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ORIGINAL ARTICLE



Effects of acute and chronic exposure to both 900 MHz and 2100 MHz electromagnetic radiation on glutamate receptor signaling pathway

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ABSTRACT

Purpose: To demonstrate the molecular effects of acute and chronic exposure to both 900 and 2100 MHz radiofrequency electromagnetic radiation (RF-EMR) on the hippocampal level/activity of some of the enzymes – including PKA, CaMKII α , CREB, and p44/42 MAPK – from N-methyl-D-aspartate receptor (NMDAR)-related signaling pathways.

Materials and methods: Rats were divided into the following groups: sham rats, and rats exposed to 900 and 2100 MHz RF-EMR for 2 h/day for acute (1 week) or chronic (10 weeks), respectively. Western blotting and activity measurement assays were used to assess the level/activity of the selected enzymes.

Results: The obtained results revealed that the hippocampal level/activity of selected enzymes was significantly higher in the chronic groups as compared to the acute groups at both 900 and 2100 MHz RF-EMR exposure. In addition, hippocampal level/activity of selected enzymes was significantly higher at 2100 MHz RF-EMR than 900 MHz RF-EMR in both acute and chronic groups.

Conclusions: The present study provides experimental evidence that both exposure duration (1 week versus 10 weeks) and different carrier frequencies (900 vs. 2100 MHz) had different effects on the protein expression of hippocampus in Wistar rats, which might encourage further research on protection against RF-EMR exposure.

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RF-EMR; CaMKII α ; PKA; CREB; p44/42 MAPK

Introduction

With the increase in the use of mobile phones, these devices are becoming one of the most essential components of contemporary life. Mobile phones generate heat and emit radiofrequency radiation in the range of 800–2200 MHz and third-generation (3G) mobile phones run on 2100 MHz radiofrequency (Bhargava et al. 2012). Mobile phones are known to be associated with potential adverse effects on human health, especially on the human brain (Narayanan et al. 2010). Several effects of radiofrequency electromagnetic radiation (RF-EMR) on the human brain have been demonstrated in experimental studies. Specifically, the effects of RF-EMR on cerebral blood flow (Aalto et al. 2006), blood-brain barrier permeability (Nittby et al. 2009), oxidant and antioxidant balance (Irmak et al. 2002), neurotransmitter balance (Tamasidze and Nikolaishvili 2007), nerve cell damage (Salford et al. 2003), genomic responses (Lai and Singh 1996), short-term memory loss and other cognitive functions (Maaroufi et al. 2014; Sakhnini et al. 2014; Singh and Kapoor 2014) have been studied. However, much less attention has been paid so far to the molecular effects of RF-EMR exposure on N-methyl-D-aspartate (NMDA) receptor-related signaling pathways on the brain.

In research of learning and memory, the hippocampus is the most studied structure in the mammalian brain because

of its well documented role in learning and memory functions, especially in spatial memory as well as its simple, tri-synaptic circuitry (Leuner and Gould 2010). Various studies have indicated that RF-EMR affected the activities (Cho et al. 2012), morphology (Hao et al. 2013; Wang et al. 2013), and chemistry (Cuccurazzu et al. 2010) of the hippocampus in animals. For example, in a study by Narayanan et al. (2009), the effects of GSM (900/1800 MHz) mobile phone exposure for 4 weeks to 50 missed calls/day on the hippocampus-dependent spatial memory performance were tested on rats and impaired spatial memory performance in the Morris water maze (MWM) test was observed on phone-exposed rats as compared to control animals.

The N-methyl-D-aspartate receptors (NMDARs) and the α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPA) are the major players for controlling synaptic plasticity and memory functions in the hippocampus (Rebola et al. 2010; Chater and Goda 2014). They often work together to produce long-lasting changes in synaptic functioning. The NMDA receptor acts differently because it is a coincidence detector of presynaptic and postsynaptic firing (Rebola et al. 2010).

Activation-dependent molecular changes at synapses are mediated by a rise in intracellular calcium which enters the postsynaptic neuron through NMDA ion channels

(Rebola et al. 2010). Calcium alone or conjugated with calmodulin acts as a powerful secondary messenger and leads to the activation of numerous synaptic proteins including Ca^{2+} /calmodulin-dependent protein kinase called CaMK that controls the function of other proteins through the phosphorylation of Ser/Thr amino acid residues on these proteins (Swulius and Waxham 2008). In the hippocampus, among many downstream signaling molecules of the NMDA receptor pathway, Ca^{2+} /calmodulin-dependent protein kinase II alpha (CaMKII α) has long been considered the key mediator in regulating the early phase of long-term potentiation (E-LTP) and memory formation (Soderling 2000; Lisman et al. 2002; Colbran and Brown 2004; Colbran 2004). Studies have shown that pharmacological inhibition of CaMKII or genetically removing it leads to the blockage of LTP induction (Lisman and McNlytre 2001). Introduction of an active form of CaMKII into the postsynaptic neuron leads to inducing potentiation which prevents subsequent LTP induction; thus, CaMKII is both necessary and sufficient for LTP induction (Lisman and McNlytre 2001).

