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Short-term electromagnetic fields treatment induces significant changes on the auditory evoked potentials in adult rats.

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Running Title: Effect of electromagnetic fields on brain activity

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Abstract

Purpose: There is a growing interest in usage of radio frequency (RF) electromagnetic field (EMF) as a noninvasive brain stimulation method. Previous reported data demonstrated that RF-EMF exposure caused a change in brain oscillations. Therefore, we aimed to investigate effects of RF-EMF on brain oscillation by measuring auditory response of different brain regions in rats.

Material Methods: Rats were randomly divided into three groups (n=12 per each group): Cage control (C), sham rats (Sh), and rats exposed to 2.1 GHz RF-EMF for 2h/day for a week. At the end of exposure, auditory evoked potentials (AEPs) were recorded at different locations in rats. Latencies and amplitudes of AEPs, evoked power, inter-trial phase synchronization, and auditory evoked gamma responses were obtained in response to auditory stimulus. Furthermore, TBARS levels and 4-HNE, GFAP, iNOS and nNOS expressions were evaluated in all groups.

Results: Peak-to-peak amplitudes of AEPs were significantly higher in EMF group compared with Sh group. There is no significant difference in peak latencies of AEPs between groups. Beside, evoked power, inter-trial phase synchronization, and auditory evoked gamma responses were significantly higher in EMF group compared with Sh group. Also, EMF group had significantly lower TBARS and 4-HNE levels than Sh group. There were no significant differences between groups for GFAP, nNOS, and iNOS levels, and between C and EMF groups for all parameters.

Conclusions: Our present observations suggest that short-term RF-EMF may have beneficial effects on neuronal networks by suppressing oxidative damage, and modulating brain oscillations, and could be used for noninvasive brain stimulation.

Introduction

There has been a great concern for the effects of electromagnetic fields (EMFs) on brain function, especially after the extensive use of mobile phones (MPs) over the last 20 years. In the literature, EMFs emitted by MPs have been found to cause some neurochemical, electrophysiological and cognitive alterations, but there is a large body of studies with conflicting results on brain function during or following the exposure to EMF (Consales et al. 2012; Carpenter 2013; Pall 2013). Therefore, there is still no definitive evidence and scientific consensus about the effects of EMF. These discrepancies might derive from differences in applied methods and parameters such as specific absorption rate (SAR) values, intensities, and exposure durations and frequencies (e.g. pulsed or continue).

Recently, the beneficial effects of EMF in treatment of several diseases have also been reported (Rasouli et al. 2012; Nelson et al. 2013; Pilla 2013). EMF treatment is capable of exerting cognitive-protective and cognitive-enhancing effects in both normal mice and transgenic mice that were destined to develop Alzheimer's-like cognitive impairment (Arendash et al. 2010; Dragicevic 2011). On the other hand, acute treatment of radio frequency (RF)/ microwave (MW) has been shown to have various significant effects on physiological and cognitive functions such as induced regional blood flow (Huber et al. 2005; Aalto et al. 2006), increased metabolic activity (Volkow et al. 2011), reduced reaction speed and increased accuracy in a working-memory task (Regel et al. 2007), altered human brain electrical activity (Curcio et al. 2005; Croft et al. 2008; Croft et al. 2010), increased alpha band power electroencephalogram (EEG) in human (Curcio et al. 2005).

Several neuropsychiatric and neurodegenerative disorders have been associated with disturbances in sensory information processing. It is well known that auditory evoked potentials (AEPs) receive major contributions from different brain areas such as temporal cortex, hippocampus, and association cortices. So, AEPs are widely used to examine the

sensory processes underlying neurological disorders as well as to evaluate treatments (Javitt et al. 2000; Shahriari et al. 2016). Lately, analyzes of brain oscillations have also provided a detailed examination of the sensory information processing and cognitive functions in both humans and animals. These oscillatory activities are evaluated through performing a decomposition of the EEG signal into phase and magnitude information over a range of frequencies from ultra-slow (0.05 Hz) to ultra-fast oscillations (500 Hz) depending on the stimulus (Buzsaki and Draguhn 2004; Buzsaki and Wang 2012). The synchronization of neuronal oscillations that either arises spontaneously or in response to an event or to a stimulus might represent distinct mechanisms in the brain. Moreover, their amplitude or power is usually modulated during different neural states. Also, an increasing number of studies support the view that modulation of brain activity has potential to affect neuronal activity, and may be used to treat neurological disorders. In light of these findings, there is growing interest on different noninvasive approaches to stimulate neural activity (Antal et al. 2008; Moliadze et al. 2012). Considering the idea of modulating brain activity, EMF treatment might be a promising method for noninvasive brain stimulation which was shown to be a safe and easy-to-use method (Rohde et al. 2010; Ceccarelli et al. 2013).

Our previous findings indicated that exposure to RF-EMF (2100 MHz, SAR 0.95 W/kg, for 2 h/day for 1 or 10 weeks) resulted in differential effects on oxidative stress pathway and visual evoked potentials (VEPs) depending on the exposure duration. The most striking finding of our former study was that the short-term (1 week) RF-EMF exposure could provide beneficial effects, while long-term (10 weeks) exposure has various harmful effects on the rat brain (Hidisoglu et al. 2016). For this reason, our aim was to investigate the effect of short-term 2.1 GHz EMF treatment on AEPs. Although there are numerous publications on electrophysiology of the auditory system, no previous study has compared detailed auditory evoked oscillatory responses after short-term 2.1 GHz EMF treatment. Unfortunately, despite

the rapid growth in interest of the noninvasive stimulation or EMF treatment, there is a little knowledge about interaction between EMF and brain oscillation. Therefore, present study provides a detailed examination of the auditory response to clarify relationship between RF-EMF and brain oscillation, By using multielectrodes recording in awake, freely moving rats, we examined how brain oscillatory activity and auditory potential changes in the rats after short-term 2.1-GHz EMF treatment, and whether short-term 2.1-GHz EMF treatment has modulatory effects on auditory processing or not. Also, to explain the likely mechanisms of EMF related effects, changes in lipid peroxidation, reactive astrogliosis and NOS expression after short-term 2.1-GHz EMF treatment were investigated in the present study.

Materials and methods

Animals

All experimental protocols conducted on rats were performed in accordance with the standards established by the Institutional Animal Care and Use Committee at Akdeniz University Medical School. Male albino Wistar rats aged 3 months, weighing 250 to 300 g were housed in stainless steel cages in groups of 4 rats per cage and given food and water *ad libitium*. Animals were maintained at 12 h light-dark cycles and a constant temperature of 23±1 ^{o}C at all times.

Surgery protocols

Rats were deprived of food for 24 h and then prepared for electrode placement. All surgery protocols were made between 09:00 am and 02.00 pm. In all rats, anesthesia was provided with a mixture of ketamine-based anesthetics (ketamine, 50 mg/kg and xylazine, 10 mg/kg; intraperitoneal, i.p.). During the anesthesia, the skull of the rats placed in stereotaxic apparatus was drilled for the implantation of electrodes. Stainless steel screw electrodes were inserted bilaterally into the regions of frontal (AP:4.5 mm, ML: +2 and -2 mm), parietal (AP:-4.5 mm, ML: +3.5 and -3.5 mm), temporal (AP:-8.0 mm, ML: +6.6 and -6.6 mm) and occipital (AP:-8.0 mm, ML: +4.1 and -4.1 mm) cortex while the reference electrode was inserted into cerebellum (AP:-12.72 mm, ML: 2.5 mm). All electrodes were embedded in dental acrylic and male pins were fixed to recording connector. After electrode placement, the incised skin was sutured, and at least 1 day was given for recovery. After surgery, an otoscopic examination was performed to evaluate tympanic membrane damage before AEP recordings. No tympanic damage was found in the rats. During recovery, the rats were housed in individual cages with free access to food and water.

