteome of cerebellum, hippocampus, and frontal lobe in Balb/c mice following long-term whole body irradiation. Three equally divided groups of animals (6 animals/group) were used; the first group was exposed to a typical mobile phone, at a SAR level range of 0.17–0.37 W/kg for 3 h daily for 8 months, the second group was exposed to a wireless DECT base (Digital Enhanced Cordless Telecommunications/Telephone) at a SAR level range of 0.012–0.028 W/kg for 8 h/day also for 8 months and the third group comprised the sham-exposed animals. Comparative proteomics analysis revealed that long-term irradiation from both EMF sources altered significantly ($p < 0.05$) the expression of 143 proteins in total (as low as 0.003 fold downregulation up to 114 fold overexpression). Several neural function related proteins (i.e., Glial Fibrillary Acidic Protein (GFAP), Alpha-synuclein, Glia Maturation Factor beta (GMF), and apolipoprotein E (apoE)), heat shock proteins, and cytoskeletal proteins (i.e., Neurofilaments and tropomodulin) are included in this list as well as proteins of the brain metabolism (i.e., Aspartate aminotransferase, Glutamate dehydrogenase) to nearly all brain regions studied. Western blot analysis on selected proteins confirmed the proteomics data. The observed protein expression changes may be related to brain plasticity alterations, indicative of oxidative stress in the nervous

Authors' contributions: AFF and LHM conceived the concept and design of the experiments, made the literature survey and the final biologically valid interpretation of the EMF impact upon the brain, wrote and finalized the manuscript. AFF carried out all animal handling, welfare, EMF exposure, part of brain dissection and immunoassays. AS performed the brain dissection and brain regions' separation, contributed to the non-EMF writing of the manuscript and together with MHA, EK and EA carried out a part of the immunoassays and contributed to the data evaluation related to neuroproteomics. AX, AP and KV were involved in 2-DE experiments, Maldi ToF/MS, protein identification and statistical analysis. DJS participated in the conception of the design and contributed to the interpretation and evaluation of the overall data. GThT participated in the experimental design and experimental protocols optimization, coordinated the proteomics study, carried out the overall differential proteomics analysis and data evaluation and contributed to the proteomics writing of the manuscript. All authors read and approved the final manuscript.

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system or involved in apoptosis and might potentially explain human health hazards reported so far, such as headaches, sleep disturbance, fatigue, memory deficits, and brain tumor long-term induction under similar exposure conditions.

Keywords Microwaves, Radiofrequencies, Wireless phones, Proteomics, Brain plasticity, Hippocampus, Frontal lobe, Cerebellum

INTRODUCTION

Wireless technology emitting electromagnetic radiation (EMR) is spread worldwide affecting directly or indirectly all social levels, all countries, and all ages since it includes mobile phones, cordless DECT telephones, Wi-Fi, wi-max, baby monitors, local TV, and FM broadcast stations. The concern about possible health hazards has led to extensive research, concerning exclusively the effects of mobile phone technology (devices and mast stations) at the cellular, lab animal, and epidemiological level, using a variety of model systems and approaches but not in a coordinated manner (Chavdoula et al., 2010; Fragopoulou et al., 2010a,b,c; Fragopoulou and Margaritis, 2010; Hardell and Carlberg, 2009; Hillert et al., 2008; Khurana et al., 2009, 2010), although there have been international efforts (i.e., interphone study; Cardis et al., 2011) to reveal the truth about the possible EMF health risks. The importance of mobile phone (MP) radiation research lies in the fact that there are currently 5 billion users on the planet and the vast majority is using the MP in contact with the brain (Frey, 1998).

A number of reports have dealt with possible changes on gene/protein expression, either at an individual gene/protein level or using the "omics" approaches. The individual approach has focused mainly on heat shock proteins and their mRNAs (French et al., 2001; McNamee and Chauhan, 2009), but other proteins and genes have also been studied with conflicting, so far, results (Fritze et al., 1997; Cleary et al., 1997; Nikolova et al., 2005; Zhao et al., 2007). In order to assess large numbers of genes and proteins, high throughput approaches have been applied in the last decade. These "omics" approaches, also used in the present work, have gained ground in the study of EMF effects mainly on cell cultures. Belyaev et al. (2006), analyzing by Affymetrix U34 Gene Chips cerebellum of brain samples after whole body 2 h exposure of rats at 915 GSM in TEM cells, revealed overexpression of 12 genes and downregulation of 1 gene. The same (Salford's) research group 2 years later applied Microarray hybridizations on Affymetrix rat2302 chips of RNA extracts from cortex and hippocampus of GSM 1800 exposed rats for just 6 h within TEM cells (Nittby et al., 2008). Using four exposed and four control animals they found that a large number of genes were altered at hippocampus and cortex. The vast majority were downregulated. In a series of publications by Leszczynski's research group, consistently using human endothelial cell lines EA.hy926 and EA.hy926v1, protein expression changes after exposure to 900 MHz were shown (Leszczynski et al., 2002, 2004; Nylund and Leszczynski, 2004, 2006; Remondini et al., 2006). These effects have been recently confirmed by the same group in the two types of mobile phone exposure protocols: GSM 900 and 1800 MHz (Nylund et al., 2009). Another "omics" group exposing human lens epithelial cells has detected heat-shock protein (HSP) 70 and heterogeneous nuclear ribonucleoprotein K (hnRNP K) to be upregulated following exposure to GSM 1800 MHz for 2 h (Li et al., 2007), whereas a third research group exposed human breast cancer cells MCF-7 to an RF generator simulating GSM 1800 MHz signal at various SAR values and duration of exposures (Zeng et al., 2006a). They analyzed the transcriptome and the proteome of the cells after continuous or intermittent exposure and concluded that EMF exposure caused distinct effects on gene and protein expression. The same authors suggested that the protein

expression changes might depend on duration and mode of exposure and therefore a number of biological processes might be affected (Zeng et al., 2006b). Since the above in vitro effects cannot be easily translated into humans, in 2008, Leszczynski's group performed a pilot study on volunteers (Karinen et al., 2008) and showed that mobile phone radiation might alter protein expression in human skin cells. Gene expression changes as revealed using transcriptomics had not effects on C3H 10T(1/2) mouse cells (Whitehead et al., 2006). However, and as previously mentioned, such a limited and non systematic number of publications using "omics" approaches does not allow for any conclusions to be drawn concerning the impact of mobile phone emitted radiation upon cell proteome, physiology and function (Nylund et al., 2009), as also pointed out by Vanderstraeten and Verschaeve (2008).

Concerning research on wireless DECT base and handset radiation exposure which is potentially harmful to millions of people, no actual experiments have been conducted, besides the clinical studies reported by Söderqvist et al. (2009a,b), Havas et al. (2010) and the epidemiological studies showing increased risk for brain tumors (Hardell and Carlberg, 2009; Khurana et al., 2009). A recently published article highlighted the importance of mobile phone epidemiology studies in properly addressing DECT phone use as a strong and likely confounder (Redmayne et al., 2010).

Given the limited available data on animal models, our objective was to investigate the effects of two sources of EMFs on the proteome of the cerebellum, hippocampus and frontal lobe in Balb/c mice.

These three brain regions were chosen since they are related to main functions of the brain, such as memory, attention, reward, planning, equilibrium, and motor control. Their common role is the correlation with cognitive functions (Okano et al., 2000), which have been reported in a number of studies to be altered after EMF exposure (for a review see Fragopoulou and Margaritis, 2010). The hippocampus mainly controls spatial memory, the cerebellum is responsible for motor learning, and the frontal lobe plays an important role in retaining longer term memories associated with emotions. The frontal lobe does not seem to be involved in any particular discrete perceptual sensory or so called motor function, but in spite of that, it seems to have a very critical role on how we use the kind of information that other parts of the brain are dedicated to determine.

Our high-throughput approach challenges the gaps in the literature investigating whether EMFs can provoke changes on the mouse brain proteome; changes that could be correlated with EMF memory impairments reported so far or with neurological diseases, such as Alzheimer's and even with brain tumor induction.

Three groups of 18 animals were used in the present study (6 animals/group): the first group was exposed to a commercially available mobile phone, operating at GSM 900 MHz configuration and frequency and at normal speaking emission mode at a SAR level range of 0.17 – 0.37 W/kg for 3 h daily for 8 months. The second group was exposed to a wireless DECT base at a SAR level range of 0.012 – 0.028 W/kg for 8 h/day during the lights-off period also for 8 months. The third group comprised the sham-exposed animals.

The novelty of this work lies in the fact that no brain proteome studies have been reported so far following EMF exposure and, in particular, of isolated brain regions in any animal model. In addition, to our knowledge this is the first experimental report of wireless DECT exposure effects on any biological model system and in particular following proteome analysis.