The short-term effects of CaMKII α activation partially overlap with those of protein kinase A (PKA) and include the insertion of new AMPARs (Lisman and McNlytre 2001; Cammarota et al. 2002) and an increase in the unitary conductance of the AMPA ionophore (Derkach et al. 1999; Lisman and McNlytre 2001). PKA serves a function similar to CaMKII, but PKA's effects are broader. Like CaMKII, PKA phosphorylates voltage-dependent calcium channels and enhance their excitability to future stimuli (Catterall 2011). It also promotes internalization of small-conductance Ca^{2+} -activated K^+ channel type 2 (SK2) reducing membrane K^+ conductance and thus increases neuron's excitability (Adelman et al. 2012). These effects demonstrate PKA's contribution to early phase of activity-dependent neural plasticity (i.e. E-LTP) (Castillo 2012). However, PKA was also shown to be critical for late phase of activity-dependent neural plasticity (i.e. L-LTP) and long-term memory (Wong et al. 1999; Kandel 2001; Castillo 2012; Mayford et al. 2012). It was reported that PKA phosphorylates intracellular AMPARs stores, facilitating their insertion postsynaptically. PKA may also enhance new AMPARs delivery via activation of the mitogen-activated protein kinase (MAPK) cascade (Pearson et al. 2001). The phosphorylated form of MAPK and catalytic subunit of PKA are transported to the nucleus, where through activation of the cyclic adenosine monophosphate (cAMP) responsive element-binding (CREB) protein, a nuclear transcription factor that modulates transcription of genes containing cAMP responsive elements (CRE sites) in their promoters and thus leads to long-term synaptic changes in the stimulated cells (Micheau and Riedel 1999; Pearson et al. 2001; Josselyn and Nguyen 2005). Increases in the concentration of either calcium or cAMP can trigger the phosphorylation and activation of CREB (Josselyn and Nguyen 2005). This transcription factor is a component of intracellular signaling events that regulate a wide range of biological functions, from spermatogenesis to circadian rhythms and memory (Silva et al. 1998). In addition to PKA, several other kinases such as Ca^{2+} /calmodulin-dependent protein kinase I (CaMKI), Ca^{2+} /calmodulin-dependent protein kinase II (CaMKII), Ca^{2+} /calmodulin-dependent protein kinase

IV (CaMKIV), protein kinase G (PKG), protein kinase C (PKC) also phosphorylate CREB (Jonannessen et al. 2004).

The major intracellular signaling pathways involved in both short- and long-term memory formations in the hippocampus are NMDA-dependent pathways including activation of CaMKII, PKA, CREB, and MAPK. In the literature, little is known about the effects of RF-EMR exposure on NMDA receptor-related signaling pathways. Therefore, in the present study we investigated the basal effects of acute and chronic exposure to both 900 and 2100 MHz RF-EMR on the hippocampal level/activity of previously mentioned enzymes from NMDA-dependent pathway including CaMKII α , PKA, CREB, and p44/42 MAPK. Additionally, in order to indirectly estimate the activity of CaMKII α and p44/42 MAPK, hippocampal levels of their phosphorylated forms (pCaMKII α and phospho-p44/42 MAPK) were also assessed.

Materials and methods

Animals

The animal care procedures and all experimental manipulations in the proposed study were pursued in accordance with the Institutional Animal Care and Use Committee in Akdeniz University, Antalya, Turkey (Protocol No: 2014.02.06). Experiments were conducted on inbred young male albino Wistar rats aged 3 months, weighing 200–250 g obtained from Akdeniz University Animal Facility. All rats were kept in home cages in groups of four under a constant temperature ($23 \pm 1^\circ\text{C}$) and a 12/12 h light/dark cycle. They were all given food and water *ad libitum*.

Electromagnetic field exposure

Rats were randomly divided into six groups ($n=13$ per group) after the adaptation process (Table 1). During the experiment, all rats in each group were placed in a plexiglass tube with air holes to facilitate breathing and minimize the rise in body temperature. The '900-1' and '900-10' group of rats were exposed to 900 MHz RF-EMR whereas the '2100-1' and '2100-10' groups of rats were exposed to 2100 MHz RF-EMR emitted from the signal generator for 2 h per day (5 days a week) for 1 and 10 weeks, respectively. Sham rats were housed in the same room under the same conditions with equal time period, except that the generator was turned off. All experimenters were blind to animal experimental group membership during data collection and analysis.

An RF generator (GSM Simulator 900 MHz; Everest Company, Adapazari, Turkey), which produces 900 MHz RF radiation, was used to represent exposure of global systems for mobile communications (GSM). The output power of the

Table 1. Experimental group of rats.

Group number	Group name	Abbrev
1	Sham-exposed rats for 1 week (acute)	S-1
2	Sham-exposed rats for 10 weeks (chronic)	S-10
3	Acute exposure to 900 MHz RFR for 1 week	900-1
4	Chronic exposure to 900 MHz RFR for 10 weeks	900-10
5	Acute exposure to 2100 MHz RFR for 1 week	2100-1
6	Chronic exposure to 2100 MHz RFR for 10 weeks	2100-10

generator was fixed at 5 W during exposure. The carrier frequency was 900 MHz, the modulation frequency was 217 Hz, pulse width was 0.577 msec, and the power range of the generator was 0–10 W. The other RF generator (UMTS Simulator 2100 MHz; Everest Company, Adapazari, Turkey), which produces 2100 MHz RF radiation, was used to represent exposure of Universal Mobile Telecommunications System (UMTS). The output power of the generator was fixed at 1.5 W during exposure. The carrier frequency was 2100 MHz, the modulation frequency was 217 Hz, pulse width was 0.577 msec, and the power range of the generator was 0–2 W. The set-up consisted of a carousel on which 13 rats, each restrained in a radially positioned tube, were exposed to the electromagnetic field emanating from antenna at the center (Figure 1). The rats were positioned with their snouts at a distance of 3.5 cm for 900 MHz and 10 cm for 2100 MHz, from the monopole antenna. The tubes restrained the movement of the rats to such an extent as to follow for well-defined exposure conditions, yet without immobilizing them. The entire carousel was placed in the shielded room to prevent other disturbance sources. The electric field strengths were measured by EMR300 with an appropriate probe (Narda, Germany). The electric field background level in the shielded room was between 0.02 and 0.2 V/m. In the 'signal-on' situation, the measured electric-field strengths over the rat's head positioned 3.5 and 10 cm away from the antenna were 35.5 and 35.2 V/m for 900 and 2100 MHz RF-EMR, respectively. The average whole-body SAR was 5.284 and 128 mW/kg for 900 and 2100 MHz, respectively. The SAR for the brain was in average of 0.66 and 0.27 W/kg for 900 and 2100 MHz, respectively. The numerical computation was performed using the Finite Difference Time Domain (FDTD) method (LeBlanc et al. 2000; Hyun and Oh 2006). The FDTD code with perfectly matched layer absorbing boundaries has been used to measure the SAR value on the brain of Wistar