Study design

After electrode implantation, all rats had five days handling. Following handling sessions, the animals were randomly divided into three groups (n=12 per each group). The rats were exposed to restraint stress due to experimental setup established in the current research as well as in previous studies (Sambucci et al. 2011; Dasdag S et al. 2012; Dasdag S, Bilgin, H.M., Akdag, M.Z., Celik, H., Aksen, F. 2014; Hidisoglu et al. 2016). As known, stress is an important factor that may affect many physiological functions including brain oscillations (Lupien 2009; Yuen et al. 2012). Therefore, unlike most of other studies, cage-control group was also used in this study to take into account the likely effects of restraint stress. Group 1: cage-control (C); Group 2: sham exposed group (Sh); Group 3: rats exposed to 2.1 GHz EMF for 7 days (EMF). During experiment, each animal in the groups of Sh and EMF was placed in a plexiglass tube (cylindrical tubes with 20-cm length and 7.5-cm diameter) with air hole to facilitate breathing and minimize rise in body temperature. EMF group rats were exposed to 2.1 GHz EMF exposure emitted from the generator for 2 h per day for 7 days. The same procedure (2 h per day for 7 days) was applied to the Sh group rats, but they were not exposed to EMF. The rats of the cage-control group were housed in their cages with equal time period without being exposed to any EMF and not placed into the plexiglass tubes.

Treatment system and EMF application

The treatment system is presented in Figure 1. In this system, a radio frequency generator (GSM Simulator 2.1-GHz type Everest Company, Adapazarı, Turkey), emitting 2.1-GHz EMF (217 Hz-pulse rates, 2-W maximum peak power) was used. The system was placed on a wooden table, and the antenna of generator was placed at the center of plexiglass carousel to provide equal exposure to the rats aligned around the antenna. The electric field strengths were measured by EMR-300 meter with the appropriate probe (Narda, Germany).

The electric field background level in the shielded room was between 0.02-0.2 V/m. In the signal-on situation, the measured electric field strengths over the rat's head positioned 10 cm away from the antenna were 35.2 V/m for 2.1 GHz RF-EMF. The average whole-body SAR was 0.128 W/kg for 2.1 GHz. The SAR value for the rat's brain was in average of 0.27 W/kg for 2.1 GHz. The numerical computation was performed using Finite Difference Time Domain (FDTD) method (Hyun and Oh 2006; LeBlanc et al. 2000). The FDTD code with perfectly matched layer absorbing boundaries has been used to measure SAR value in 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 were taken from the previous studies (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; Schonborn et al. 2004).

Electrophysiological recordings

On the recording day, the rats were adapted to the sound-attenuated recording room conditions for 10 min. To reveal possible regional and temporal differences, the EEG was recorded bilaterally from frontal, parietal, temporal and occipital locations between 9 am and 2 pm in a dimly lit, soundproof, and electromagnetically shielded-room. For the recordings from free-moving rats, chronically implanted electrodes were used. All electrode impedances were less than 10 kOhm. The EEG signal was amplified (Brainamp EEG/EP Amplifier, Brain Products, Munich, Germany), band-pass filtered (0.1-300 Hz) and digitized at a 1000 Hz sampling rate (Brainvision Recorder, Brain Products, Munich, Germany). AEPs were recorded using tones of 8000 Hz at the 85 dB. The repetition rate of auditory stimulus was 1 Hz. The duration of the 85-dB tones was 50 ms and the tones were presented through a loudspeaker at a distance of 15 cm from the ear of the rat. The EEG data were processed in 1000 ms epochs (500 ms pre-stimulus/500 ms post-stimulus). The averaging of 100 responses

were performed with a BrainVision Analyzer (Brain Products GmBH). Peak latencies of the components were measured from the stimulus artifact to the peak in milliseconds. Amplitudes were measured as the voltage between successive peaks. Measurements were made on one negative (N1) and two positive (P1, P2) potentials which are seen in all groups.

Data analysis

Spectral analysis of AEPs

Time-frequency analysis was applied for all epochs between 4 to 48 Hz using Morlet-based wavelets transform with 3 cycles and within 2 ms- sliding windows between -500 to 500 ms (EEGLAB, (Delorme and Makeig 2004)). Spectral analysis was computed on the wavelet-transformed epochs for each stimulus at each time point and wavelet frequency to yield time-frequency maps. The color at each image pixel indicates amplification (in dB) at a given frequency and latency to the time locking stimulus. Spectral analysis was used to determine the dominant frequencies in the AEPs during the experiment. The peak powers at 28-48 Hz individual frequency were extracted for statistical assessment. Therefore, we used each rat's peak power at the gamma frequency band.

Inter-trial coherence (ITC)

Inter-trial coherence (ITC) indicates that the EEG activity at a given time and frequency in single trials becomes phase-locked. The ITC measure takes values between 0 and 1. A value of 0 represents absence of synchronization between EEG data and the time locking stimulus; a value near 1 indicates phase synchronization. Here, we calculated the ITC using EEGLAB (Delorme and Makeig, 2004) as follows: For j=1 to N trials,

$$ITC\ (t,f) = \left\| \frac{1}{N} \sum_{i=1}^{N} e^{j\varphi j(t,f)} \right\|$$

where $\varphi j(t,f)$ is the phase of the wavelet at time t and frequency f. All ITC values were baseline corrected over -300 ms to -50 ms and were computed each rat for grand average. We used each rat's peak-frequency at 28-48 Hz frequency band.

Digitally filtered auditory evoked gamma oscillatory responses

Digital filtering of AEPs was performed with BrainVision Analyzer (Brain Products GmBH). Each rat's averaged evoked responses were digitally filtered in the 28-48 Hz frequency range. The maximum peak-to-peak amplitudes for each rat's averaged gamma (28-48 Hz) responses were analyzed. The largest peak-to-peak value in these frequency ranges in terms of microvolt found in the time window between 0 and 200 ms.

Biochemical investigations

After electrophysiological recordings, animals were sacrificed the next day at the same time interval (9:00 AM and 2:00 PM). At the end of the exposure period, the animals were killed by an overdose injection of anesthetic agent. Brain tissues were immediately removed and temporal cortex was isolated. The isolated temporal cortex tissues (n=8) were homogenized separately in 3 ml of 50mM potassium phosphate per gram tissue, pH 7.0, containing 1mM EDTA at 0-4 ^{0}C for TBARS assay, and 4 frozen temporal cortex samples were crushed at liquid nitrogen and were resuspended in lysis buffer (50 mM Tris–HCl [pH 7.4], 100 µM EDTA, 100 µM EGTA, 1% NP-40, 0.1% SDS, and 0.1% deoxycholic acid) supplemented with protease inhibitor cocktail tablets (Roche, NJ, USA) for western blotting assay. The samples were centrifuged at 10.000g for 10 min, and supernatants obtained were collected and stored at −80 °C. All tissues were rapidly sonicated in a thermally regulated sonicator (Branson Sonifier 250, G. Heinemann Ultraschall- und Labortechnik, Germany) for 1min. The sonicated samples were stored frozen at -80 ^{0}C until assay determinations.

TBARS assay

A part of sonicated samples was centrifuged at 14,000 g for 10 min at 4^0C in an eppendorf microcentrifuge (Biofuge 15R, Heraeus Sepatech, Osterode, Germany). The supernatant of centrifuged samples was used for the assay of TBARS measurements. TBARS levels were measured by a fluorimetric method described by (Wasowicz et al. 1993), using 1,1,3,3-tetraethoxypropane as a standard. The results are given as μmol/g protein.