MATERIALS AND METHODS

Animals

A total of 18 healthy adult male mice *Mus musculus*, strain *Balb/c*, were obtained from the Hellenic Pasteur Institute Animal Facility and then transferred to our animal facility in the Department of Cell Biology and Biophysics of Athens University where they were left for two weeks to get acclimatized. Animals were housed equally divided into 3 groups in Techniplast, USA Plexiglas cages, 1290D Eurostandard Type III, 425 × 266 × 155 mm - floor area 820 cm^2. The first and the second study group were exposed to a commercially available dual band mobile phone and a wireless DECT base, respectively. Free moving mice were exposed within their cages, as reported previously (Fragopoulou et al., 2010b). The third group comprised the sham-exposed group. All animals were kept under standard laboratory conditions: (22 ± 2)°C, (40–60)% relative humidity, 12 h:12 h light/dark cycle (lights on at 7:00 am) and received food (pellets) and water *ad libitum*. Taking into consideration the welfare of the animals, enrichment material was used within their home cages, i.e., paper and plastic tubes. All experimental procedures were carried out in agreement with the ethical recommendations of the European Communities Council Directive of 24 November 1986 (86/609/EEC) and with the ethical rules of the Bioethics Committee of the Faculty of Biology of Athens University. The 3R's concept of Russell and Burch (Refinement, Reduction and Replacement) was seriously taken into consideration (Russell and Burch, 1959).

EMF Exposure Conditions and Field Measurements

Since the objective of this work is the exploration of any changes in the brain proteome, special attention was given to ensure that the only factor affecting the animals would be the radiation emitted from mobile phone or the base of the DECT wireless device. Therefore, other fields or noise (i.e., magnetic field, other RFs of various frequencies and noise levels) were measured and negligible and in any case they were the same quantitatively and qualitatively with the sham-exposed group.

Mobile Phone Exposure

The animals of this group (n = 6) were exposed to radiation within their home cage three hours per day for 8 months. The exposure protocol of "3 h/day × 8 months" has been chosen in order to mimic a daily typical mobile phone operation by an active person. The mobile phone was placed underneath the cage. A semi-Faraday cage was specially constructed having one open surface to allow mobile phone communication and at the same time to prevent radiation leakage towards sham-exposed animals. The GSM 900 MHz electrical field intensity of the radiation emitted by the mobile phone was measured using the Smartfieldmeter, EMC Test Design, LLC, Newton, MA, USA placing the dual band omni directional probe (900, 1800 MHz) inside a similar cage housing the animals positioned at the same place either at the end or in the beginning of exposure. The obtained measurements were reproducible on a daily basis (minimum-maximum value depending on the sound intensity). In order to simulate the conditions of human voice and activate mobile phone ELF modulated EMF emission, radio station was playing as a source of auditory stimulation throughout the exposure time. The measured electrical field intensity was below ICNIRP's recommendations (ICNIRP, 1998) within the range of 15–22 V/m in the various areas within the cage with the animals following also the typical GSM power modulation by the sound intensity. The SAR value (SAR = σ*E^2/ρ) calculated as previously described (Fragopoulou et al., 2010a,b) was between 0.17 and 0.37 W/kg. This is a rough estimation of the whole body

average SAR of individual animals. The aim was to achieve similar exposure conditions occurring to a human user when holding the mobile phone next to his/her ear with the only difference that the mice were receiving whole body and not head-only exposure.

Wireless DECT Base Exposure

The animals of this group were exposed to a commercially available wireless DECT base, which constantly emits radiation at a bandwidth of 1880–1900 MHz, very close to the GSM1800 band, scanning all 10 allocated RF channels without any handset communicating with the base. The DECT base was placed close to the mouse cage and was programmed to operate for 8 h per day during the lights-off period for 8 months. This exposure protocol of 8 h/day has been chosen to correspond to human occupational or home DECT base exposure. A semi-Faraday cage was specially constructed to prevent radiation leakage towards sham-exposed animals. Electrical field levels were measured with Smartfieldmeter as described above and the values recorded were from 4–6 V/m depending on the position within the cage. No voice modulation is required for DECT operation, but the same radio station was playing for comparative purposes to the mobile phone exposure. Therefore, SAR value calculated, as described above, ranged from 0.012–0.028 W/Kg.

Sham-exposed Group

Mice were kept in a similar room as the exposed groups, under the same conditions of living. The cages of the animals were inside a Faraday cage to prevent radiation entry from the mobile phone and DECT base when in operation. A radio was playing at the same station and the same volume as the one in the rooms of the exposed animals. Non significant levels of Radio-frequency (RF) field deriving from the exposure sources was detected inside the cage with the animals, as measured by the Smartfieldmeter.

BRAIN TISSUE REMOVAL AND HOMOGENIZATION

At the end of the experiment, mice were euthanized according to the bioethical rules of the European Committee for animal protection, with cervical dislocation followed by rapid brain tissue removal between 8 and 10 am. Parts of the brain (frontal lobe, hippocampus, and cerebellum) were quickly separated, immediately frozen in liquid nitrogen, and then stored at -80^{o}C until sample processing for further manipulation.

TWO-DIMENSIONAL ELECTROPHORESIS

The tissue was homogenized in a glass Wheaton (tight) homogenizer in a buffer consisting of 8 M urea, 40 mM Tris-HCL (pH 8.5), 2 M thiourea, 4% CHAPS, 1% dithioerythritol (DTE), 0.2% IPG buffer pH 3–10 (Amersham Biosciences) and 1 mg/mL of a mixture of protease inhibitors (1 mM PMSF and 1 tablet (Roche Diagnostics) per 50 mL of wash buffer and phosphatase inhibitors (0.2 mM Na_3VO_3 and 1 mM NaF)). The homogenate was left at room temperature for 1 h and centrifuged at 13,000 rpm for 30 min. The protein content of the supernatant was determined using the Bradford quantification method.

Two-dimensional gel electrophoresis was performed as previously reported (Anagnostopoulos et al., 2010). Samples of 1 mg total protein were applied on 18 cm IPG strips with *pI* 3–10 NL or 4–7 L (Bio-Rad Lab, Hercules, CA), at their basic and acidic ends, using sample cups. IPG strips had been prepared for IEF by 20 h rehydration in a buffer of 8 M urea, 4% CHAPS and 1% DTE.

First dimension focusing, for separation by two-dimensional gel electrophoresis, started at 250 V and voltage was gradually increased to 8000 V, with 3 V/min, kept constant for 25 h (approximately 150,000 Vh totally). IEF was conducted in a PROTEAN IEF Cell, Bio-Rad apparatus. After focusing, IPG strips were equilibrated first in 6 M urea, 50 mM Tris-HCL (pH 8.8), 2% (w/v) SDS, 30% (v/v) glycerol, and 0.5% (w/v) DTE for 15 min then in the same buffer containing 4% (w/v) iodoacetamide instead of DTE, for 15 more min. Second dimensional electrophoresis was performed on 12% SDS-polyacrylamide gels (180 × 200 × 1.5 mm) with a run of 40 mA/ gel, in PROTEIN-II multi-cell apparatuses (Bio-Rad, Hercules, CA).

PROTEIN VISUALIZATION AND IMAGE ANALYSIS

After vertical electrophoresis, gels were fixed in 50% methanol containing 5% phosphoric acid for 2 h. The fixative solution was washed off by agitation in distilled water for 45 min. Protein spots were visualized by application of Coomassie Blue G-250 staining solution (Novex, San Diego, CA) on 2-DE gels for 12 h. Gel images were scanned in a GS-800 Calibrated Densitometer (Bio-Rad Laboratories, Hercules, CA) using the scanning application/tool of the PD-Quest v8.0 software (Bio-Rad, Hercules, CA). Protein spots of all gels contained in the analysis, were detected, aligned, matched, and quantified using the PD-Quest v8.0 image processing software, according to the manufacturer's instructions. Manual inspection of the spots was used to verify the accuracy of matching. Spot volume was used as the analysis parameter to quantify protein expression. Normalization of each individual spot was performed according to the total quantity of the valid spots in each gel, after subtraction of the background values. Optical Density (O.D.) level (%) of each protein from the sham-exposed or exposed groups was determined separately and calculated as the sum of the volume % of all spots from all gels containing the same protein. Selection of protein spots or entire gel regions for MS analysis was based upon O.D. alteration between the two groups analysed. A minimum of 1.25 fold change in the expression level was used as the selection criterion.

PEPTIDE MASS FINGERPRINTING AND IDENTIFICATION OF PROTEINS

Peptide mass fingerprinting analysis was essentially performed as described previously (Mavrou et al., 2008). Briefly, all spots on the gels were annotated semi-automatically using the Melanie 4.02 software, excised with a Proteiner SPII robot (Bruker Daltonics, Bremen, Germany) and placed into 96-well microtiter plates. The excised spots were destained using 180 μl of 100 mM ammonium bicarbonate in 30% ACN and the gel piece was dried in a speed vacuum concentrator (MaxiDry Plus, Heto, Denmark). The dried gel piece was rehydrated with 5 μL of 20 μg/mL recombinant trypsin (proteomics grade, Roche diagnostics, Basel, Swiss) solution. After 16 h at room temperature, 10 μL of 50% acetonitrile containing 0.3% trifluroacetic acid were added, and the gel pieces were incubated for 15 min with gentle shaking. Sample application to a target plate and analysis as well as peptide matching and protein searching were carried out as described previously (Mavrou et al., 2008). Briefly, tryptic peptide mixtures (1 μL) were applied on an anchor chip MALDI plate with 1 μL of matrix solution, consisting of 0.08% CHCA (Sigma), the internal standard peptides des-Arg-bradykinin (Sigma, 904.4681 Da), and adrenocorticotropic hormone fragment 18–39 (Sigma, 2465.1989 Da) in 65% ethanol, 50% CAN, and 0.1% TFA. Peptide mixtures were analysed in a MALDI-ToF mass spectrometer (Ultraflex II, Bruker Daltonics). Laser shots (n = 1000) of intensity between 40% and 60% were collected and summarized and the peak list was created

using the FlexAnalysis v2.2 software (Bruker). Peptide matching and protein searches were performed automatically with MASCOT Server 2 (Matrix Science). Peptide masses were compared with the theoretical peptide masses of all available proteins of *Mus musculus* in the SWISS-PROT database. Stringent criteria were used for protein identification with a maximum allowed mass error of 10 ppm and a minimum of four matching peptides. Probability score with $p < 0.05$ was used as the criterion for affirmative protein identification. Monoisotopic masses were used, and one missed trypsin cleavage site was calculated for proteolytic products.