rats. The electric field strength, the conductivity and the density of the model for each voxel have been used for the estimation of the SAR value. Electrical properties, conductivity and dielectric constant were taken from the literature (Peyman et al. 2001, Gabriel 2005). The applied carousel set-up procedure of the present study was compatible with the set-up procedure of the other studies in the literature (Burkhardt et al. 1997; Fritze et al. 1997; Schönborn et al. 2003).

Tissue sample preparation

All rats were anesthetized under urethane and brains were perfused transcardially with heparinized saline. Then, the perfused brains were rapidly removed, placed on dry ice and the hippocampi dissected. For the Western blotting assay, total hippocampal tissue of rats was immediately frozen in liquid nitrogen and then stored at -80°C until use. For the activity measurement assays, total hippocampal tissue of rats was immediately homogenized, and after centrifuge process supernatant was removed and stored at -80°C until use.

Western blot protein analysis

Hippocampal tissues of rats were weighed and homogenized by ultrasonication in the presence of protease inhibitors. Tissue samples were transferred to a centrifuge tube containing 800 μl of Triton lysis buffer (1% Triton X-100, 20 mM Tris-HCL pH 7.4, 150 mM NaCl, 5 mM EGTA, 10 mM EDTA, 10% glycerol, 20 mM HEPES and protease inhibitor cocktail [Roche]). Samples were then subjected to sonication. Sonication was performed with a sonic dismembrator in an ice-bath with a microtip probe set to a power output of 40 W for three cycles of 5 sec each (15 sec interval between

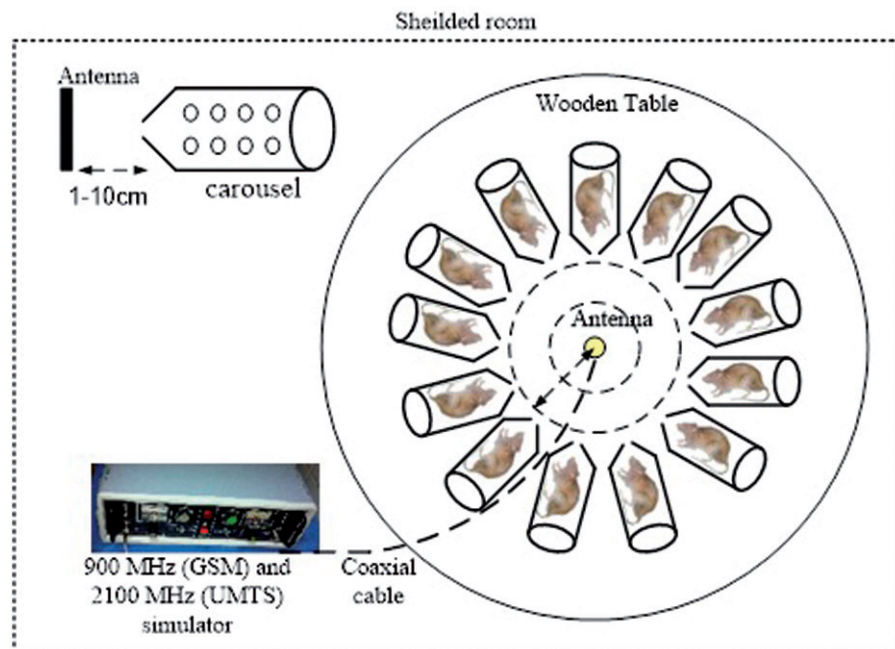


Figure 1. Carousel-type electromagnetic field exposure system.

sonications). Afterwards they were centrifuged at 20,000 *g* for 30 min at 4 °C, then supernatant was removed and stored at –80 °C until use.

For determination of CaMKII α , pCaMKII α , PKA, CREB, p44/42 MAPK, phospho-p44/42 MAPK expressions, 7.5 μ g (for pCaMKII α , CREB) and 5 μ g (for CaMKII α , PKA, p44/42 MAPK, phospho-p44/42 MAPK) of hippocampal samples were run on 6–7.5% polyacrylamide gels. The separated proteins were transferred onto PVDF membrane by means of electroblotting with Mini Protean Tetra Cell equipment (Bio-Rad Laboratories, Richmond, CA, U.S.A.). Afterwards the membranes were incubated at room temperature in blocking solution made of 5% dried non-fat milk in TBS-T buffer (20 mM Tris-HCl, 0.5 M NaCl and 0.05% Tween 20, pH 7.6). Blots were then incubated for 2 h at room temperature with primary antibodies. Primary antibodies were applied at a dilution of 1:1000 (CaMKII α , Santa Cruz; p44/42 MAPK, Cell Signaling), 1:500 (CREB, ABCAM), 1:2000 (phospho-p44/42 MAPK, Cell Signaling), 1:5000 (PKA, BD Bioscience) and 1:250 (pCaMKII α , Santa Cruz). After being rinsed with TBS-T buffer, the membranes were incubated for 1 h at room temperature with the secondary alkaline phosphatase conjugated antibody (Santa Cruz) used at a dilution of 1:1000 (pCaMKII α), 1:2000 (CREB, p44/42 MAPK) and 1:5000 (CaMKII α , PKA, phospho-p44/42 MAPK) in 5% dried non-fat milk in TBS-T buffer. A monoclonal antibody directed against β -actin and α -tubulin was used as a control to normalize protein expression levels. Blots were rinsed with TBS-T buffer and incubated with an AP conjugate substrate kit (Bio-Rad) to visualize the specifically bound antibodies. The final images were photographed using a computer-based gel imaging instrument (Vilber Lourmat) with Infinity-Capt version 12.9 software. Immunoreactive protein bands were then quantified by densitometric scanning method using an Image J software package.