Western blotting

Expressions of 4HNE adduct proteins, GFAP, nNOS and iNOS proteins were determined by performing western blotting. Protein concentrations in all samples were measured spectro-photometrically by a protein assay reagent kit (Pierce, Rockford, IL, USA) via a modified Bradford method. Bovine serum albumin was used as a standard. Equal amounts of protein (20 μg) were loaded per lane and were separated by performing SDS-polyacrylamide gel electrophoresis at 100 V and 30 mA for approximately 2 hours. Next, the separated proteins were transferred to PVDF membranes in 25 min by using a fast transfer system (Trans- Blot Turbo; Bio-Rad) at 25 V and 2.0 mA. The membranes were blocked with 3-4% non-fat dry milk and were incubated with the recommended dilutions of anti-4-HNE (1:500 dilution, ab46545, Abcam), anti-GFAP (1:10000 dilution, ab7260, Abcam), anti-nNOS (1:2000 dilution, ab1376, Abcam), anti-iNOS (1:2000 dilution, ab15323, Abcam), and anti-GAPDH (1:10000 dilution, Thermo Scientific) antibodies. Next, the membranes were incubated with horseradish peroxidase-conjugated horse anti-mouse or horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibodies (Vector Laboratories). Finally, bound antibodies were detected using a chemiluminescence-based HRP substrate system (Pierce, USA). The membranes were exposed to Hyperfilm (Amersham, UK) that was subsequently scanned and analyzed using ImageJ software. Protein expression is expressed as the ratio of target protein expression to GAPDH expression.

Statistical analysis

All statistical analyses of the obtained data were calculated using SPSS 18.0 (SPSS, Chicago, IL, USA) software of Windows. The differences between groups for latencies, peak-to-peak amplitudes of AEPs, spectrograms, ITC and filtered auditory evoked gamma responses were assessed by means of repeated measures of ANOVA. In the analysis, repeated measures of ANOVA included the between-subjects factor as group (C, Sh and EMF); and within-subject factors as 8 electrode sites (FrA1, FrA2, PtA1, PtA2, TeA1, TeA2, V1 and V2). Greenhouse-Geisser corrected p values were reported. TBARS values were analyzed using ANOVA. Post-hoc comparisons were analyzed with Bonferroni test. The differences in western-blotting (4-HNE, GFAP, nNOS and iNOS) were analyzed using Kruskal–Wallis test and all pairwise multiple comparisons were performed using the Mann-Whitney U test. Results are expressed as mean ± standard deviations (SD). The significance levels were set at $p < 0.05$. All experimenters were blind to animal experimental group membership during data collection and analysis.

Results

The general health of the animals such as grooming, appearance and behavioral status did not appear different. No significant difference could be observed in the body weight change among different experimental groups (data not shown).

P1, N1 and P2 components of auditory evoked potentials (AEP) for all experimental groups are presented in Figure 2. Measurement was made on one negative and two positive potentials, which were seen in all of the groups. The means and SD of peak latencies and peak-to-peak amplitudes of AEP components of the three groups are shown in Table 1 and Table 2, respectively. In the present study, there was no main group effect in terms of peak latencies of AEP components [P1: $F_{2.30} = 0.11$, $p > 0.05$; N1: $F_{2.30} = 0.52$, $p > 0.05$; P2: $F_{2.30} = 1.83$, $p > 0.05$]. Furthermore, there was no significant group x location effect [P1: $F_{14.210} = 1.60$, $p > 0.05$; N1: $F_{14.210} = 1.71$, $p > 0.05$; P2: $F_{14.210} = 1.33$, $p > 0.05$]. However, there was significant location effect independent of groups for N1 latencies [N1: $F_{7.210} = 16.96$, $p < 0.001$]. Post-hoc comparisons showed that frontal and parietal electrodes elicited longer N1 latencies in comparison to temporal and occipital electrodes for all groups ($p < 0.05$ for all comparisons).

Mean ± SD of peak-to-peak amplitudes of AEP components (P1N1 and N1P2) in response are shown in Table 2. There was a main group effect in maximum peak-to-peak P1N1 amplitudes in all electrode regions [$F_{2.28}$=144.71, $p < 0.001$], with lower P1N1 values in Sh group compared to C, and higher P1N1 values in EMF compare to Sh group ($p < 0.001$). There was also significant location [$F_{7.196}$=35.62, $p < 0.001$] effect in terms of P1N1 responses. Post-hoc comparisons showed that occipital electrodes elicited lower P1N1 responses in comparison to other electrodes ($p < 0.05$). Furthermore, there was also a significant interaction effect for location × group [$F_{14.196} = 6.52$, $p < 0.001$. In the post-hoc comparisons, it was found that Sh group had lower values for the maximum P1N1 amplitudes

compared with C group, and EMF group had higher values for the maximum P1N1 amplitudes compared to Sh group over frontal, parietal and temporal locations ($p < 0.01$ for all comparisons).

There was a main group effect in maximum peak-to-peak N1P2 amplitudes in all electrode regions [$F_{2.28}$=6.42, $p < 0.01$], with lower N1P2 values in Sh group compared to C group, and with higher N1P2 values in EMF group compared to Sh group ($p < 0.05$). There was also significant location effect [$F_{7.196}$=18.79, $p < 0.001$] in terms of N1P2 responses. Post-hoc comparisons showed that occipital electrodes elicited lower N1P2 responses in comparison to other electrodes ($p < 0.05$). Furthermore, there was an interaction effect for location × group [$F_{14.196} = 3.52$, $p < 0.001$]. In the post-hoc comparisons, it was found that Sh group had lower values for the maximum N1P2 amplitudes compared with C group, and EMF group had higher values for the maximum N1P2 amplitudes compared to Sh group over frontal, parietal and temporal locations ($p < 0.05$ for all comparisons).

Maximum peak to peak auditory evoked gamma response for all experimental groups is presented in Figure 3 and Table 3. There was a main group effect on maximum peak-to-peak gamma response, with decreased amplitude values in Sh group versus the C group ($p < 0.001$), and with increased values in EMF group compared to Sh group ($p < 0.05$). Moreover, there was a main location effect [$F_{7.210}$=16.50, $p < 0.001$]. Post-hoc comparisons showed that frontal electrodes elicited lower gamma oscillatory responses in comparison to parietal and occipital ones ($p < 0.001$) and temporal electrodes had higher gamma response values than occipital electrodes ($p < 0.05$).

Auditory evoked gamma band spectrograms (28-48 Hz) and maximum gamma band spectrograms values for all experimental groups are presented in Figure 4 and Table 4, respectively. There was a main group effect in maximum gamma band spectrogram values in all electrode regions [$F_{2.46} = 12.32$; $p < 0.001$], with lower values in Sh group compared to C

group, and with higher values in EMF group compared to Sh group ($p < 0.001$ for all comparisons). There were no location [$F_{7.322}$=1.91, $p > 0.05$] and location × group [$F_{14.322}$ = 1.05, $p > 0.05$] effects for gamma responses.

Inter-trial coherence (ITC) in gamma band (28-48 Hz) are shown in Figure 5 and Table 5. There was a main group effect in gamma band ITC values in all electrode regions [$F_{2.45}$=4.24, $p < 0.05$], with lower values in Sh group compared to C group, and with higher values in EMF group compared to Sh group ($p < 0.05$). Moreover, there was a main location effect [$F_{7.315}$ = 2.84, $p < 0.01$], with higher gamma ITC values in frontal and parietal regions than in other regions. Furthermore, there was also location × group effect [$F_{14.315}$ = 2.07, $p < 0.05$] for gamma band ITC values. In the post hoc comparisons, it was found that Sh group had lower ITC values compared with C group, whereas EMF group had higher ITC values compared with Sh group over frontal, parietal and temporal electrodes ($p < 0.05$ for all comparisons).

