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Possible effects of different doses of 2.1 GHz electromagnetic radiation on learning, and hippocampal levels of cholinergic biomarkers in Wistar rats

Çiğdem Gökçek-Saraç ^a, Güven Akçay ^b, Serdar Karakurt^c, Kayhan Ateş^d, Şükrü Özen^d, and Narin Derin ^b

^aFaculty of Engineering, Department of Biomedical Engineering, Akdeniz University, Antalya, Turkey; ^bFaculty of Medicine, Department of Biophysics, Akdeniz University, Antalya, Turkey; ^cFaculty of Science, Department of Biochemistry, Selçuk University, Konya, Turkey; ^dFaculty of Engineering, Department of Electrical and Electronics Engineering, Akdeniz University, Antalya, Turkey

ABSTRACT

The present study evaluated whether short-term exposure to different doses of 2.1 GHz radio-frequency electromagnetic radiation (RF-EMR) has different effects on rats' behaviour and hippocampal levels of central cholinergic biomarkers. Animals were divided into three equal groups namely; group 1 was sham-exposed group, group 2–3 were exposed to 45 V/m and 65 V/m doses of 2.1 GHz frequency for 1 week respectively. Numerical dosimetry simulations were carried out. Object location and Y-maze were used as behavioural tasks. The protein and mRNA expression levels of AChE, ChAT, and VACHT, in the hippocampus were tested using Western Blotting and Real-Time PCR. The impairment performance of rats subjected to 65 V/m dose of 2.1 GHz RF-EMR in both object location and Y-maze tasks was observed. The hippocampal levels of AChE, ChAT, and VACHT, were significantly lower in rats exposed to 65 V/m dose of 2.1 GHz RF-EMR than others. The stronger effect of "65 V/m" dose on both rat's hippocampal-dependent behavioural performances and hippocampal levels of cholinergic biomarkers may be due to the stronger effect of "65 V/m" dose where rats' snouts were located at the nearest distance from the monopole antenna. Furthermore, the simulated SAR values were high for 65 V/m electric-field strengths. For the first time, we report the potential dose-dependent effects of short-term exposure to 2.1 GHz radiation on rat's behavioural performances as well as hippocampal levels of cholinergic biomarkers. Further studies are needed to understand the mechanisms by which RF-EMR influences the function of the central cholinergic system in the brain.

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Rats; radiofrequency electromagnetic radiation; behaviour; hippocampus; cholinergic biomarkers

Introduction

The unstoppable developments in the cell phone technology have made cell phones as an indispensable part of our daily lives. We spend most of our time using our cell phones but without totally knowing the possible harmful effects. The possible harmful effects of the nonionizing form of radiofrequency electromagnetic radiation (RF-EMR) emitted by cell phones on human health have still become a hot topic for scientists (Altun et al. 2018; Birks et al. 2017; Langer et al. 2017; Singh and Kapoor 2014). There are many experimental animal studies examining cellular and molecular effects of RF-EMR from cell phones on several types of cancer (Morgan et al. 2015), genetic damage (Alkis et al. 2019; Megha et al. 2015; Ruediger 2009), oxidative stress (Alkis et al. 2019; Chauhan et al. 2017; Megha et al. 2015), chromosome abnormalities (Kim et al. 2008), the blood-brain barrier permeability (Nittby et al. 2009), nerve cell damage (Salford et al. 2003), neurological

diseases (Banaceur et al. 2013), neurotransmitter balance (Tamasidze and Nikolaishvili 2007), in addition to cognition and behavior (Calvente et al. 2016; Maaroufi et al. 2014; Sakhnini et al. 2018; Singh and Kapoor 2014; Son et al. 2018). However, scientific published data have been contradictory in their results according to experimental conditions (Cassel et al. 2004; Esmaili et al. 2017; Hao et al. 2013; Kim et al. 2019; Narayanan et al. 2009), so it is insufficient to get a solid answer to possible risks by RF-EMR. One of these risks is that the human brain absorbs RF-EMRs emitted by cell phones to a degree that may affect neuronal functions (Birks et al. 2017; Kim et al. 2019; Vander Vorst et al. 2006). Because, during cellular phone use, cell phones are kept close cranial proximity to the user's head, which causes 4–6 cm deep penetration of RF-EMR into the human brain (Jeong et al. 2015; Jiang et al. 2016; Kesari et al. 2014; Kleinlogel et al. 2008). Therefore, there is a need to gain detailed information regarding

the mechanism of neurobiological effects by RF-EMRs.