PKA activity

PKA activity in tissues was determined using PKA Activity Kit Assay (ab139435) which is based on a solid phase enzyme-linked immune-absorbent assay (ELISA) that utilizes a specific synthetic peptide as a substrate for PKA and a polyclonal antibody that recognizes the phosphorylated form of the substrate.

CREB activity

CREB activity in tissues was measured via Cayman's CREB (Phospho-Ser¹³³) Transcription Factor Assay Kit (Item No: 10009846). To isolate nuclear proteins, Cayman's Nuclear Extraction Kit (Item No: 10009277) was used. In brief, CREB contained in a nuclear extract binds specifically to the cAMP response element (CRE). The activated CREB transcription factor complex is detected by addition of a specific primary antibody directed against Phospho-Ser¹³³ on CREB. A secondary antibody conjugated to HRP is added to provide a sensitive colorimetric readout at 450 nm.

Protein measurements

Protein concentrations were measured at 595 nm by a modified Bradford assay using Coomassie Plus reagent with bovine serum albumin as a standard (Pierce Chemical).

Statistical analysis

To evaluate the Western blotting and activity assay data, one-way ANOVA with all pairwise multiple comparison procedure done by Tukey's Honestly Significant Difference (HSD) post hoc test was applied. In addition, two-way ANOVA was conducted on these data to assess the main effects of radiation frequency and duration of exposure as well as a potential radiation frequency $\times$ duration of exposure interaction. A *p* value less than or equal to 0.05 was considered statistically significant. The statistical package SPSS v.20 was used for statistical analyses.

Results

Figure 2 illustrates the representative immunoreactive protein bands for all proteins under study obtained from the acute (S-1) and chronic (S-10) groups of sham-exposed rats, from the acute (900-1) and chronic (900-10) groups of 900 MHz RF-EMR-exposed rats, and from the acute (2100-1) and chronic (2100-10) groups of 2100 MHz RF-EMR-exposed rats. CaMKII α and pCaMKII α were detected as bands of 50 kDa, PKA was detected as bands of 53 kDa, CREB was detected as 43 kDa band, and p44/42 MAPK and phospho-p44/42 MAPK were detected as bands of 42, 44 kDa. Relative protein

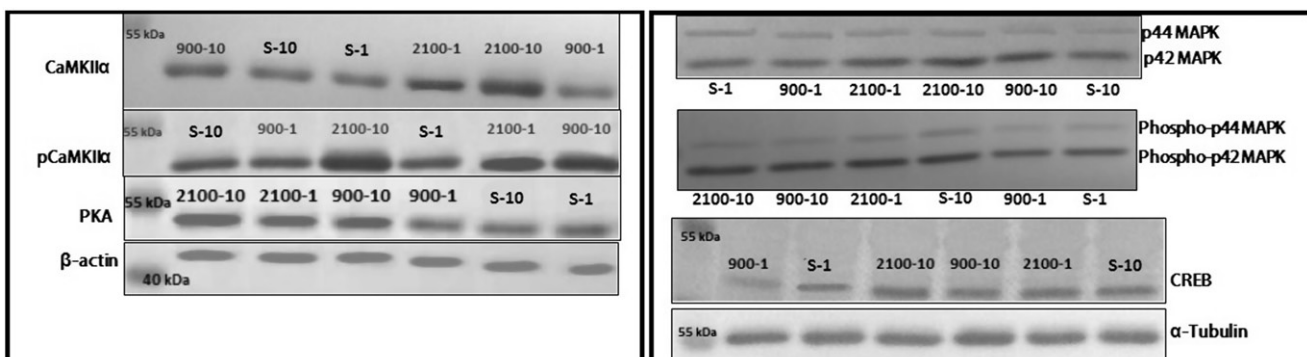


Figure 2. Representative immunoreactive protein bands for CaMKII α , pCaMKII α , PKA, p44/42 MAPK, phospho-p44/42 MAPK, and CREB, from hippocampus of the S-1, S-10, 900-1, 900-10, 2100-1 and 2100-10 rat groups. The β -actin and α -tubulin bands were used as an internal control for each gel.

expressions were determined by comparing band intensities with that of β -actin and α -tubulin which were detected at the position corresponding to a molecular weight of 42 and 55 kDa, respectively, and were used as the internal control.