WESTERN BLOT ANALYSIS

Frozen tissues were sonicated in RIPA (radioimmunoprecipitation) lysis buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 1% NP-40, 5 mM EDTA, 0.5% sodium deoxycholate, and 0.1% SDS), in the presence of protease inhibitors on ice. The homogenate was centrifuged at 20,000 rpm for 20 min at 4°C. The protein concentration of each brain extract was determined by Bradford assay and 50 μg was loaded onto 12% SDS-PAGE (sodium dodecyl sulphate polyacrylamide gel electrophoresis) after boiling in SDS sample buffer, and electroblotted onto nitrocellulose membrane (Bio-Rad). The membrane was blocked in 5% dried non fat milk diluted in PBS-T (0.1%) for 60 min at room temperature and probed with primary antibodies, mouse monoclonal anti-GMF (diluted at 1:100), goat polyclonal anti-ApoE (sc-6384, diluted at 1:1000), and rabbit polyclonal anti-GFAP (ab7260, diluted at 1:4000) using standard immunoblotting techniques. After the 1 h RT application of species-specific HRP- (horseradish peroxidase) conjugated secondary antibodies (anti-rabbit, Amersham-Pharmacia Biotechnology, Piscataway, NJ, USA, at 1:8.000, anti-mouse, Dako, Denmark at 1:10.000 and anti-goat, Sigma, Germany at 1:14000) appropriately diluted in blocking solution, the immunoblots were developed using an enhanced chemiluminescence (ECL) reagent kit (Amersham Biosciences, Piscataway, NJ, USA) or ECL Plus (GE Healthcare, Amersham Biosciences) western blotting detection reagent. Unspecific protein bands were used as internal loading controls. The molecular weight (MW) definition of unknown bands was identified against a lane of MW protein standards (Fermentas, Hanover, MD, USA).

Following exposure and development the negatives were scanned and processed through image analysis "Gel analyzer" software (v.1.0, Biosure, Ltd, Greece) to quantitatively estimate band densities. The immunoblots shown are derived from different animals randomly selected.

NETWORK ANALYSIS

All protein identifications, both the ones solely expressed in exposed regions, and those differentially expressed among exposed and sham-exposed regions, were used for Pathway Analysis. For this purpose, the Swiss-Prot accession numbers were inserted into the Ingenuity Pathway Analysis (IPA) software (Ingenuity Systems, Mountain View, CA). This software categorizes gene products based on the location of the protein within cellular components and suggests possible biochemical, biological, and molecular functions. Furthermore, proteins were mapped to genetic networks available in the Ingenuity database and ranked by score. These genetic networks describe functional relationships between gene products based on known interactions in literature. Through the IPA software, the newly formed networks are associated with known biological pathways.

STATISTICAL ANALYSIS

To ensure confidence in our experimental approach we employed a design which involved duplicate 2-DE gels per sample (i.e., to determine analytical variation) and separate preparations for each replicate sample per experiment (i.e., to determine biological variation), summing up to 36 2-DE gels in total.

Mean densitometry values of all spots corresponding to a specific protein from each group were first checked for normal distribution using the Kolmogorov-Smirnov/Lilliefor test (StatPlus 2007 software, AnalystSoft, Vancouver, Canada). Data with normally distributed densitometric values were exported to Microsoft Excel 2007 software and compared with the two pair *t*-test assuming unequal variances. Means of spot intensities for proteins with not normally distributed values were compared for statistical significance with the Mann-Whitney non parametric test (GraphPad Instat 3 software, GraphPad software Inc, La Jolla, CA). Statistical significance (a-level) was defined as $p < 0.05$. In order to control the False Discovery Rate (FDR), individual a-levels for each spot were adjusted following the FDR correction procedure (Benjamini and Hochberg, 1995).

The above analysis was performed in order to increase the sensitivity without compromising the accuracy of the statistical output. As such, all the normally distributed populations were tested using a t-test. If these had been tested using Mann-Whitney some statistically significant differentiations would have been missed. FDR was used to correct for multiple comparisons.

RESULTS

In this study we examined the protein expression levels in different mouse brain regions after whole body exposure of Balb/c mice, separately to mobile phone and wireless DECT base electromagnetic radiation.

Protein expression was estimated by proteomics analysis using 2-DE with broad (3–10 NL) and narrow (4–7 L) IPG strips. All brain tissue samples were analyzed in duplicate. Hippocampi were pooled in order to assure the protein quantity (1 mg total protein per 2-DE gel) needed for the analysis. In total, 36 gels were performed in this study. Coomassie blue staining revealed a mean number of 843 ± 73 and 587 ± 45 protein spots within the pH range 3–10 and the pH range 4–7, respectively. Areas of interest with reproducible spot intensity and/or pattern differences observed in pI 3–10 2-DE gels, were mainly monitored in the acidic regions. Further examination therefore, using 4–7 IPG strips guaranteed greater detail of spot analysis in the specific areas.

A total of 432 proteins were found expressed in the studied materials. Concretely, 149 single gene products were identified in the cerebellum, 136 single gene products were identified in the frontal lobe, and 147 single gene products were identified in the hippocampus. These results seem to be in accordance with recent findings in a rat hippocampus proteomics analysis (Fountoulakis et al., 2005).

TABLE 1 Number of differentially expressed proteins across three major brain regions, following long-term electromagnetic radiation exposure to conventional mobile phone (M) and DECT wireless base (B).

	Hippocampus		Frontal lobe		Cerebellum	
Proteins	B	M	B	M	B	M
Upregulated	11	37	12	19	8	36
Downregulated	11	33	11	18	10	18
Total number of proteins changed	**22**	**70**	**23**	**37**	**18**	**54**

Statistical analysis under the criteria described above, revealed that 143 single gene products were found differentially expressed among the studied brain tissue samples, as shown in Suppl. Table 1. This table summarizes the identified proteins, gives the spot numbers under which the proteins appeared on the 2-DE gels, their identity, SwissProt accession numbers, theoretical pI, molecular weight, MASCOT score, the number of peptides used per identification, protein coverage, and the expression level, as calculated with the PD Quest 8.0 software. Proteins with difference in expression at a level of 1.25 were considered upregulated, while a 0.75 difference designated downregulated proteins.