In the 1990s, the GSM operators used a 900 MHz frequency band (2 G), today it is developed up to the 2100 MHz (3 G), 2600 MHz (4.5 G) and is moving further towards the higher frequency bands (Kim et al. 2019). The specific energy absorption rate (SAR) is defined as the rate at which energy is absorbed in body tissues and is measured in terms of watts per kilogram (W/kg) (Vander Vorst et al. 2006). According to international standards recommended by the International Commission on Non-Ionizing Radiation Protection (ICNIRP) Guidelines, the dose-limits for 900 MHz, 1800 MHz, and 2100 MHz frequency bands are 41 V/m, 57 V/m, and 61 V/m, respectively (ICNIRP 2009) as well as the recommended SAR limit of 2 W/kg for the local head region (ICNIRP 2020). Although there are several studies investigating possible effects of different frequencies and different duration time of RF-EMR exposure on brain functions (Cassel et al. 2004; Esmaili et al. 2017; Hao et al. 2013; Kesari et al. 2014; Narayanan et al. 2009), studies investigating the dose-limit-dependent effects of RF-EMR exposure at a constant band frequency on brain functions, especially on cognitive functions and behaviours are very limited.

The hippocampus is associated primarily with cognition, spatial memory, and learning processes in the mammalian brain (Lisman et al. 2017; Voss et al. 2017). In rodents, RF-EMR exposure might affect the activity (Cho et al. 2012; Gokcek-Sarac et al. 2017; Kim et al. 2018b), morphology (Hao et al. 2013; Salford et al. 2003), and chemistry (Cuccurazzu et al. 2010; Kim et al. 2018a) of the hippocampus in addition to effects on behavioural performances in the hippocampal-dependent tasks (Gokcek-Sarac and Er 2017; Hao et al. 2013; Narayanan et al. 2010). For instance, the Morris water maze (MWM) and the radial-arm maze tests showed impaired hippocampus-dependent learning and memory functions in rats exposed for 4 weeks to 900/1800 MHz RF-EMR (Narayanan et al. 2009) and for 10 days to 2450 MHz EMF (0.6 W/kg SAR) (Wang and Lai 2000). Similarly, reduce excitatory synaptic activity in cultured hippocampal neurons has been detected by 1800 MHz (2.40 W/kg SAR) exposure for 8 days (Xu et al. 2006).

Cholinergic neurons by having an intense projection of fibers through the hippocampus (Prado et al. 2017; Wevers 2011) are involved in many cognitive functions such as learning, memory, arousal, movement, sleep, attention (Rima et al. 2020), and that the loss of central cholinergic neurons is observed in some of the neurodegenerative diseases including Alzheimer's and Parkinson's diseases (Parhizkar et al. 2017; Rima et al. 2020). Acetylcholine, the primary neurotransmitter of

the cholinergic system, is synthesized by choline acetyltransferase (ChAT) by the choline and acetyl-CoA (Deiana et al. 2011; Oda 1999). In the synaptic terminals, acetylcholine that is stored in the synaptic vesicles by vesicular acetylcholine transporters (VACHT), is released into the synaptic cleft as a result of an action potential and it binds either to post-synaptic receptors on cell or pre-synaptic receptors on the neurons that it is released (Ferreira-Vieira et al. 2016). Upon binding to its receptor, a biological response is generated. Acetylcholinesterase (AChE) that is located at the post-synaptic membrane of the synapse catalyses the conversion of acetylcholine molecule and water into acetic acid and choline (Oda 1999). Products of this reaction can be returned to the presynaptic terminal via the re-uptake process (Oda 1999).

It has been reported that the main central cholinergic biomarkers in the cholinergic pathway are involved in the behavioral responses of rats (Deiana et al. 2011; Gokcek-Sarac et al. 2013; Klinkenberg et al. 2011; Martyn et al. 2012; Prado et al. 2006; Sun et al. 2020; Wang et al. 2018; Zhang et al. 2018). The significantly lower hippocampal expression levels of ChAT, AChE, and VACHT in Vascular dementia (VaD) rat model are correlated with the rats' impaired learning and memory performances in the Morris water maze task (Sun et al. 2020). In addition, Zhang et al. (2018) reported that anesthesia/surgery leads to a significant decrease in the protein levels of ChAT, AChE, and VACHT in the pre-frontal cortex, and leads to working memory deficits in adult mice. Furthermore, VACHT-deficient mice show impaired performance in object and social recognition tasks (Prado et al. 2006). In the literature, the relationship between the radiation and the cholinergic system has also been reported in rats (Goncalves et al. 2012; Obajuluwa et al. 2017). But, there is little knowledge about the effects of radiofrequency electromagnetic radiation on cholinergic functions as well as hippocampal-dependent learning and memory responses in rats. Hence, this study attempted to investigate the dose-dependent (45 V/m, 65 V/m) effects of a certain band frequency (2.1 GHz) of RF-EMR exposure for a short-term (1 week) on hippocampal expression levels of main cholinergic biomarkers including AChE, ChAT, and VACHT, and their mRNA levels as well as on learning and spatial memory performances in male Wistar rats.