TBARS values of the temporal cortex of all experimental groups are given in Figure 6. There was a statistically significant difference between groups [$F_{2.21}$ = 6.12, $p < 0.001$]. Brain TBARS levels were significantly increased in the Sh group compared to C group ($p < 0.05$). TBARS levels were significantly decreased in the EMF group versus the Sh group ($p < 0.05$). No significant difference was observed in TBARS levels between EMF and C groups.

Western blots and densitometric analysis of temporal cortex homogenates for 4-HNE, GFAP, nNOS, and iNOS are given in Figure 7. There was a statistically significant difference between groups for 4-HNE levels [$U(2) = 6.48$, $p < 0.05$]. 4-HNE levels were decreased in the EMF group compared with the Sh group. Although it did not reach the significant level, an increment in 4-HNE level was observed in Sh group versus the C group. No significant differences were found between groups for GFAP, nNOS, and iNOS levels.

Discussion

In this study, we aimed to investigate in detail the possible short-term RF-EMF effects on the brain, more specifically on auditory sensory processing. To date, we encountered no studies applying several analytical methods (sensory evoked power, phase locking and filtered oscillatory responses) to the analysis of auditory evoked potentials (AEPs) to identify effects of RF-EMF on the brain. Therefore, we used these combined analysis procedures in order to understand the EEG responses in the present study and obtained a number of interesting results. So, the present study is the first to compare AEPs and auditory evoked gamma oscillations after short-term RF-EMF treatment in awake rats.

Numerous reports indicated that EMF exposure affects brain activity and cortical excitability during both awake and sleep states as reflected by EEG recordings (Croft et al. 2008; Lowden et al. 2011; Loughran et al. 2012; Schmid et al. 2012; Roggeveen et al. 2015). AEPs are sensitive and reliable to determine stimulus-dependent hearing levels, auditory perception, and early auditory information processing (Salisbury et al. 2010; Ford et al. 2012). Our current findings suggest that RF-EMF markedly impacts the auditory system. In this study, there were significant differences at the peak-to-peak amplitudes, but not in latency, of AEPs between all groups. In Sh group, peak-to-peak amplitudes of AEPs were significantly decreased compared with C group. Earlier physiological studies showed that restraint stress exposure, per se, (up to 10 days), accompanied by elevation of corticosterone levels, cause structural and functional alterations in neurons and changes in auditory response (Dagnino-Subiabre et al. 2009; Bose et al. 2010). Hence, it is likely that reductions in AEP amplitudes observed in Sh group might be due to restraint stress induced by placing rats in plexiglass tubes during treatment condition (not exposure to RF-EMF). Additionally, it was shown that amplitudes of AEPs in EMF group have almost reached to control level after short-term RF-EMF treatment. Our results demonstrated that short-term RF-EMF treatment provides to

recovery effects on the brain by eliminating effects of restraint stress. Consistently, the present study provides experimental data showing that short-term RF-EMF treatment may exert beneficial effects on auditory sensory processing via eliminating oxidant damage induced by restraint stress. Moreover, to evaluate lipid peroxidation (LPO) after short-term EMF treatment, we measured TBARS levels and 4-HNE expressions that are known as oxidative stress biomarkers, in the temporal cortex in all groups. Likewise our previous findings (Hidisoglu et al. 2016), we determined that TBARS levels and 4-HNE expressions significantly decreased in EMF group compared with Sh group. These findings show that short-term EMF treatment suppressed oxidative damage induced by restraint stress. As a possible explanation, short-term EMF treatment may lead to the suppressing of oxidative damage by inducing antioxidant defense system as shown in our previous study (Hidisoglu et al. 2016). Therefore, short-term EMF treatment may have beneficial effects via providing a balance between oxidative stress and antioxidant defense system. Furthermore, in our sham treatment condition, restraint stress which can disrupt astrocytic functions, have modulatory effects on neuronal synaptic plasticity. In the literature, it could be suggested that stress inhibits gliogenesis in the brain (Czeh et al. 2007), results in impaired activity of glial cells (Hu et al. 2012), and a profound shortening of astrocytic branching and process length (Tynan et al. 2013). On the other hand, astrogliosis is a nonspecific consequence of many injuries including oxidative damage in the brain. For this reason, glial cell responses were investigated by testing GFAP protein expression in the temporal cortex after short-term RF-EMF treatment. Some studies showed that acute RF/MW-EMF treatment induced reactive astrogliosis (Mausset-Bonnefont et al. 2004; Brillaud et al. 2007; Ammari et al. 2008), however the others suggested that short-term RF-EMF treatment has no significant effect on reactive astrogliosis (Thorlin et al. 2006; Grafstrom et al. 2008). In our present study, no significant difference was determined in GFAP protein expression between all groups, but we

observed that the GFAP protein expression was tended to decrease in the EMF group compared with Sh group. Our present study clearly differs from previous studies (Mausset-Bonnefont et al. 2004; Brillaud et al. 2007; Ammari et al. 2008) which all used GSM 900 MHz signals and had different exposure duration and SAR values. However, our results do not exclude potentially deleterious effect of RF-EMF at high SAR values. Consequently, our findings suggest that short-term RF-EMF treatment at low SAR values might affect glial cells function, and produce beneficial effects by decreasing reactive astrogliosis in the brain.

On the other hand, we investigated the effects of short-term RF-EMF treatment on nitric oxide synthase (NOS) enzymes, both neuronal and inducible isoforms (nNOS and iNOS, respectively) in the temporal cortex. As known, nNOS expression is regulated by both physiological and path physiological stimuli, while iNOS expression is regulated by activated microglia. These is forms are responsible for nitric oxide (NO) production, which is an important biological messenger, highly diffusible, that plays a prominent role in the physiology of the CNS (Yun et al. 1996). After short-term RF-EMF treatment, we did not observe any significant changes in expression of these isoforms between all groups. However, in our previous study (Hidisoglu et al. 2016), we observed significant increment NO generation in the total brain. Therefore, it could be concluded that short-term RF-EMF treatment might produce noticeable effect on the brain as a whole, not on specific brain regions.

It has been documented that the gamma band activity is modulated by sensory inputs, including auditory stimulus (Basar et al. 1991; Tallon-Baudry and Bertrand 1999). Also, we observed significant changes in slow gamma band activity among all groups. For these reasons, we analyzed sensory evoked response for this band activity at all electrode locations. Consistent with these, the frequency differences between all groups were more pronounced at auditory evoked gamma responses. Auditory evoked gamma response was significantly

increased in power and in inter-trial coherence at all electrode locations for EMF group compared with Sh group, indicating an augmentation in the phase coherence and in the consistency of response to stimuli in the brain. As known, typically characterized by an early onset (50-70 ms post-stimulus) and a frequency of around 40 Hz, these phase-locked oscillations have been associated with integrative processing and plastic changes in sensory networks (Knief et al. 2000). Therefore, the potential explanation for our findings is that stress-induced synaptic failure resulted in the reduction of synchronization activity and the generation of gamma oscillation in Sh group, however, gamma band power and inter-trial coherence have almost reached to control level in EMF group. From these findings, it could be concluded that RF-EMF treatment has modulatory effects on synaptic transmission and restore synchronization activity within neuronal assemblies. Hence, previous reports supported our conclusion since they have reported significant changes in various neurotransmitters in the rat brain after exposure to EMF. In one of these studies, it was demonstrated that exposure to EMF increased catecholamine levels and decreased acetylcholine esterase activity (Lai 1994). Some authors evaluating the relationship between AEPs and neurotransmitter systems showed that alterations in the neurotransmitter systems cause a change in some AEP amplitudes (Manjarrez et al. 2005; Klinkenberg et al. 2013). In agreement with these findings, we also found that EMF treatment increased P1N1 and N1P2 amplitudes of AEPs in our present study. On the other hand, alterations in neuromodulatory systems induced by EMF treatment could increase cortical excitability and synchronization of neural networks that are related to auditory processing. Because earlier studies indicated that neurons in the neural assemblies interact with each other through their synaptic connections and thereby gamma oscillation emerges from the synchronization of excitatory-inhibitory interactions (Traub et al. 1999; Belluscio et al. 2012; Buzsaki and Wang 2012). Consequently, the present results are consistent with the idea that short-term RF-EMF treatment affects

neural oscillatory patterns that are related to important mechanisms for the auditory processing.