The differences in the hippocampal levels of both CaMKII α and pCaMKII α among rat groups were demonstrated by

Figure 3(A). There was a significantly higher expression levels of both CaMKII α and pCaMKII α in the 2100-1 groups as compared to the S-1 groups ($p \leq 0.001$ and $p \leq 0.001$, respectively), whereas, both CaMKII α and pCaMKII α levels were significantly lower in the 900-1 groups as compared to the S-1 groups ($p = 0.004$ and $p = 0.029$, respectively). The comparison of

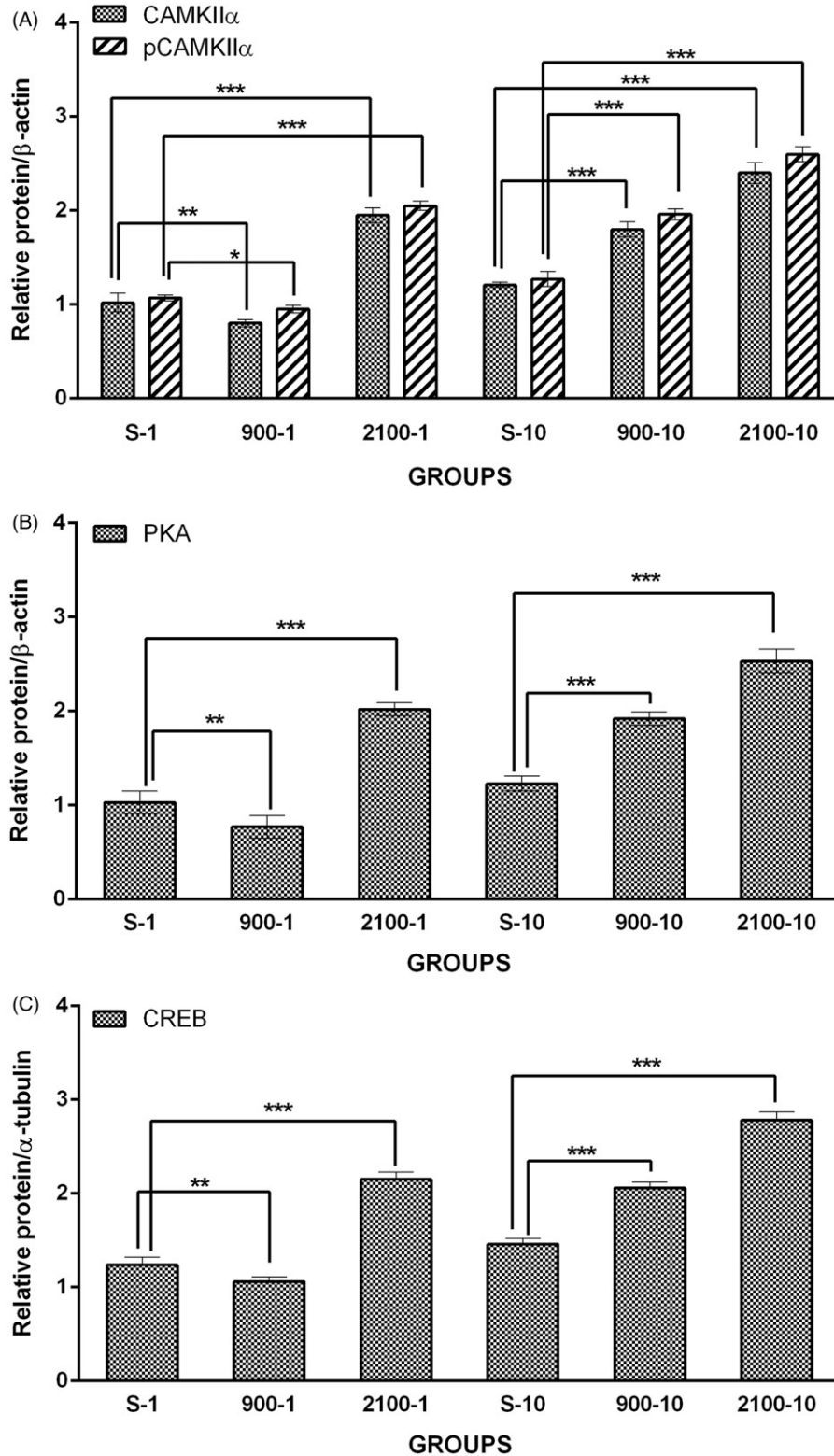


Figure 3. Comparison of the levels of (A) CaMKII α and pCaMKII α ; (B) PKA; (C) CREB; and (D) p44/42 MAPK and phospho-p44/42 MAPK in the hippocampus of S-1, S-10, 900-1, 900-10, 2100-1 and 2100-10 rat groups. Band quantifications are expressed as the mean $\pm$ SD of the relative intensity in respect to that of β -actin and α -tubulin, used as the internal control. Error bars denote $\pm$ SD. Asterisks denote the level of significance: *** $p < 0.001$.

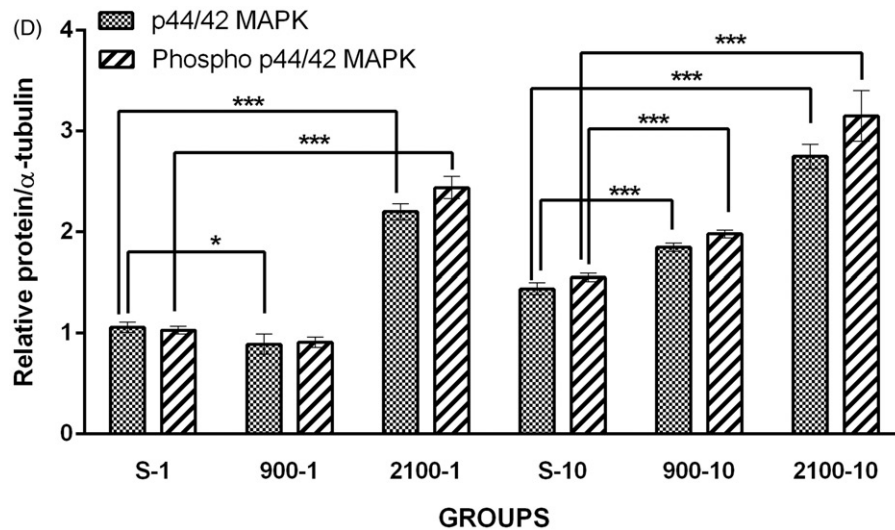


Figure 3. Continued.