Materials and methods

Animals

At the first stage of the experiments, 3 months old inbred albino male Wistar rats (N = 33) weighing

between 200 to 300 g served as subjects. All rats were housed under a constant temperature ($23 \pm 1^\circ\text{C}$) and a 12/12 h light/dark cycle. Rats were subjected to an *ad libitum* access to water and commercial rat pellet. All experimental manipulations and procedures for animal care were approved by the Institutional Animal Care and Use Committee in Akdeniz University (Protocol No: 2016.12.14).

After 5 days of the adaptation period, rats were randomly divided into three equal groups ($n = 11$ per group). They were identified as Group 1: Sham-exposed group, Group 2: 45 V/m dose of 2.1 GHz RF-EMR exposed group, and Group 3: 65 V/m dose of 2.1 GHz RF-EMR exposed group.

Electromagnetic field exposure

A RF generator (UMTS Simulator 2.1 GHz; Everest Company, Adapazarı, Turkey), which produces 2.1 GHz RF radiation, was used to represent the exposure of the Universal Mobile Telecommunications System (UMTS). The output power of the generator was fixed at 1.5 W during exposure. The carrier frequency was 2.1 GHz, the modulation frequency was 217 Hz, the pulse width was 0.577 msec, and the power range of the generator was 0–2 W. During exposure, rats were placed in a specially designed plexiglass tubes with air holes to assist breathing and prevent the rise in body temperature. Group 2 and Group 3 were exposed to RF-EMR emitted by the signal generator for 2 h/day for

1 week. The same procedure was applied to the sham-exposed group, except that the generator was turned off.

The exposure system was presented in Figure 1. The entire carousel was placed on the anechoic chamber in the shielded room to prevent other disturbance sources. Each rat was restrained in a radially positioned tube with their snouts that were located at a distance of 8 cm (d_1) and 4 cm (d_2) away from the monopole antenna (Figure 1). The coaxial cable has been used to provide a connection between the RF generator and the antenna. In the experimental setup, the electric-field strengths measured by EMR300 with appropriate probe (Narda, Germany) in the “signal-on” condition have been recorded as 45 V/m and 65 V/m for d_1 and d_2 , respectively.

SAR measurements

Numerical dosimetry analyses have been done through the finite integration technique (FIT) which was introduced by Weiland (1977). The gridding process is applied as a finite integration technique like a finite difference time domain (FDTD) method. However, unlike the other techniques, FIT uses an integral form of Maxwell's equations (Zhabiz 2011). In the present study, the rat model used in simulations has consisted of voxels. The voxel is defined as the smallest unit of the three-dimensional objects. The resolution of one voxel is defined as $1.827 \times 1.827 \times 2.015 \text{ mm}^3$ in the voxel rat model used. Dielectric properties of biological structures