In conclusion, our current findings are consistent with some studies in the literature that short-term RF-EMF treatment might exert beneficial effects on the brain by modulating the neuronal networks, and it could be used as a noninvasive brain stimulation technique. However, further studies are still needed to clarify the relationship between effects of short-term RF-EMF treatment and brain activity.

Declaration of interest

The authors declared no conflicts of interest.

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Author Contributions

The first author, Enis Hidisoglu, contributed to all aspects of the study (i.e., conception and design; data collection, analysis, and interpretation). The second author, Deniz Kantar-Gok, contributed substantially to the statistical analysis and interpretation of data. The third author, Sukru Ozen, contributed to the calculation of dosimeter in the rat. The principal investigator, Piraye Yargicoglu, contributed substantially to the conception and design of the study and data interpretation. Enis Hidisoglu and Piraye Yargicoglu wrote the manuscript. All authors give their approval of the final version to be published.

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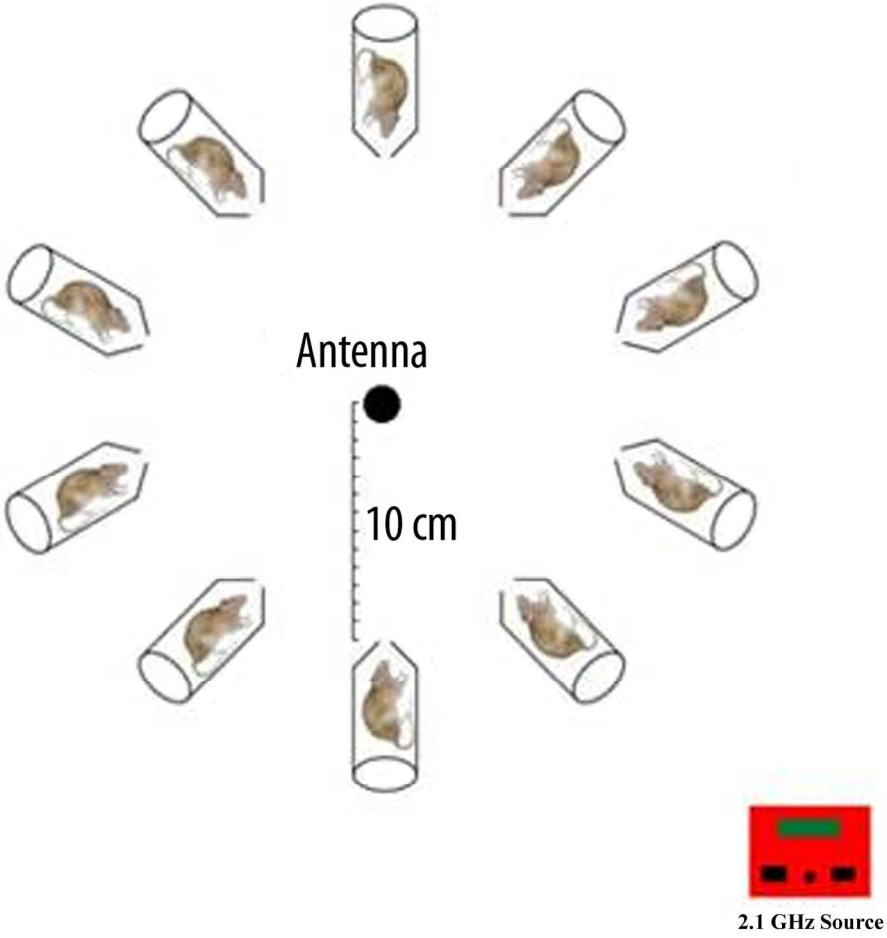

Figure 1. Exposure setup emitting 2.1 GHz EMFs from monopole antenna placed at the center of plexiglass carousel. Rats were aligned circularly around the antenna at same distance (10 cm) to ensure equal exposure of EMFs.

99x101mm (600 x 600 DPI)

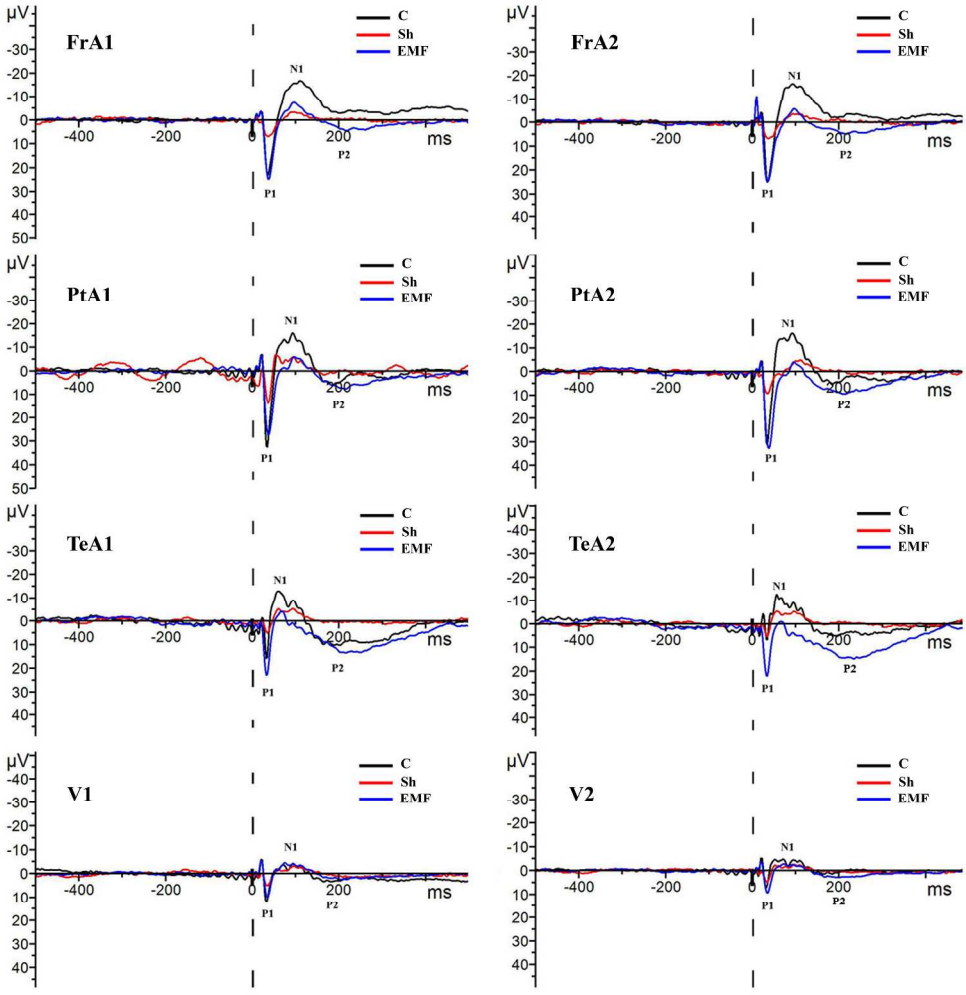

Figure 2. Grand average of AEPs waveforms evoked by auditory stimulus at FrA1, FrA2, PtA1, PtA2, TeA1, TeA1, V1 and V2 electrode locations. Black, red and blue lines represent the AEPs of C, Sh and EMF groups, respectively. Time 0 is the stimulus onset and N1 is the first negative wave.