both CaMKII α and pCaMKII α levels in the 2100-10 groups as compared to the S-10 groups showed significantly higher expression of both proteins in the 2100-10 groups ($p \leq 0.001$ and $p \leq 0.001$, respectively). In addition, both CaMKII α and pCaMKII α levels in the 900-10 groups were significantly higher than the S-10 groups ($p \leq 0.001$ and $p \leq 0.001$, respectively). In the 900-1, 2100-1, 900-10 and 2100-10 rat groups, the mean numbers of pCaMKII α level (0.95 ± 0.02 , 2.05 ± 0.03 , 1.96 ± 0.06 and 2.6 ± 0.03 , respectively) were significantly ($p \leq 0.001$) higher than the mean numbers of CaMKII α level (0.8 ± 0.02 , 1.95 ± 0.04 , 1.8 ± 0.06 and 2.4 ± 0.06 , respectively) (Figure 3(A)). Two-way ANOVA applied to Western blot data of CaMKII α and pCaMKII α revealed a highly significant main effect of both, the radiation frequency ($p \leq 0.001$ and $p \leq 0.001$, respectively), and the duration of exposure ($p \leq 0.001$ and $p \leq 0.001$, respectively), and a significant radiation frequency $\times$ duration of exposure interaction ($p \leq 0.001$ and $p \leq 0.001$, respectively).

The hippocampal level of PKA was significantly higher in the 2100-1 groups as compared to the S-1 groups and lower in the 900-1 groups as compared to the S-1 groups ($p \leq 0.001$ and $p = 0.006$, respectively) (Figure 3(B)). On the other hand, PKA level was significantly higher in both 2100-10 and 900-10 groups as compared to the S-10 groups ($p \leq 0.001$ and $p \leq 0.001$, respectively) (Figure 3(B)). There was a significant increase in the PKA activity of the 900-1 and 2100-1 rat groups as compared to the S-1 rat groups ($p = 0.002$ and $p = 0.008$, respectively). Similarly, an increase in the PKA activity was observed in the 900-10 and 2100-10 rat groups as compared to S-10 rat groups ($p = 0.006$ and $p \leq 0.001$, respectively) (Figure 4(A)). Two-way ANOVA applied to activity data of PKA revealed a highly significant main effect of both, the radiation frequency and the duration of exposure ($p \leq 0.001$ and $p \leq 0.001$, respectively), and moderately significant radiation frequency $\times$ duration of exposure interaction ($p = 0.053$). As shown in Figure 3(C), quantitative immunoblot analysis of CREB levels revealed significantly higher expression of CREB in the 2100-1 groups ($p \leq 0.001$)

and significantly lower expression of CREB in the 900-1 groups ($p = 0.007$) as compared to the S-1 groups. Both 2100-10 and 900-10 groups exhibited higher levels of CREB as compared to the S-1 groups ($p \leq 0.001$ and $p \leq 0.001$, respectively) (Figure 3(C)). There was a significant increase in the CREB activity in the hippocampus of the 2100-1 rat groups as compared to S-1 rat groups ($p \leq 0.001$). In addition, an increase in the CREB activity was observed in the hippocampus of 900-10 and 2100-10 rat groups as compared to the S-10 rat groups ($p = 0.005$ and $p \leq 0.001$, respectively) (Figure 4(B)). Two-way ANOVA applied to activity data of CREB revealed a highly significant main effect of both, the radiation frequency and the duration of exposure ($p \leq 0.001$ and $p = 0.005$, respectively), and a radiation frequency $\times$ duration of exposure interaction ($p \leq 0.001$). In addition, two-way ANOVA conducted on Western blot data of PKA and CREB confirmed a highly significant main effect of both, the radiation frequency ($p \leq 0.001$ and $p \leq 0.001$, respectively) and the duration of exposure ($p \leq 0.001$ and $p \leq 0.001$, respectively), and a significant radiation frequency $\times$ duration of exposure interaction ($p \leq 0.001$ and $p \leq 0.001$, respectively).

Figure 3(D) illustrates the differences in the levels of both p44/42 MAPK and phospho-p44/42 MAPK among rat groups. One-way ANOVA applied to these data yielded a significantly higher expression of both p44/42 MAPK and phospho-p44/42 MAPK in the 2100-1 groups as compared to S-1 groups ($p \leq 0.001$ and $p \leq 0.001$, respectively), whereas, the p44/42 MAPK level was significantly lower in the 900-1 groups as compared to S-1 groups ($p = 0.021$). One-way ANOVA performed on these data revealed the difference in the level of phospho-p44/42 MAPK between the 900-1 and S-1 groups insignificant. On the other hand, the comparison of levels of both p44/42 MAPK and phospho-p44/42 MAPK in the 2100-10 groups as compared to S-10 groups showed significantly higher expression of both proteins in the 2100-10 groups ($p \leq 0.001$ and $p \leq 0.001$, respectively). In addition, both p44/42 MAPK and phospho-p44/42 MAPK levels in the 900-10