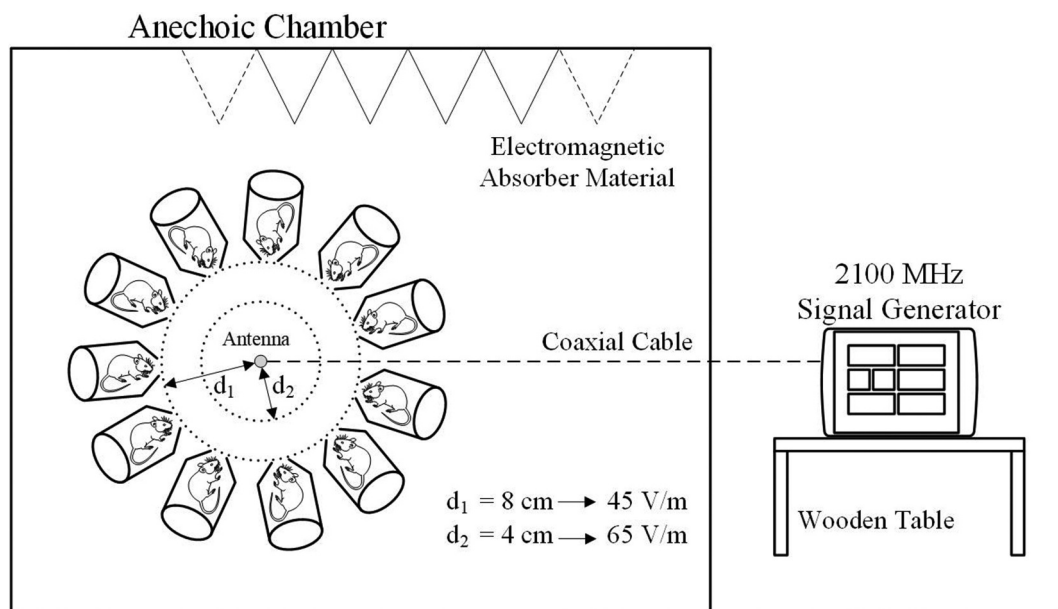


Figure 1. Setup of the electromagnetic exposure system.

Table 1. Simulated SAR values of the brain and average whole body at 2.1 GHz frequency for 45 V/m and 65 V/m electromagnetic fields for 10 g average tissue.

Simulated SAR values at 2.1 GHz for 10 g average tissue	45 V/m	65 V/m
Brain	0.41 W/kg	1.3 W/kg
Average whole body	0.47 W/kg	2.17 W/kg

might be obtained through the complex permittivity measurement at the desired frequency (Gabriel et al. 1996). In this way, the interaction between the incident electromagnetic wave and the biological tissue can be explained.

Dosimetry simulations have been implemented using a FIT based simulation program called CST Microwave Studio (3DEXPERIENCE®, Dassault Systemes, Hamburg). According to the simulation scenario, a monopole antenna is fed by 1.5 W stimulation power through a signal generator which radiates an electric field into the environment. The distance between the antenna and the rat model is 4 cm (d_2) and 8 cm (d_1), respectively. The rat volumetric pixel (voxel) model is crucial for SAR simulations. In this model, each tissue has its own electrical and thermal features that can be measured at the frequency of the mobile phone.

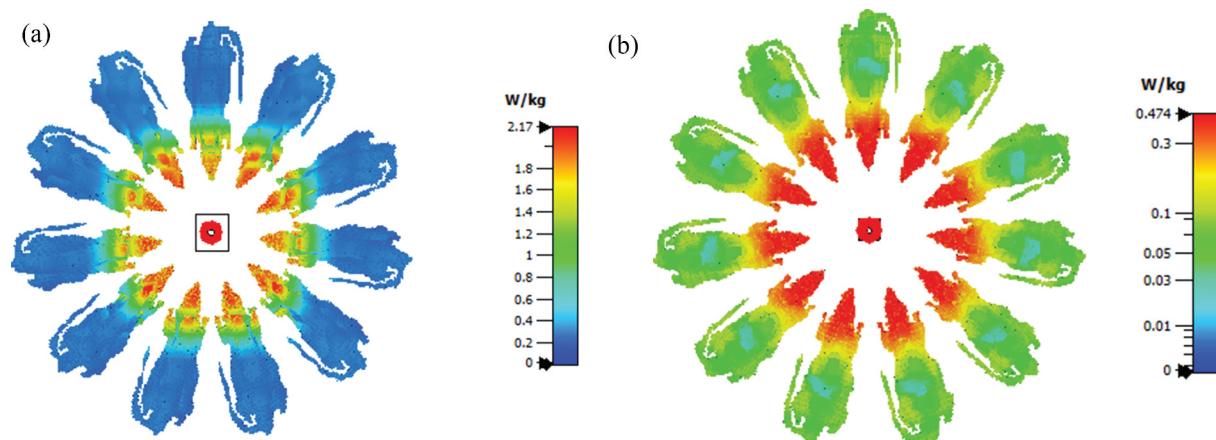
Figure 2 represents the whole body average induced SAR values for 10 g average tissue at 2.1 GHz where the distance is 4 cm (d_2) and 8 cm (d_1), respectively. The maximum exposures have been observed at the closest point of the model to the antenna and the head region of the body. The simulated average whole-body SAR values at 2.1 GHz for 45 V/m and 65 V/m were 0.47 W/kg and 2.17 W/kg, respectively. The SAR values for the brain were on average of 0.41 W/kg and 1.3 W/kg at 2.1 GHz for 45 V/m and 65 V/m, respectively.

The simulated SAR values of the brain and the simulated whole-body average induced SAR values for 10 g average tissue at 2.1 GHz are summarized in Table 1. As seen in Table 1, as the electric-field strengths increase, the simulated SAR values for 10 g average tissue also increase.