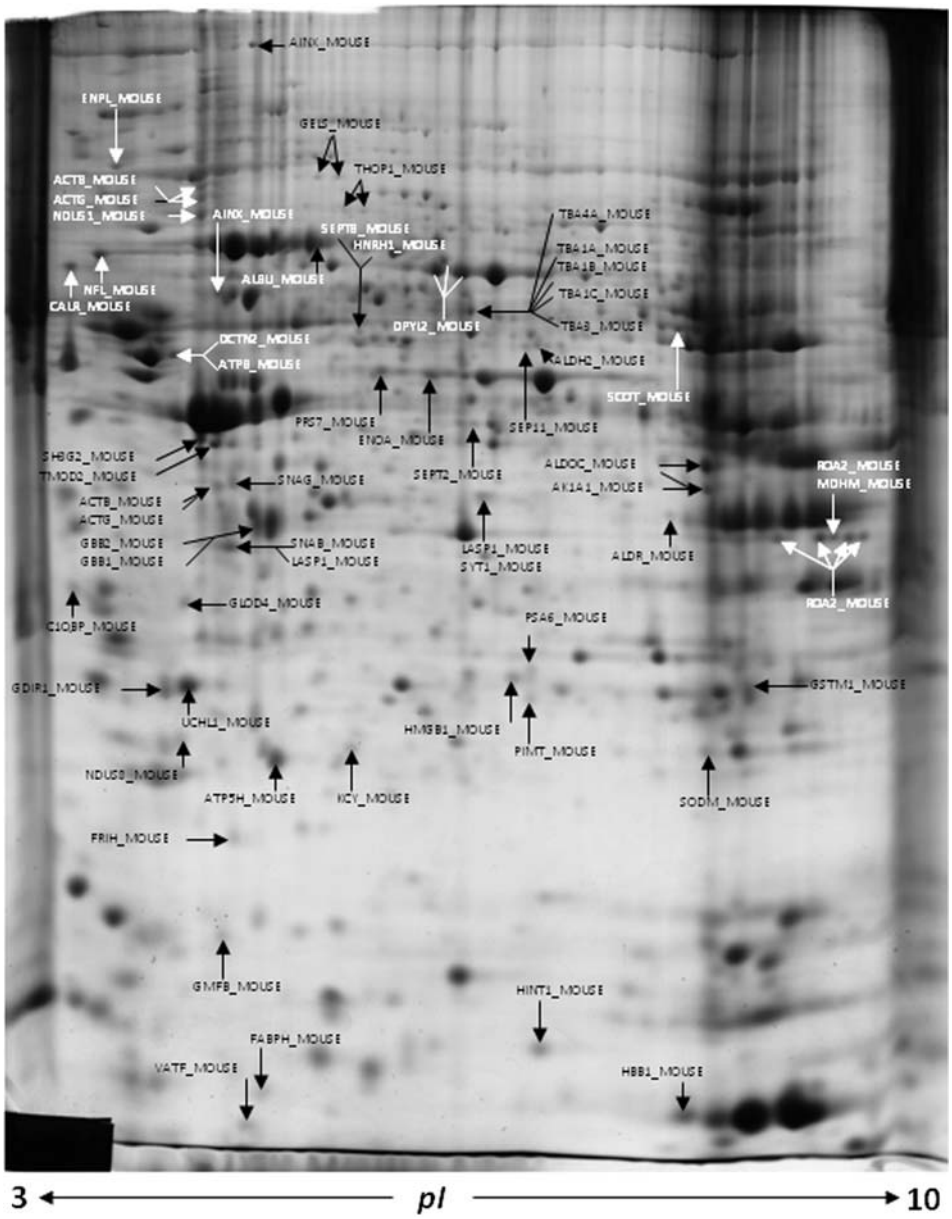

FIGURE 1 Representative 2-DE gel of mouse hippocampus. Arrows indicate the proteins downregulated after the exposure of the mice to mobile (black arrows) and to base (white arrows) compared to the sham-exposed animals.

TABLE 2 Differentially expressed proteins in the mouse brain after exposure to base and mobile phone radiation.

		Hippocampus		Frontal Lobe		Cerebellum	
Accession Name	Protein Name	B	M	B	M	B	M
1433E_MOUSE	14-3-3 protein epsilon - Mus musculus (Mouse)	↓					
AATC_MOUSE	Aspartate aminotransferase, cytoplasmic - Mus musculus (Mouse)	↑	↑			↑	↑
ACTY_MOUSE	Beta-centractin - Mus musculus (Mouse)						↑
AHSA1_MOUSE	Activator of 90 kDa heat shock protein ATPase homolog 1 - Mus musculus (Mouse)		↑				
AINX_MOUSE	Alpha-internexin - Mus musculus (Mouse)	↑					↓
ALDH2_MOUSE	Aldehyde dehydrogenase, mitochondrial precursor - Mus musculus (Mouse)		↓				
ALDOA_MOUSE	Fructose-bisphosphate aldolase A - Mus musculus (Mouse)				↑		
ALDOC_MOUSE	Fructose-bisphosphate aldolase C - Mus musculus (Mouse)	↓				↑	↑
ALDR_MOUSE	Aldose reductase - Mus musculus (Mouse)	↑	↑				
ANXA5_MOUSE	Annexin A5 - Mus musculus (Mouse)		↓				
APOA1_MOUSE	Apolipoprotein A-I precursor - Mus musculus (Mouse)		↑				
APOE_MOUSE	Apolipoprotein E precursor - Mus musculus (Mouse)		↑				↑
ATP5H_MOUSE	ATP synthase subunit d, mitochondrial - Mus musculus (Mouse)	↑			↓		
ATPA_MOUSE	ATP synthase subunit alpha, mitochondrial precursor - Mus musculus (Mouse)		↑			↑	↑
ATPB_MOUSE	ATP synthase subunit beta, mitochondrial precursor -Mus musculus (Mouse)		↓				↑
ATPG_MOUSE	ATP synthase subunit gamma, mitochondrial precursor - Mus musculus (Mouse)				↓		
BACH_MOUSE	Cytosolic acyl coenzyme A thioester hydrolase - Mus musculus (Mouse)				↓		
BLVRB_MOUSE	Flavin reductase - Mus musculus (Mouse)					↓	
C1QBP_MOUSE	Complement component 1 Q subcomponent-binding protein, mitochondrial - Mus musculus (Mouse)		↓				
CAH2_MOUSE	Carbonic anhydrase 2 - Mus musculus (Mouse)		↑	↓	↑	↓	
CALR_MOUSE	Calreticulin precursor - Mus musculus (Mouse)		↓	↑			
CH60_MOUSE	60 kDa heat shock protein, mitochondrial precursor - Mus musculus (Mouse)					↓	
CISY_MOUSE	Citrate s ynthase, mitochondrial precursor - Mus musculus (Mouse)				↑		
CLPP_MOUSE	Putative ATP-dependent Clp protease proteolytic subunit, mitochondrial - Mus musculus (Mouse)		↑				
CNTN2_MOUSE	Contactin-2 precursor - Mus musculus (Mouse)						↑
CPNE6_MOUSE	Copine-6 - Mus musculus (Mouse)		↑				
CRYM_MOUSE	Mu-crystallin homolog - Mus musculus (Mouse)			↑			
CSN4_MOUSE	COP9 signalosome complex subunit 4 - Mus musculus (Mouse)		↑				
DCTN2_MOUSE	Dynactin subunit 2 - Mus musculus (Mouse)	↓					
DDAH1_MOUSE	N(G),N(G)-dimethylarginine dimethylaminohydrolase 1 - Mus musculus (Mouse)					↓	
DHE3_MOUSE	Glutamate dehydrogenase 1, mitochondrial precursor - Mus musculus (Mouse)		↑			↑	↓
DHPR_MOUSE	Dihydropteridine reductase - Mus musculus (Mouse)						↑

DLDH_MOUSE	Dihydrolipoyl dehydrogenase, mitochondrial precursor - Mus musculus (Mouse)			↓				
DNM1L_MOUSE	Dynamin-1-like protein - Mus musculus (Mouse)				↓			
DPYL2_MOUSE	Dihydropyrimidinase-related protein 2 - Mus musculus (Mouse)	↑	↑	↑			↑	
DREB_MOUSE	Drebrin - Mus musculus (Mouse)			↓	↓			
DYN1_MOUSE	Dynamin-1 - Mus musculus (Mouse)					↓	↓	
EFHD2_MOUSE	EF-hand domain-containing protein D2 - Mus musculus (Mouse)				↑		↓	
ENOA_MOUSE	Alpha-enolase - Mus musculus (Mouse)		↓	↑		↑	↑	
ENOG_MOUSE	Gamma-enolase - Mus musculus (Mouse)						↑	
ENPL_MOUSE	Endoplasmin precursor - Mus musculus (Mouse)		↑					
ERP29_MOUSE	Endoplasmic reticulum protein ERp29 precursor - Mus musculus (Mouse)					↓		
EZRI_MOUSE	Ezrin - Mus musculus (Mouse)		↑					
FABPH_MOUSE	Fatty acid-binding protein, heart - Mus musculus (Mouse)		↓					
FRIH_MOUSE	Ferritin heavy chain - Mus musculus (Mouse)	↑						
FUBP1_MOUSE	Far upstream element-binding protein 1 - Mus musculus (Mouse)						↑	
FUMH_MOUSE	Fumarate hydratase, mitochondrial precursor - Mus musculus (Mouse)	↓	↑		↑		↑	
G3P_MOUSE	Glyceraldehyde-3-phosphate dehydrogenase - Mus musculus (Mouse)		↑				↓	
GABT_MOUSE	4-aminobutyrate aminotransferase, mitochondrial precursor - Mus musculus (Mouse)		↑				↑	
GBB1_MOUSE	Guanine nucleotide-binding protein G(I)/G(S)/G(T) beta subunit 1- Mus musculus (Mouse)		↓		↓		↓	
GBB2_MOUSE	Guanine nucleotide-binding protein G(I)/G(S)/G(T) beta subunit 2 - Mus musculus (Mouse)		↓	↓	↓		↓	
GDIB_MOUSE	Rab GDP dissociation inhibitor beta - Mus musculus (Mouse)		↑					
GDIR1_MOUSE	Rho GDP-dissociation inhibitor 1 - Mus musculus (Mouse)	↑			↑	↑		
GELS_MOUSE	Gelsolin precursor - Mus musculus (Mouse)		↓					
GFAP_MOUSE	Glial fibrillary acidic protein - Mus musculus (Mouse)			↑	↑			
GLOD4_MOUSE	Glyoxalase domain-containing protein 4 - Mus musculus (Mouse)		↓					
GLRX3_MOUSE	Glutaredoxin-3 - Mus musculus (Mouse)		↑				↓	
GMFB_MOUSE	Glia maturation factor beta - Mus musculus (Mouse)	↓	↓					
GPDM_MOUSE	Glycerol-3-phosphate dehydrogenase, mitochondrial precursor - Mus musculus (Mouse)						↑	
GRB2_MOUSE	Growth factor receptor-bound protein 2 - Mus musculus (Mouse)				↓	↓	↑	
GRP75_MOUSE	Stress-70 protein, mitochondrial precursor - Mus musculus (Mouse)			↓	↓		↓	
GRP78_MOUSE	78 kDa glucose-regulated protein precursor - Mus musculus (Mouse)						↓	
GSTM1_MOUSE	Glutathione S-transferase Mu 1 - Mus musculus (Mouse)	↑						
HINT1_MOUSE	Histidine triad nucleotide-binding protein 1 - Mus musculus (Mouse)		↓					
HMGB1_MOUSE	High mobility group protein B1 - Mus musculus (Mouse)	↑						
HNRH1_MOUSE	Heterogeneous nuclear ribonucleoprotein H - Mus musculus (Mouse)		↓					