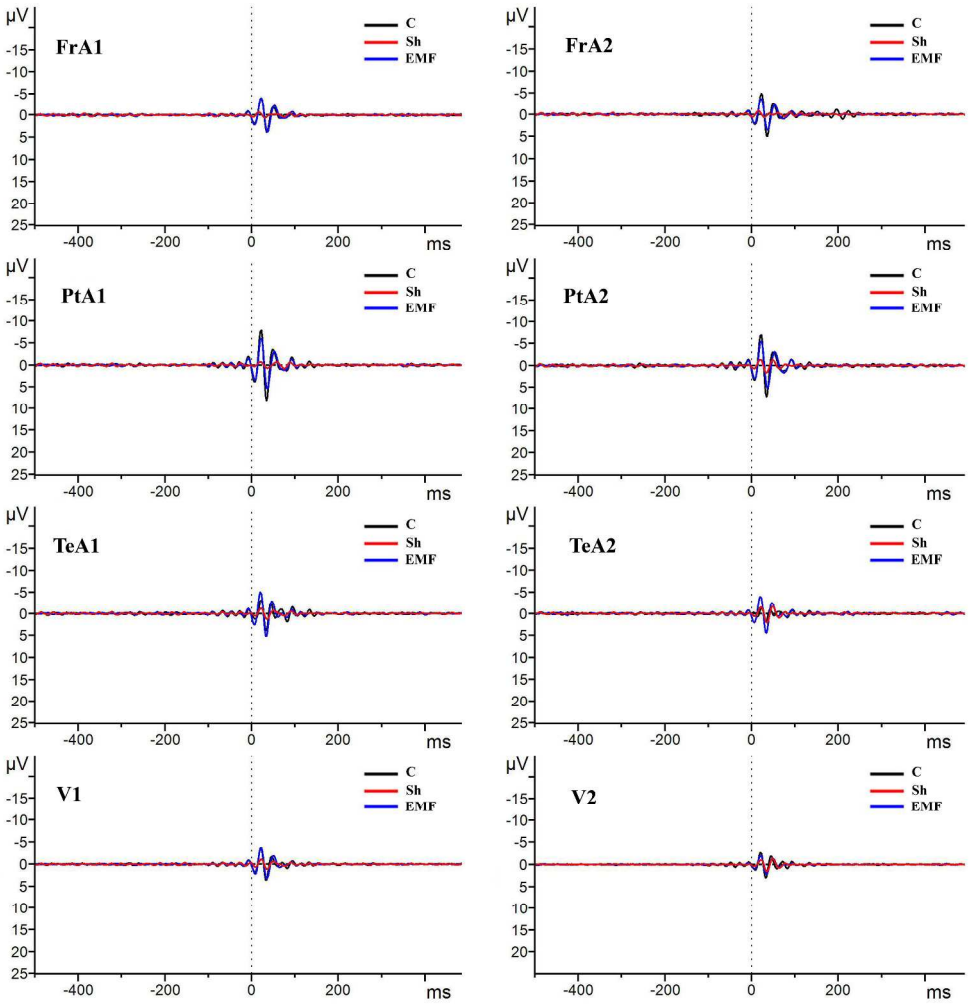

Figure 3. Grand average of filtered (28-48 Hz) auditory evoked gamma oscillatory responses of all groups at all electrode locations. Black, red and blue lines represent the filtered gamma responses of C, Sh and EMF groups, respectively. Time 0 is the stimulus onset.

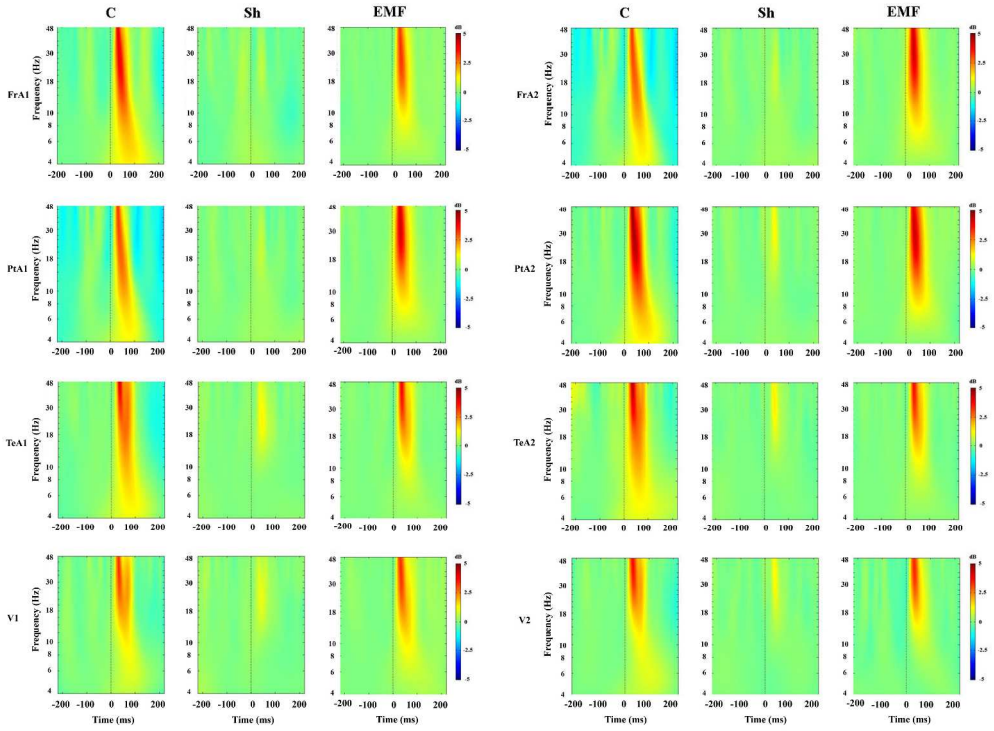

Figure 4. Grand average of ERSP time-frequency matrices calculated by wavelet-based analysis of the averaged evoked potentials showing relative changes in total power compared with the baseline period at all electrode locations. Power (in dB) is indicated by color code with warmer colors representing higher power values (color bar at the right side of the each panel). It was found that exposure to EMF leads to increase gamma band power compared to Sh group.