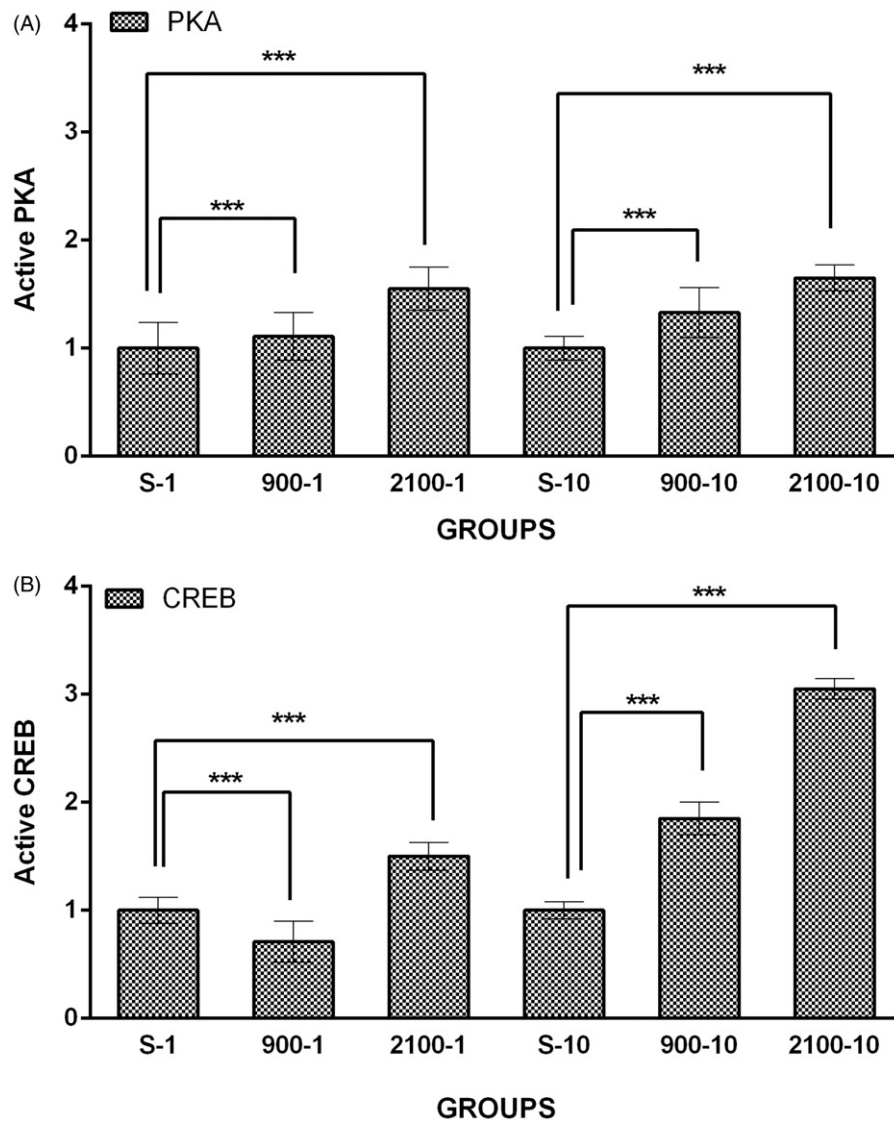


Figure 4. Enzymatic activity of PKA based on solid phase enzyme-linked immune-absorbent assay (ELISA) (A) and enzymatic activity of CREB based on detection of activated CREB TF complex by a primary antibody directed against Phospho-Ser¹³³ (B) in the hippocampus of S-1, 900-1, 2100-1, S-10, 900-10 and 2100-10 rat groups. Values were represented as mean $\pm$ SD. Asterisks denote the level of significance: *** $p < 0.001$.

groups were significantly higher than of the S-10 groups ($p \leq 0.001$ and $p \leq 0.007$, respectively). In the 2100-1, 900-10 and 2100-10 rat groups, mean numbers of phospho-p44/42 MAPK level (2.44 ± 0.06 , 1.98 ± 0.01 and 3.15 ± 0.2 , respectively) were significantly ($p \leq 0.001$) higher than mean numbers of p44/42 MAPK level (2.2 ± 0.05 , 1.85 ± 0.03 and 2.75 ± 0.07 , respectively) (Figure 3(D)). Two-way ANOVA applied to Western blot data of p44/42 MAPK and phospho-p44/42 MAPK revealed a highly significant main effect of both, the radiation frequency ($p \leq 0.001$ and $p \leq 0.001$, respectively) and the duration of exposure ($p \leq 0.001$ and $p \leq 0.001$, respectively), and a significant radiation frequency $\times$ duration of exposure interaction ($p \leq 0.001$ and $p = 0.015$, respectively).

Discussion

While talking, mobile phones are typically in close proximity to the brain. All of the brain regions, including hippocampus,

are detrimentally affected from RF-EMR produced by mobile phones due to this exposure. The most prominent neurotransmitter associated with learning and memory functions in the hippocampus is glutamate. Glutamate exerts its signaling function by binding to hippocampal glutamate receptors (Rebola et al. 2010). One of them is called the NMDA receptor and it acts as a coincidence detector of presynaptic and postsynaptic firing via its role in the induction of activity-dependent long-term potentiation (LTP) and long-term depression (LTD) (Rebola et al. 2010). This property of the NMDA receptor is linked to both NMDA receptor channels blockage by Mg^{2+} and their permeability to Ca^{2+} . NMDA receptor-dependent calcium influx results in activation of kinases, notably CaMKII, PKA, and MAPK (Lau and Zukin 2007). Activation of PKA also activates CREB, which binds the cAMP response element and activates the gene transcription and Ginty 2002). Although, there have been considerable concerns about the potential influences of RF-EMR exposure on

brain, more comprehensive studies are required to elucidate the possible effects on the NMDA receptor-related signaling pathways. Therefore, the major aim of the present study was to demonstrate the molecular effects of RF-EMR exposure on hippocampal level/activity of some of the enzymes – including PKA, CaMKII α , CREB, and p44/42 MAPK – from N-methyl-D-aspartate receptor (NMDAR)-related signaling pathways.