TABLE 2 (*continued*)

		Hippocampus		Frontal Lobe		Cerebellum	
Accession Name	Protein Name	B	M	B	M	B	M
HNRPK_MOUSE	Heterogeneous nuclear ribonucleoprotein K - Mus musculus (Mouse)		↑				↓
HS90A_MOUSE	Heat shock protein HSP 90-alpha - Mus musculus (Mouse)		↑				
HS90B_MOUSE	Heat shock protein HSP 90-beta - Mus musculus (Mouse)		↑				
HSP7C_MOUSE	Heat shock cognate 71 kDa protein - Mus musculus (Mouse)				↓		
IMMT_MOUSE	Mitochondrial inner membrane protein - Mus musculus (Mouse)			↓			↑
KAD1_MOUSE	Adenylate kinase isoenzyme 1 - Mus musculus (Mouse)		↑		↑		
KCRB_MOUSE	Creatine kinase B-type - Mus musculus (Mouse)			↑			↓
KCRU_MOUSE	Creatine kinase, ubiquitous mitochondrial precursor - Mus musculus (Mouse)						↑
KCY_MOUSE	UMP-CMP kinase - Mus musculus (Mouse)		↓				↓
KPYM_MOUSE	Pyruvate kinase isozymes M1/M2 - Mus musculus (Mouse)	↓	↑		↑		↑
LASP1_MOUSE	LIM and SH3 domain protein 1 - Mus musculus (Mouse)		↑				
LDHA_MOUSE	L-lactate dehydrogenase A chain - Mus musculus (Mouse)		↑				
LGUL_MOUSE	Lactoylglutathione lyase - Mus musculus (Mouse)	↑					
LIS1_MOUSE	Platelet-activating factor acetylhydrolase IB subunit alpha - Mus musculus (Mouse)		↑				
MDHC_MOUSE	Malate dehydrogenase, cytoplasmic - Mus musculus (Mouse)				↑		
MDHM_MOUSE	Malate dehydrogenase, mitochondrial precursor - Mus musculus (Mouse)		↑				
MLRB_MOUSE	Myosin regulatory light chain 2-B, smooth muscle isoform - Mus musculus (Mouse)			↑			
NDKB_MOUSE	Nucleoside diphosphate kinase B - Mus musculus (Mouse)						↓
NDUAA_MOUSE	NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 1 - Mus musculus (Mouse)				↓		
NDUBA_MOUSE	NADH dehydrogenase [ubiquinone] 1 beta subcomplex subunit 10 - Mus musculus (Mouse)				↑		
NDUS1_MOUSE	NADH-ubiquinone oxidoreductase 75 kDa subunit, mitochondrial - Mus musculus (Mouse)		↓				
NDUS3_MOUSE	NADH dehydrogenase [ubiquinone] iron-sulfur protein 3, mitochondrial - Mus musculus (Mouse)		↑				
NDUS8_MOUSE	NADH dehydrogenase [ubiquinone] iron-sulfur protein 8, mitochondrial - Mus musculus (Mouse)		↓				
NDUV1_MOUSE	NADH dehydrogenase [ubiquinone] flavoprotein 1, mitochondrial - Mus musculus (Mouse)		↑		↑		↑
NFL_MOUSE	Neurofilament light polypeptide - Mus musculus (Mouse)	↓	↓			↑	↑
NFM_MOUSE	Neurofilament medium polypeptide - Mus musculus (Mouse)				↑		
OAT_MOUSE	Ornithine aminotransferase, mitochondrial precursor - Mus musculus (Mouse)						↑

ODO1_MOUSE	2-oxoglutarate dehydrogenase E1 component, mitochondrial precursor - Mus musculus (Mouse)				↑		
ODP2_MOUSE	Dihydrolipoyllysine-residue acetyltransferase component of pyruvate deh ydrogenase complex, mitochondrial- Mus musculus (Mouse)		↑	↑			
ODPB_MOUSE	Pyruvate dehydrogenase E1 component subunit beta, mitochondrial - Mus musculus (Mouse)			↓	↓		↓
PDC6I_MOUSE	Programmed cell death 6-interacting protein - Mus musculus (Mouse)						↑
PDIA3_MOUSE	Protein disulfide-isomerase A3 precursor - Mus musculus (Mouse)						↑
PEBP1_MOUSE	Phosphatidylethanolamine-binding protein 1 - Mus musculus (Mouse)		↑			↑	
PGAM1_MOUSE	Phosphoglycerate mutase 1 - Mus musculus (Mouse)						↓
PGK1_MOUSE	Phosphoglycerate kinas e 1 - Mus musculus (Mouse)				↑		
PIMT_MOUSE	Protein-L-isoaspartate(D-aspartate) O-methyltransferase - Mus musculus (Mouse)	↓					
PPCKM_MOUSE	Phosphoenolpyruvate carboxykinase [GTP], mitochondrial - Mus musculus (Mouse)		↑				
PRDX1_MOUSE	Peroxiredoxin-1 - Mus musculus (Mouse)				↑		
PRDX3_MOUSE	Thioredoxin-dependent peroxide reductase, mitochondrial precursor - Mus musculus (Mouse)				↓		↑
PRDX6_MOUSE	Peroxiredoxin-6 - Mus musculus (Mouse)		↑				↑
PRS7_MOUSE	26S protease regulatory subunit 7 - Mus musculus (Mouse)		↓	↑			
PSA6_MOUSE	Proteasome subunit alpha type-6 - Mus musculus (Mouse)	↑					
PSB4_MOUSE	Proteasome subunit beta type-4 precursor - Mus musculus (Mouse)		↑				
PURA_MOUSE	Transcriptional activator protein Pur-alpha - Mus musculus (Mouse)				↓		
PYC_MOUSE	Pyruvate carboxylase, mitochondrial precursor - Mus musculus (Mouse)						↑
QCR1_MOUSE	Cytochrome b-c1 complex subunit 1, mitochondrial precursor - Mus musculus (Mouse)			↑	↑		↓
QCR2_MOUSE	Cytochrome b-c1 complex subunit 2, mitochondrial precursor - Mus musculus (Mouse)						↑
ROA2_MOUSE	Heterogeneous nuclear ribonucleoproteins A2/B1 - Mus musculus (Mouse)		↓				
SAM50_MOUSE	Sorting and assembly machinery component 50 homolog - Mus musculus (Mouse)		↑				
SCOT_MOUSE	Succinyl-CoA:3-ketoacid-coenzyme A transferase 1, mitochondrial - Mus musculus (Mouse)		↓				
SEP11_MOUSE	Septin-11 - Mus musculus (Mouse)		↓				
SEPT2_MOUSE	Septin-2 - Mus musculus (Mouse)		↓				
SEPT5_MOUSE	Septin-5 - Mus musculus (Mouse)				↓		
SEPT8_MOUSE	Septin-8 - Mus musculus (Mouse)		↓				
SH3G2_MOUSE	Endophilin-A1 - Mus musculus (Mouse)		↓	↓			
SNAB_MOUSE	Beta-soluble NSF attachment protein - Mus musculus (Mouse)		↓				
SNAG_MOUSE	Gamma-soluble NSF attachment protein - Mus musculus (Mouse)		↓				

TABLE 2 (*continued*)

		Hippocampus		Frontal Lobe		Cerebellum	
Accession Name	Protein Name	B	M	B	M	B	M
SODM_MOUSE	Superoxide dismutase [Mn], mitochondrial precursor - Mus musculus (Mouse)		↓			↓	↓
SRR_MOUSE	Serine racemase - Mus musculus (Mouse)		↑				
STXB1_MOUSE	Syntaxin-binding protein 1 - Mus musculus (Mouse)			↑	↑		
SUCB1_MOUSE	Succinyl-CoA ligase [ADP-forming] beta-chain, mitochondrial - Mus musculus (Mouse)			↓			↓
SYN2_MOUSE	Synapsin-2 - Mus musculus (Mouse)		↑		↑	↓	↑
SYT1_MOUSE	Synaptotagmin-1 - Mus musculus (Mouse)	↓	↓				
SYUA_MOUSE	Alpha-synuclein - Mus musculus (Mouse)			↓	↑		
TAGL3_MOUSE	Transgelin-3 - Mus musculus (Mouse)				↓		
TCPB_MOUSE	T-complex protein 1 subunit beta - Mus musculus (Mouse)					↓	↑
THIL_MOUSE	Acetyl-CoA acetyltransferase, mitochondrial precursor - Mus musculus (Mouse)						↑
THOP1_MOUSE	Thimet oligopeptidase - Mus musculus (Mouse)		↓				
TKT_MOUSE	Transketolase - Mus musculus (Mouse)		↑				↑
TMOD2_MOUSE	Tropomodulin-2 - Mus musculus (Mouse)		↓				↓
TPIS_MOUSE	Triosephosphate isomerase - Mus musculus (Mouse)					↓	
UCHL1_MOUSE	Ubiquitin carboxyl-terminal hydrolase isozyme L1 - Mus musculus (Mouse)	↑	↓	↑			↑
VATA_MOUSE	Vacuolar ATP synthase catalytic subunit A - Mus musculus (Mouse)				↓		
VATB2_MOUSE	Vacuolar ATP synthase subunit B, brain isoform - Mus musculus (Mouse)		↑				
VATF_MOUSE	Vacuolar proton pump subunit F - Mus musculus (Mouse)		↓				
VDAC1_MOUSE	Voltage-dependent anion-selective channel protein 1 - Mus musculus (Mouse)			↓	↓		↑
VINC_MOUSE	Vinculin - Mus musculus (Mouse)						↑

The exposure conditions as explicitly described in "Materials and Methods" had an impact in the differential protein expression of a large number of brain proteins as follows.