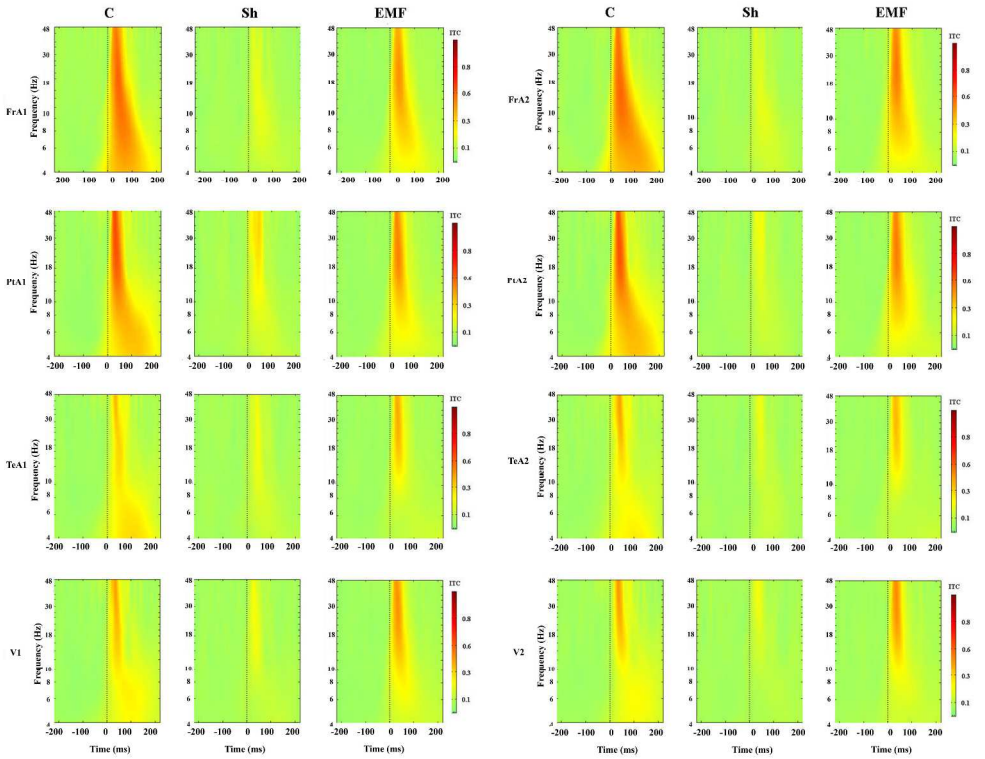

Figure 5. Grand average of time-frequency plots showing phase-locking (ITC) values for auditory stimulus at all electrode locations. Phase-locking is indicated by color code with warmer colors representing higher phase stability across trials (color bar at the right side of the each panel). ITC was found to be affected by treatment of RF-EMF.

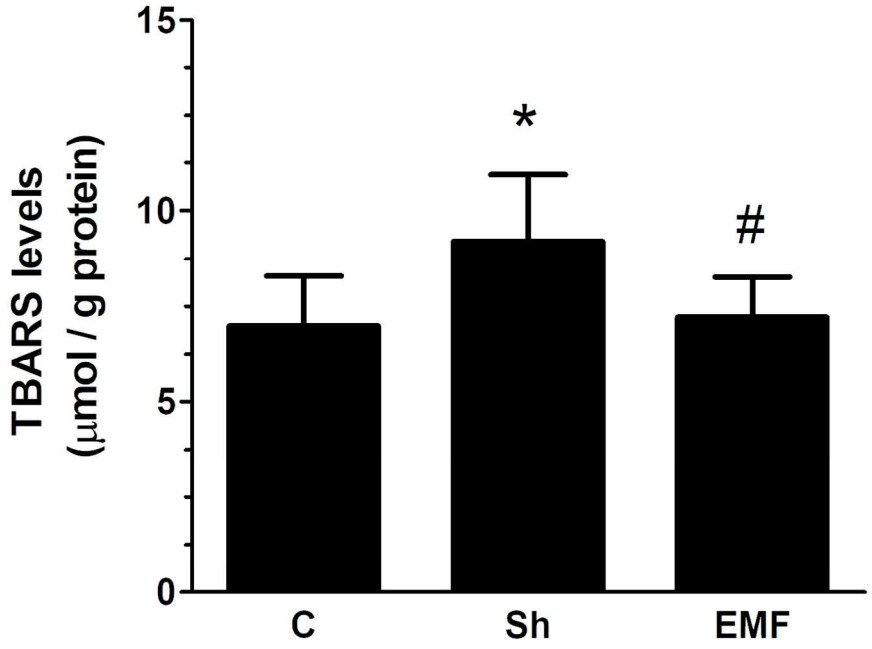

Figure 6. TBARS values of temporal cortex in C, Sh and EMF groups. Values are expressed as means ± standard deviations. *p < 0.05 vs. C. #p < 0.05 vs. Sh (n=8 for all groups).

134x94mm (300 x 300 DPI)

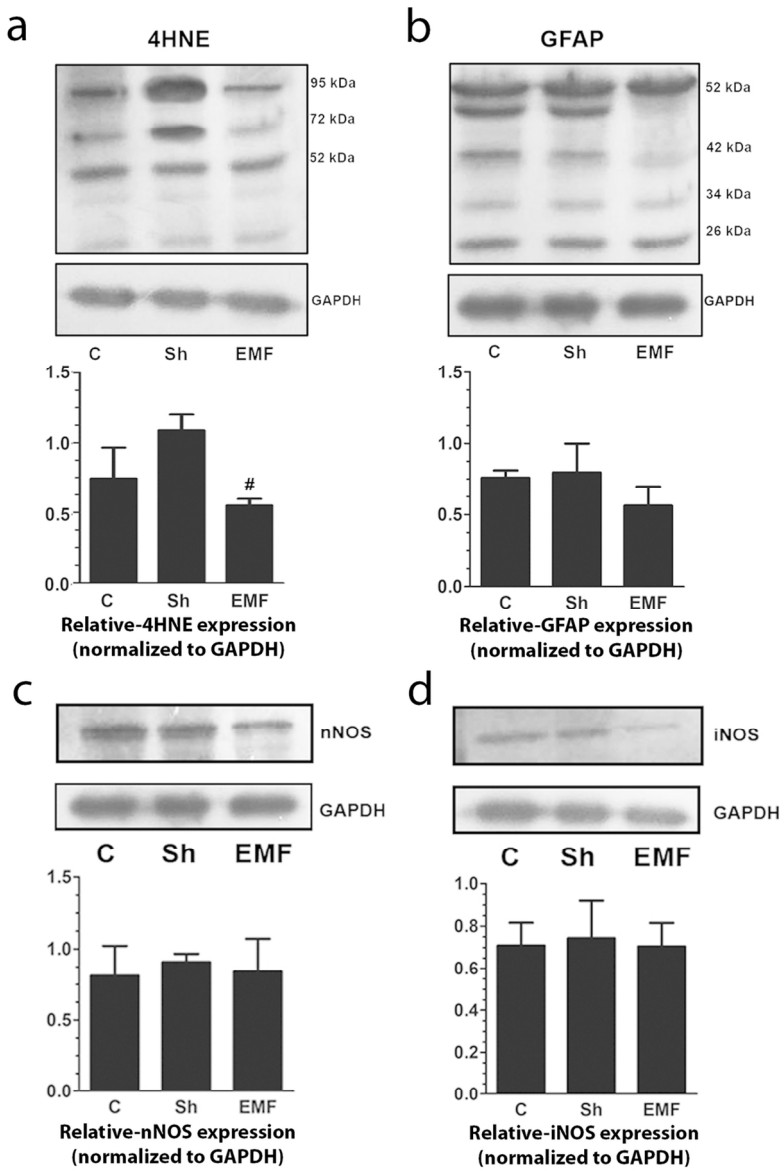

Figure 7. Expression analyses and representative western blotting images of 4-HNE, GFAP, nNOS and iNOS proteins in the temporal cortex of rats in C, Sh and EMF groups. Expression of each protein was normalized to that of GAPDH. Values are expressed as means ± standard deviations. #$p < 0.05$ vs. Sh ($n = 4$ for all proteins).