There are several animal studies – mainly behavioral – showing differential effects of different waveforms and different duration time of exposure (Nittby et al. 2008; Deshmukh et al. 2013; Hao et al. 2013). In Nittby et al. (2008), rats were exposed for 2 h each week for 55 weeks to RF-EMR (GSM-900) at different SAR levels (0.6 and 60 mW/kg at the initiation of the experimental period) emitted by a test phone and results indicated that GSM-exposed rats had impaired memory for objects and their temporal order of presentation, compared to sham-exposed controls. In another study, animals were subjected to long-term (30 days) microwave exposure of 900 MHz and results showed that there was a significant impairment in cognitive function in elevated plus and Morris water maze (Deshmukh et al. 2013). Hao et al. (2013) reported that the 916 MHz electromagnetic field (EMF) exposure for 6 h a day, 5 days a week, for 10 weeks adversely influenced the learning and memory performances of the rats in the radial arm maze task to some extent in a period during exposure, and the rats could adapt to long-term EMF exposure. With regard to the above studies, different waveforms and different duration times of RF-EMR exposure had different behavioral effects. So, the other aim of the present study was to investigate basal effects of both acute (1 week) and chronic (10 weeks) exposure to both 900 and 2100 MHz RF-EMR on rats as compared to a sham group of rats.

In the present study, the comparison of hippocampal level/activity of PKA and CaMKII α between acute and chronic groups of 900 and 2100 MHz RF-EMR revealed that hippocampal level/activity of these enzymes were significantly higher in the chronic (10 weeks) groups than the acute (1 week) groups. In a study by Manikonda et al. (2007), long-term exposure (90 days) of rats to 50 Hz extremely low frequency (ELF) caused increased intracellular Ca²⁺ levels together with increased activities of PKA and calcineurin in hippocampal regions compared to control groups. Another research data showed that expression of CaMKII gamma was significantly up-regulated both in hippocampal tissues and nerve growth factor (NGF)-differentiated PC12 cells, which can be activated by the increased Ca²⁺ influx signal through postsynaptic NMDARs that arise from following 30 mW/cm² microwave exposure (Xiong et al. 2015). In the present study, the α form of CaMKII was studied since it is the predominant form which is specifically localized in the forebrain structures. The increased level/activity of the PKA and CaMKII α found in the present study is consistent with the results of the above studies and might be related to the increased intracellular Ca²⁺ and calcineurin levels (Manikonda et al. 2007; Xiong et al. 2015).

A significantly high level/activity of CREB found in the present study in chronic groups (10 weeks) compared to acute (1 week) groups of both 900 and 2100 MHz RF-EMR is consistent with activation of transcription factors including

phospho-CREB (p-CREB) by activation of CaMKII triggered by glutamate-mediated Ca²⁺ entry (Xiong et al. 2015). In the Xiong et al. (2015) study, rats were exposed to 30 mW/cm² microwave for 10 min every other day for three times. The central nervous system (CNS), especially the hippocampus, is sensitive to microwave exposure from mobile phones. Regarding cognitive function, learning and memory impairments have been reported following microwave exposure (Lu et al. 2012; Deshmukh et al. 2013; Wang et al. 2013).

Experiments carried out on both acute and chronic groups of 900 and 2100 MHz RF-EMR revealed significantly higher level/activity of p44/42 MAPK in chronic groups (10 weeks) than acute (1 week) groups. In a study by Hidisoglu et al. (2016), the levels of brain thiobarbituric acid reactive substances (TBARS) and 4-hydroxy-2-nonenal (4-HNE), which are known as oxidative stress biomarkers, increased significantly in the 10 weeks group that was exposed to 2100 MHz EMF. Another study (Kesari et al. 2014) reported that 3G mobile phone exposure (2 h a day for 60 days) on rats induced the oxidative stress which caused a transient increase in the level of p38 MAPK. It has been known that major signal transduction pathway activated by oxidative stress is mitogen-activated protein kinases (MAPK) in addition to phosphoinositide 3 kinase (PI-3K), and nuclear factor-kB signaling (NF-kB) (Johnson and Lapadat 2002). In light of these findings, the increased level/activity of p44/42 MAPK observed in the present study might be explained by oxidative stress induction via mobile phone exposure.

Overall results of the present study have revealed that the hippocampal level/activity of selected enzymes were significantly higher in both acute (1 week) and chronic (10 weeks) groups of rats exposed to 2100 MHz RF-EMR than rats exposed to 900 MHz RF-EMR. Previously, different effects of different carrier-frequencies (900 vs. 1800 MHz) on protein expression were observed on primary human endothelial cell lines by using proteome profiling (Nylund et al. 2010). In contrast to our results, Nylund et al. (2010) reported that exposure of cells to 1800 MHz GSM signal had only a very small effect on cell proteome, as compared to 900 MHz GSM exposure. The discrepancy may arise from the different experimental procedures used in the studies.

To our knowledge, this is the first study investigating basal effects of acute (1 week) and chronic (10 weeks) exposure to both 900 and 2100 MHz RF-EMR to hippocampal level/activity of selected enzymes from the NMDA-dependent pathway. Our experimental approach involved an examination of the potential effects of RF-EMR exposure on the hippocampal NMDA-dependent signaling pathway. The majority of the mobile phone studies have investigated the effects of second-generation (2G) mobile phone exposure, while fewer researches have focused on the investigation of the effects of the third-generation (3G) mobile phone exposure on the brain functions. When considered from this point of view, our findings might contribute greatly to the literature.

In conclusion, our results provide experimental data suggesting that both duration (1 week vs. 10 weeks) and different carrier-frequencies (900 vs. 2100 MHz) had different effects on protein expression of hippocampus in Wistar rats

which might be used to encourage further research on protection against the RF-EMR exposure.

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