- **Hippocampus**. 11 proteins were upregulated, whereas another 11 were downregulated after the animal exposure to a wireless DECT base, compared to the sham-exposed animals. In addition, 37 proteins were found upregulated and 33 downregulated after the exposure of the animals to a mobile phone compared to the sham-exposed (Table 1).
- **Frontal lobe**. 12 proteins were upregulated and 11 proteins were downregulated after exposure of the animals to a wireless base. The mobile phone exposure caused 19 proteins to become upregulated and 18 proteins downregulated (Table 1).
- **Cerebellum.** 8 proteins were upregulated and 10 proteins were downregulated after exposure of the animals to a wireless base, whereas 36 proteins were upregulated, and 18 proteins were downregulated in the mobile phone exposed animal group (Table 1).

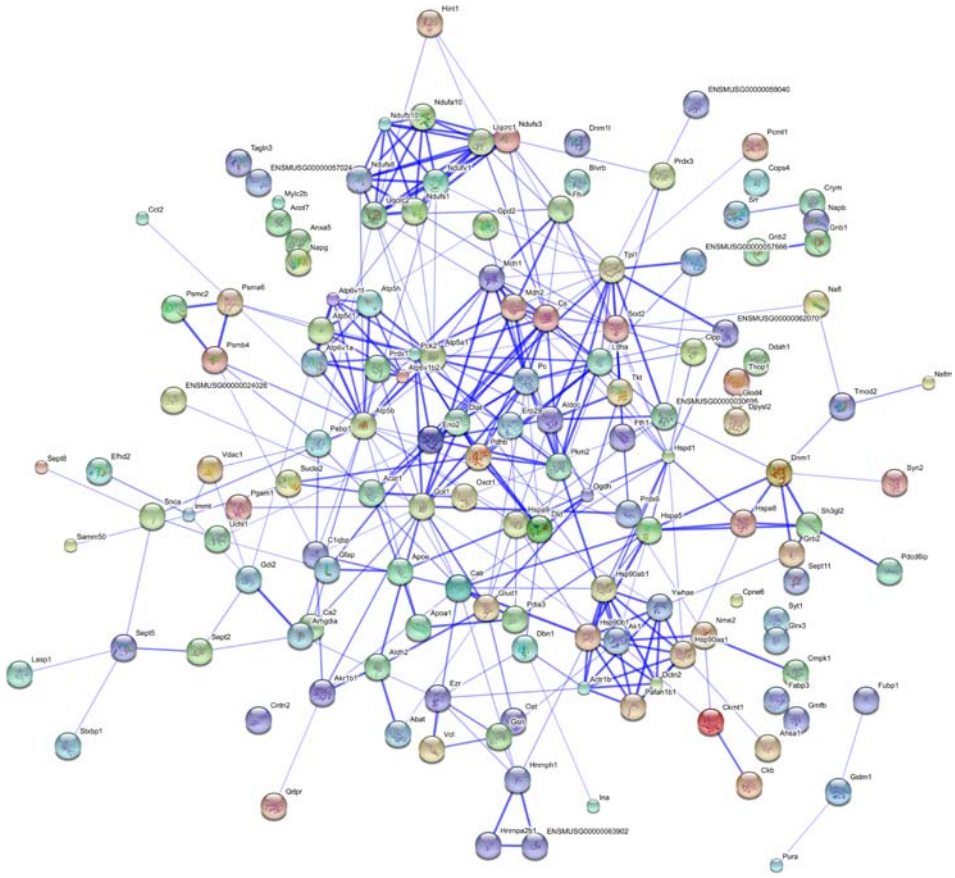

FIGURE 2 Total Pathway network for differentially expressed proteins in the three brain regions (hippocampus, cerebellum, frontal lobe) after mice exposure to EMFs taking into account Table 2 data. The diagram was constructed with the use of the Ingenuity Pathway Analysis software as described in the "Materials and Methods" section. The more references existing in literature about the functional relationship of the shown proteins, the more intense the interconnecting blue lines appear. Major protein categories altered after EMF exposure are HSPs and proteins of the brain metabolism.

- Summarizing, it seems that the mobile phone has a higher impact to all three brain regions isolated and studied, compared to the wireless DECT base, in the specific frequencies and intensities used. Furthermore, it is interesting that approximately, the same number of proteins becomes upregulated or downregulated for a given brain region except the cerebellum where the vast majority of affected proteins (36) have been upregulated.

Fig. 1 is a representative image of a 2-DE gel 3–10 pI of hippocampus sample showing proteins that were downregulated after exposure of mice to wireless DECT base (white arrows) and mobile phone (black arrows) compared to sham-exposed. Differentially expressed proteins are shown annotated by their SwissProt symbols and arrows. Corresponding images of the rest analyzed samples are given as Suppl. Figures 1, 2, and 3.

Table 2 shows in detail the differentially expressed proteins between the studied samples and different exposure protocols (whole body exposure of mice to a wireless DECT base 8 h/day × 8 months or whole body exposure of mice to a mobile phone 3 h/day × 8 months). The status of each differentially expressed protein is indicated by arrows as the mean expression level from the samples reproducibly analyzed.

The pathways which engaged all differentially expressed proteins in exposed and sham-exposed mouse brain regions were studied using the IPA software. The IPA analysis revealed four statistically significant networks between the exposed and sham-exposed regions (Fig. 2). A thorough look in the obtained results indicates that a group of plasticity-related neural proteins have been affected in the different brain regions, which were identified and their detailed expression levels have been calculated by the appropriate software as described in the "Materials and Methods" section (Table 3 and Suppl. Figure 4). Protein spots significantly upregulated in brain regions obtained from mice that have been exposed either to wireless DECT base or to a mobile phone were identified as contactin-2 precursor, glial fibrillary acidic protein, neurofilament medium, and syntaxin. From the proteins that were downregulated after irradiation, it is worth mentioning GMF (glia maturation factor beta), which was found by proteomics to be downregulated 300 fold (0,003 fold decrease) in the hippocampus after wireless base exposure and just 8 fold downregulated (0,125 fold decrease) in mobile phone exposed animals (Table 3).

Western Blot analysis using the appropriate antibodies in both exposed and sham-exposed regions of single animals was applied to confirm the differential expression of two upregulated proteins (apoE and GFAP) and one downregulated (GMF). Optical density measurements of the bands revealed that there was a 1.42 and 2.48 fold increase in the amount of GFAP after mobile phone radiation in frontal lobe for each one of the two randomly chosen animals tested compared to the mean value of the protein for two sham-exposed animals (randomly chosen). Similarly, a 3.53 and 3.04 fold increase in the amount of apoE in cerebellum after wireless DECT base radiation was found and a 0.29 and 0.36 fold decrease in the amount of GMF after wireless DECT base exposure was detected for each animal, respectively, as compared to sham-exposed animals (Fig. 3a, b, and c).

DISCUSSION

This is the first report not only on mouse brain proteome effects induced by EMF, but also on three major regions, namely the hippocampus, cerebellum, and frontal lobe. Therefore, there is no reference baseline to compare the actual results. The closest reports but at the gene level from Slalford's-Belyaev's groups have analyzed expression changes firstly in cerebellum (GSM 900 MHz, Belyaev et al., 2006) and

TABLE 3 Quantitative differential proteomics has revealed the degree of under/over-expression of selected proteins. ± values indicate standard deviations (SD). B: wireless DECT base exposed animals, M: mobile phone exposed animals. Parentheses indicate probability. According to FDR correction, a-level = 0.003571429 and corresponds to $p < 0.05$. Under this level, the under- or over-expression is considered statistically significant.