101x147mm (300 x 300 DPI)

Table 1. The means and standard deviations of peak latencies of AEP components in C, Sh and EMF groups. There was no main group effect in terms of peak latencies of AEPs components between all groups.

Groups		FrA1 (ms)			FrA2(ms)			PtA1(ms)			PtA2(ms)			TeA1(ms)			TeA2(ms)			V1(ms)			V2(ms)		
		P1	N1	P2	P1	N1	P2	P1	N1	P2	P1	N1	P2	P1	N1	P2	P1	N1	P2	P1	N1	P2	P1	N1	P2
C	Mean ±SD	37.00 ±4.55	90.17 ±17.61	194.00 ±26.06	37.67 ±7.67	89.83 ±12.97	196.33 ±24.30	35.17 ±3.86	82.67 ±14.97	186.83 ±25.45	35.83 ±.46	79.17 ±19.62	186.00 ±35.12	35.33 ±7.55	70.67 ±17.90	182.83 ±33.38	37.00 ±8.55	72.80 ±18.38	188.40 ±34.02	32.50 ±2.11	72.33 ±18.74	174.00 ±20.32	35.50 ±8.01	66.00 ±18.47	172.67 ±36.55
Sh	Mean ±SD	35.33 ±6.56	85.11 ±17.75	188.00 ±16.81	36.89 ±7.56	88.67 ±11.18	196.44 ±39.15	36.67 ±7.00	78.89 ±11.67	182.22 ±35.57	36.67 ±8.49	85.11 ±13.61	188.22 ±29.23	34.22 ±2.54	73.56 ±6.84	187.78 ±33.07	34.00 ±3.61	72.67 ±5.66	189.22 ±29.27	35.78 ±6.12	88.22 ±12.39	189.56 ±32.94	33.56 ±5.27	76.67 ±4.47	182.89 ±27.82
EMF	Mean ±SD	37.57 ±3.25	96.29 ±20.45	206.71 ±22.24	37.43 ±2.87	97.00 ±16.51	207.29 ±21.22	37.14 ±2.44	94.86 ±21.34	206.00 ±10.55	37.29 2.43	91.00 ±21.06	204.71 ±19.82	32.86 ±2.57	73.29 ±17.69	210.14 ±24.73	33.14 ±3.11	70.29 ±18.66	211.14 ±21.05	35.57 ±3.44	83.71 ±22.21	194.00 ±23.85	33.71 ±4.56	76.00 ±20.80	196.57 ±19.59

Table 2. The means and standard deviations of peak-to-peak amplitudes of AEPs in C, Sh and EMF groups. There was main group effect in maximum peak-to-peak amplitudes P1N1 and N1P2 values amplitudes in all electrode locations, with lower P1N1 and N1P2 values in Sh group compared to C and EMF groups. * : $p < 0.05$ versus C group; ** : $p < 0.01$ versus C group; $^{\#}$: $p < 0.05$ versus Sh group ; $^{\#\#}$: $p < 0.01$ versus Sh group.

Groups		FrA1 (μV)		FrA2 (μV)		PtA1 (μV)		PtA2 (μV)		TeA1 (μV)		TeA2 (μV)		V1 (μV)		V2 (μV)	
		P1N1	N1P2	P1N1	N1P2	P1N1	N1P2	P1N1	N1P2	P1N1	N1P2	P1N1	N1P2	P1N1	N1P2	P1N1	N1P2
C	Mean ±SD	45.99 ±16.05	21.14 ±17.24	43.12 ±13.34	18.14 ±7.40	51.96 ±14.11	27.89 ±11.91	56.13 ±18.20	31.47 ±13.27	45.41 ±23.94	41.22 ±21.04	35.54 ±13.23	31.21 ±11.62	19.40 ±6.54	12.30 ±6.09	16.22 ±6.33	11.20 ±4.38
Sh	Mean ±SD	14.24 ±8.12**	8.25 ±4.73*	14.31 ±9.04**	7.87 ±4.69*	11.88 ±8.91**	7.53 ±6.13*	14.03 ±10.05**	7.76 ±8.29*	11.21 ±7.57**	10.80 ±5.66*	12.31 ±8.17**	10.03 ±6.99*	8.20 ±6.36	6.43 ±4.27	9.05 ±7.17	6.59 ±5.08
EMF	Mean ±SD	34.11 ±18.72$^{\#\#}$	19.12 ±11.18$^{\#}$	32.34 ±17.50$^{\#\#}$	17.91 ±11.80$^{\#}$	38.51 ±21.83$^{\#\#}$	19.28 ±12.39$^{\#}$	39.65 ±18.75$^{\#\#}$	22.53 ±14.17$^{\#}$	33.10 ±17.59$^{\#\#}$	27.06 ±21.39$^{\#}$	30.22 ±15.45$^{\#\#}$	26.48 ±17.92$^{\#}$	18.82 ±13.33	11.68 ±9.48	13.63 ±7.38	12.00 ±8.36

Table 3. The means and standard deviations of maximum peak to peak gamma response of AEPs in C, Sh and EMF groups. There was a main group effect on maximum peak-to-peak gamma response, with lower amplitude values in Sh group versus the C and EMF groups. **: $p < 0.001$ versus C group; $^{\#}$: $p < 0.05$ versus Sh group.

Groups		FrA1 (μV)	FrA2 (μV)	PtA1 (μV)	PtA2 (μV)	TeA1 (μV)	TeA2 (μV)	V1 (μV)	V2 (μV)
C	**Mean**	10.48	10.86	16.90	15.53	12.43	14.38	9.12	7.13
	±SD	±6.19	±6.15	±5.96	±7.53	±5.12	±6.90	±4.01	±4.16
Sh	**Mean**	2.84	2.94	3.61	4.90	2.93	4.54	2.77	4.20
	±SD	±1.05**	±1.65**	±1.18**	±4.05**	±1.43**	±5.23**	±2.02**	±5.41**
EMF	**Mean**	7.85	7.54	12.02	11.46	10.80	9.17	7.40	6.24
	±SD	±3.16 $^{\#}$	±2.34 $^{\#}$	±5.59 $^{\#}$	±4.02 $^{\#}$	±6.00 $^{\#}$	±5.37 $^{\#}$	±3.72 $^{\#}$	±3.24 $^{\#}$

Table 4. The means and standard deviations of maximum gamma band spectrograms values of AEPs in C, Sh and EMF groups. There was a main group effect in maximum gamma band spectrogram values in all electrode regions, with lower values in Sh group compared to other groups. ** $p < 0.001$ versus C group; $^{\#\#}$ $p < 0.001$ versus Sh group.

Groups		FrA1	FrA2	PtA1	PtA2	TeA1	TeA2	V1	V2
C	**Mean**	4.31	3.89	5.18	5.06	4.35	4.89	3.99	4.24
	±SD	±1.53	±3.28	±2.21	±2.05	±1.72	±2.41	±2.12	±2.01
Sh	**Mean**	1.21	1.08	1.57	1.82	1.67	1.87	1.60	1.84
	±SD	±0.77**	±0.68**	±1.76**	±1.29**	±1.15**	±1.83**	±1.21**	±1.84**
EMF	**Mean**	3.55	4.50	4.08	4.66	3.93	3.82	3.87	3.85
	±SD	±1.78$^{\#\#}$	±4.29$^{\#\#}$	±2.00$^{\#\#}$	±2.06$^{\#\#}$	±1.75$^{\#\#}$	±1.71$^{\#\#}$	±1.89$^{\#\#}$	±3.03$^{\#\#}$