	Hippocampus		Frontal lobe		Cerebellum	
Protein	B	M	B	M	B	M
ApoE		**2.82 ± 0.24 (0.0002004)**			**1.67 ± 0.14 (0.00248)**	**1.7 ± 0.13 (0.002801)**
Contactin-2 precursor						**1.72 ± 0,12 (6,93E-07)**
Dynactin subunit 2	**0.75 ± 0.06 (0,0003472)**					
Drebrin			**0.56 ± 0.03 (6.30E-005)**	**1.25 ± 0.098 (2.85E-005)**		
Dynamin-1					**0.18 ± 0.07 (2,75E-07)**	**3.46 ± 0.36 (3.99E-011)**
Glial fibrillary acidic protein			**11.6 ± 0.54 (4.24E-007)**	**114.2 ± 3.82 (3.77E-007)**	**1.56 ± 0.26 (0,0016803)**	**1.68 ± 0.19 (0,00198)**
Glia maturation factor beta	**0.003 (1.57E-005)**	**0.13 ± 0.05 (2.02E-006)**				
Neurofilament light polypeptide	**0.65 ± 0.87 (3.96E-006)**	**0.61 ± 0.096 (6,87E-005)**		**1.37 ± 0.18 (4.62E-006)**		**2.62 ± 0.027 (4.10E-007)**
Neurofilament medium polypeptide				**1.87 ± 0.12 (2.03E-005)**		
Syntaxin			**2.13 ± 0.43 (0,00180)**	**11.1 ± 0.96 (1.40E-006)**		
Synapsin-2		**3.6 ± 0.56 (4.89E-006)**		**3.7 ± 0.62 (0.00266)**	**0.21 ± 0.007 (2,44E-007)**	**1.4 ± 0.08 (0.000169)**
Synaptotagmin-1	**0.12 ± 0.004 (3.82E-011)**	**0.16 ± 0.03 (1.01E-05)**				
Alpha-synuclein			**0.42 ± 0.06 (1.28E-006)**	**1.2 ± 0.08 (1.68E-006)**		

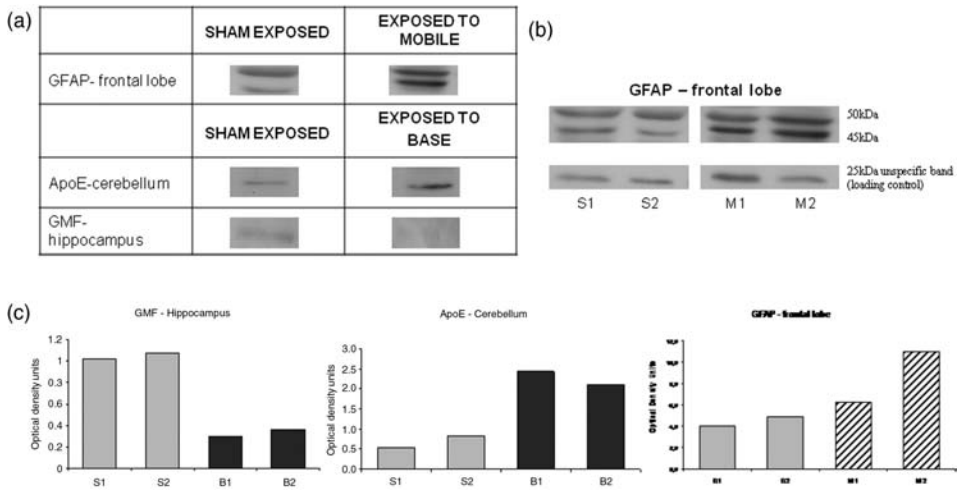

FIGURE 3 Western blot-detected expression of ApoE, GFAP, and GMF proteins in sham-exposed (S), mobile phone exposed (M), and DECT base exposed animals (B). Representative samples are shown. (a) Equal protein amounts of homogenized brain tissues were separated by gel electrophoresis and immunoblotted with antibodies against apoE, GFAP, and GMF. Representative bands from all the membranes and samples are shown. ApoE and GFAP are overexpressed after radiation whereas GMF is greatly downregulated. (b) Detailed GFAP detection in frontal lobe is presented for two single animals. Unspecific band was used as an internal loading control. A clear overexpression is shown following exposure of the animals to mobile phone radiation. (c) Quantification of protein content for GMF, ApoE, and GFAP in different brain tissues using scanning densitometry. Each bar represents amount of protein for a single animal. Two animals are shown for each group.

secondly in hippocampus and cortex (GSM 1800 MHz, Nittby et al., 2008) in rats. They found significant alterations after a single 2-h and 6-h exposure, respectively.

In this work, we investigated separately the effects of chronic (8 months) daily whole body exposure of mice Balb/c to electromagnetic radiation from: (a) a typical-medium SAR level mobile phone (MP) GSM 900 MHz (3 h per day) and (b) the base of a wireless DECT (8 h per day), on the proteome of brain tissues. We showed that a large number of proteins become overexpressed or downregulated in three selected brain regions, namely, the frontal lobe, hippocampus, and cerebellum. Most of these changes occur in the hippocampus, whereas, the majority of the changes have been induced by MP, as shown in Table 1. This first observation could be explained by the fact that there are more concentrated functions in the hippocampus compared to the other two regions and that the hippocampal region may be more active metabolically. There is also a possibility for existence of SAR hot spots in the hippocampus formation relative to the other brain regions (Lai, 1994; Belyaev, 2010). The second fact (MP $\gg$ B) may be explained by the higher SAR value of the MP radiation, albeit the exposure duration was less (3 h vs. 8 h). As shown in Table 2, the overexpression/downregulation profile of the 143 proteins in the three brain regions may be helpful in understanding the behavioral and physiological effects reported for electromagnetic radiation on brain function including blood brain barrier disruption, memory malfunction, oxidative stress, etc.

In an attempt to group the 143 changed proteins we could conclude that:

- 11 of them have changed in all 3 brain regions, more distinctly, synapsin-2 and NADH dehydrogenase. Some of them are indicative of oxidative stress in the nervous system (Martin-Romero et al., 2002). Recently, a detailed molecular mechanism involving NADH oxidase, by which mobile phone radiation exerts its

effects, has been proposed (Friedman et al., 2007). By using Rat1 and HeLa cells, it was shown that EMF exposure resulted in rapid activation of ERK/MAPKs (mitogen-activated protein kinase) mediated in the plasma membrane by NADH oxidase, which rapidly generates ROS.

- 50 of them are hippocampus-specific including, hsp90, septin 8, anexin, ezrin, dynactin (all synapses related proteins), GMFbeta, proteasome subunits alpha and beta involved in apoptosis (Singh and Khar, 2006).
- 25 proteins were specifically changed only in cerebellum, including beta centractin (Weng et al., 2008), mitochondrial hsp60, contactin 2, dynamin, programmed cell death interacting protein, and vinculin.
- 22 proteins are frontal lobe-specific, including drebrin (involved in neuronal plasticity; drebrin A regulates dendritic spine plasticity and synaptic function in mature cultured hippocampal neurons), Neurofilament Medium, and a number of metabolic proteins.
- Lastly, some proteins have been affected by radiation simultaneously in two brain regions namely hippocampus-cerebellum, hippocampus-frontal lobe and frontal lobe-cerebellum (16, 7 and 10 proteins, respectively). These include ApoE (hippocampus-cerebellum, related to memory function), NFL (also hippocampus-cerebellum, related to neuronal integrity), and a number of mitochondrial and metabolic proteins (Aspartate aminotransferase, Glutamate dehydrogenase and others) that could be related to the recent observation on human brain after exposure to 50 min cell phone exposure in which the non thermal effects were associated with increased brain glucose metabolism in the region closest to the MP antenna (Volkow et al., 2011; Lai and Hardell, 2011).

On the lack of any similar work available in the literature it may be useful to provide an overall discussion of the possible scenarios related to the non targeted action of electromagnetic radiation upon the mouse brain proteome; the observed changes in protein expression in a number of mouse neuronal tissue-related proteins following long-term exposure to EMFs reflect the interaction of the microwaves (directly or indirectly) with brain tissue constituents. Considering some of the affected proteins we note the following.

(1) The impressive protein downregulation of the nerve growth factor glial maturation factor beta (GMF) (300 fold in DECT base and 8 fold in mobile phone), which is considered as an intracellular signal transduction regulator in astrocytes (Zaheer et al., 2007), may have an effect in the maintenance of the nervous system. As mentioned by the same authors, since "overexpression of GMF leads to interactions between neural cells, astrocytes, microglia and oligodentrocytes", we speculate that severe downregulation induced by DECT and MP radiation may inhibit the normal function of these cells. In addition, since this protein causes differentiation of brain cells, stimulation of neural regeneration, and inhibition of proliferation of tumor cells, its decrease could perhaps lead in the long run to a tumor induction. Immunoblotting, in GMF, confirmed in general the proteomics data.
(2) GFAP overexpression by 15 fold in both types of radiation is in line with other single protein expression reports following MP exposure of animals (see below) and is indicative of glial intermediate filament overproduction. This may in turn cause neurotransmitter uptake dysfunction and induction of gliosis (Ammari et al., 2008), which is a key step towards the epidemiologically suggested brain tumor increase on long use of mobile phones (Hardell and Carlberg, 2009; Khurana et al., 2009). The glial cells support neurons, release growth factors, and

remove debris after injury or neuronal death. Astrocytes help form the blood brain barrier that prevents toxic substances circulating in the blood from entering the brain. It was proposed many years ago that overexpression of GFAP is the response of astrocytes to oxidative stress (Morgan et al., 1997), which is being reported to take place in brain tissues after exposure of guinea pigs to mobile phone radiation (Meral et al., 2007). Since GFAP is a sensitive biomarker for neurotoxicity, our findings may indicate neuronal tissue injury caused by electromagnetic radiation or a probable injury of the blood brain barrier, reported to be an effect of exposure (Nittby et al., 2009; Sirav and Seyhan, 2009). Immunoblotting with anti-GFAP confirmed in general the proteomics data.

(3) ApoE is a class of apolipoprotein found in the chylomicron and LDLs that bind to a specific receptor on liver cells and peripheral cells. It has been studied for its role in several biological processes not directly related to lipoprotein transport, including Alzheimer's disease (AD), immunoregulation, and cognition. So, the overexpression in the cerebellum and hippocampus after mobile exposure might be related to the memory deficits reported by our group (Fragopoulou et al., 2010b; Fragopoulou and Margaritis, 2010; Ntzouni et al., 2011). This is in agreement with the observation that ApoE4 knock-in mice exhibit an age-dependent decrease in hilar GABAergic interneurons correlated with the extent of learning and memory deficits as found by the Morris water maze task (Andrews-Zwilling et al., 2010).
(4) Synapsin-2 and syntaxin-1 overexpression by both radiation types (MP and DECT) in hippocampus may indicate a compensatory neuronal response to radiation by making more synapses.
(5) Synaptotagmin levels in the hippocampus are in line with the above-mentioned GMF dramatic downregulation. This protein species is known to function as a calcium sensor in the regulation of neurotransmitter release and hormone secretion.

The significance of the present results may be noticeable in relation to the epidemiological, clinical, and other experimental data reported so far concerning behavioral deficits and brain structural/functional alterations induced by EMF in rodents. Although at the epidemiological level Schüz et al. (2006) found as an outcome of the Interphone study no overall increased risk of glioma or meningioma observed among cellular phone users, however, for long-term cellular phone users, the same authors suggested that the results need to be reconsidered before firm conclusions can be drawn. In fact, recent data by Hardell's group have provided solid evidence for a long term effect on brain tumors (Hardell and Carlberg, 2009; Khurana et al., 2009) which might be supported by the protein expression changes found in our results. Along the same lines, reports dealing with EMF-induced brain networking dysfunction can be explained. For instance, in a clinical study with 41 volunteers participating, it was reported that 890 MHz mobile phone-like signal alters the integrity of the human blood–brain and blood–cerebrospinal fluid barriers (Söderqvist et al., 2009). There is also a relationship of MP radiation with behavioral problems in prenatally exposed children (Divan et al., 2008).

Our data using the cordless DECT base as a source of EMF may appear surprising due to the low SAR level, as deduced by measuring the field within the animal cage, approximately 20 mW/Kg, but one explanation could be the intensity windows effect (Blackman, 2009; Belyaev, 2010). Interestingly, Salford's work with rats, applying similar low SAR value (0.6 and 60 mW/Kg), but using mobile phone radiation for just 2 h per week for 55 week, demonstrated significantly altered performance during an episodic-like memory test (Nittby et al., 2008).

It is well established that, in general, the primary action of EMF on living tissue involves an increase of reactive oxygen species (ROS) as demonstrated in exposed sperm (Agarwal et al., 2009; De Iuliis et al., 2009) and under continuous stress conditions in Drosophila flies (Irmak et al., 2002). The ROS accumulation and induced oxidative stress may lead to a signal transduction pathway (ERKs kinases) (Friedman et al., 2007; Lee et al., 2008), whereas at the same time ion channels are disturbed (Friedman et al., 2007; Minelli et al., 2007), Heat Shock Proteins are activated (Friedman et al., 2007; Blank and Goodman, 2009) and conformational change of enzymes (Barteri et al., 2005) is taking place. Thus, on the basis of the literature data and our findings an EMF-impact mechanism can roughly be proposed involving ROS formation followed by stress activation, which may lead to the overexpression of HSPs (Fig. 4). Through this event several indirect changes may occur that alter the physiology of the brain cells, including DNA damage (Lai and Singh, 1996), translation-transcription interference through protein conformation changes (Challis, 2005), a possible cellular metabolism dysfunction, membrane dyspermeability (McNamee and Chauhan, 2009), and memory deficits (Fragopoulou and Margaritis, 2010). It is clear that the effects of EMFs are very difficult to predict in the cells, and that SAR values do not provide any information about the molecular mechanisms likely to take place during exposure.

Unlike drugs, EMFs are absorbed in a variety of different, diverse, and nonlinear ways depending on the "microenvironment" receiving the radiation, the orientation of the molecular targets and their shape, the metabolic state at the moment of exposure, the energy absorbance at the microscale of the cell, and the modulation of the waves. On this basis it is rather difficult to replicate experiments under different conditions and cell

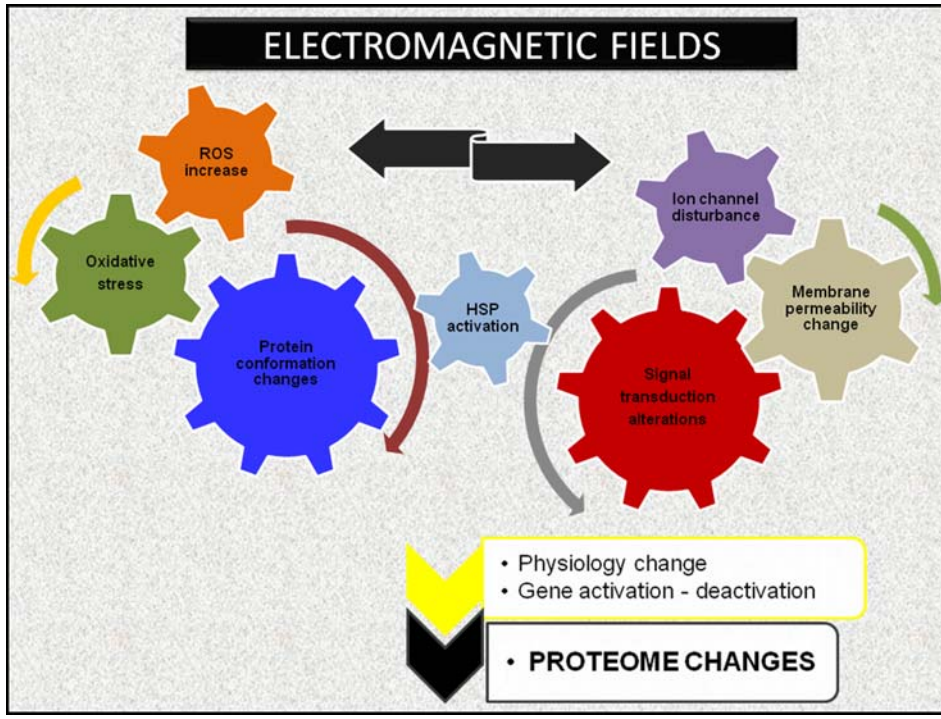

FIGURE 4 Schematic drawing depicting a suggested mechanism of EMF interaction with living matter. It is considered, on the basis of the available data and the present work, that the end result of protein expression changes may have derived through a cascade of events starting from ROS increase and ion channel disturbance, followed by oxidative stress and signal transduction changers. Key role in the events may be played by the heat shock proteins activation.

systems, which may explain the discrepancy of the results among research groups. Especially in the case of proteomics analysis used to approach the effects of EMFs in this article, it is even more difficult, since 2D electrophoresis is inherently variable from one run to the next, especially when being performed in different labs. However, the 2-DE approach is still largely refractory to high-throughput methods due to a number of reasons and can be judiciously coupled to several types of biological experiments to provide meaningful data. Indeed, efforts to improve reproducibility have largely centered on robotics, improved visualization methods as better spot-detection algorithms (Ong and Pandey, 2001; Rogers and Graham, 2007) and as clearly stated by Ong and Pandey (2001), 2-DE-based approaches can still be effectively used when applied with a clear under-standing of its strengths and limitations.

CONCLUSIONS

Our differential proteomic analysis results suggest that conventional MP and DECT base EMFs affect the proteome of hippocampus, cerebellum, and frontal lobe following whole body exposure of Balb/c mice. Since this is the first report showing mouse brain proteome changes induced by EMFs, there is no reference baseline to compare the actual results. However, it is more likely that the observed proteome changes reflect EMF impact and not variability between individual mice, since it has been found just recently that genetic background in both out-bred mouse stocks and inbred mouse strains has a negligible effect on the brain proteome profile (Földi et al., 2011). Based on the currently available literature it is assumed that EMF may function as a stress factor creating ROS and inducing oxidative stress, whereas at the same time ion channels are disturbed and Heat Shock Proteins are activated. This, in turn, may affect gene over/under-expression, possibly through transcription factor activation/deactivation (Kar et al., 2011) in a random manner, since EMF impact is non targeted although stress-related events within the cell are most likely affected. As an end result, functions related to stress response may be triggered. The altered protein expression in this report may reflect such a cascade of events, in which some proteins are related to neural plasticity whereas others belong to the general metabolic processes. The reported herein effects can be considered non thermal since the actual SAR values calculated are well below ICNIRP's (1998) guidelines. In any case, it is seriously considered by pioneers in the topic of EMFs that the relatively low field strengths capable to affect biochemical reactions is a further indication that cells are in a position to sense potential danger long before there is an increase in temperature (Blank and Goodman, 2009).

Further work is underway to reveal the onset of the proteome changes after short term exposure conditions (data under analysis). Also, it is necessary to use multidisciplinary and multilevel approaches in order to delineate the mechanisms of EMF interaction with living organisms.

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Supplementary table and figures available online.