Behavioral tasks

Object location test

The object location test is used to examine both short-term and spatial memory of rats. It consists of three stages: habituation, training and retention. During habituation, rats were placed in the center of the arena (40 cm high, 80 × 80 cm in size) and allowed to explore for 5 min without any objects. During the training phase, rats were placed in the center of the arena and allowed 5 min to examine two similar objects, which were placed roughly equidistant from each other. The time spent exploring each object was recorded. The maze was cleaned with 70% ethanol to eliminate odor traces between trials. In the retention phase, one of the objects has been relocated and rats were allowed to explore objects for 5 min. The time delay between phases is an hour. Subjects are expected to spend more time exploring the relocated object. The total exploration time and the total number of touches to the relocated object were recorded. The rats' behaviour was monitored by a video camera system (EthoVision 3.1 System by

**Figure 2.** SAR distribution for 10 g average tissue where the distance is 4 cm (a), and 8 cm (b), respectively.

Noldus Information Technology, Holland) fixed at the ceiling.

Y-maze test

The Y-maze test is widely used to assess both short-term and spatial memory of rats. Testing occurs in a Y-shaped maze with three black identical plastic arms (50 cm long, 20 cm wall high, 10 cm wide) at a 120° angle from each other in a room including a variety of distinct distal cues. During the first session of the experiment, one arm called as “novel arm” was completely closed and rats were allowed to explore the other two open arms for 15 min. At the end of the session, rats were removed from the maze and kept in the home cages for an hour. The area was cleaned before each test with 70% ethanol to prevent the movement of rats with odor traces. One hour after the first session, all arms were opened and rats were allowed to freely explore the three arms for 5 min. During this second session, subjects with intact spatial memory are expected to make the first turn into the “novel arm” and spend more time exploring this arm. The number of entries to the new arm and the time spent to explore the novel arm were recorded. The rats’ behaviour was monitored by a video camera system (EthoVision 3.1 System by Noldus Information Technology, Holland) fixed at the ceiling.

Biochemical assays

Tissue sample preparation

At the end of behavioural tests, 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 then hippocampi dissected. For the biochemical assays, the total hippocampal tissues of rats were immediately frozen in the liquid nitrogen and then stored at −80°C until use.

Western blotting

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

For determination of acetylcholinesterase (AChE), choline acetyltransferase (ChAT), and vesicular acetylcholine transporter (VACHT) expressions, 15 µg of hippocampal samples were run on 7.5% polyacrylamide gels. The separated proteins were transferred onto the PVDF membrane using electroblotting with Mini Protean Tetra Cell equipment (Bio-Rad Laboratories, Richmond, CA, USA). Afterwards, the membranes were incubated at room temperature in a 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 (ChAT, ABCAM; AChE, ABCAM), and 1:250 (VACHT, NOVUS Biologicals). After being rinsed with TBS-T buffer, the membranes were incubated for 1 h at room temperature with the secondary alkaline phosphatase-conjugated antibody (ABCAm) used at a dilution of 1:3000 (ChAT, AChE) and 1:4000 (VACHT) in 5% dried non-fat milk in TBS-T buffer. A monoclonal antibody directed against β-actin 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 (VilberLourmat) with Infinity-Capt version 12.9 software. Immunoreactive protein bands were then quantified by densitometric scanning method using an Image J software package.

cDNA synthesis and quantitative mRNA expression by real-time PCR

Quantitative real-time PCR (qRT-PCR) was used to examine differences in the expression levels of mRNA of AChE, ChAT, and VACHT genes with some modifications of Karakurt et al. (2019). Total RNA isolation of hippocampal tissues of rats was performed using TRIZOL and quantity and quality was measured using NanoDrop™ 2000 (Thermo Scientific). Reverse transcription of RNA to cDNA was carried out using Moloney-Murine Leukemia Virus Reverse Transcriptase and then, cDNA was stored at −20°C for further use. Quantitative Real Time PCR (qRT-PCR) was carried out using CFX Connect system (Bio-Rad, USA). The final reaction mixture (25 µl) contained 200 ng cDNA, 0.5 µM reverse and forward primers, 12.5 µl 1X Maxima® SYBR Green qPCR Master Mix (Qiagen, Hilden, Germany) and 9.5 µl RNase free ddH₂O. During the reaction, NTC (No template control) was used to detect DNA contamination. Housekeeping gene, GAPDH, was used as an internal standard. DNA was

Table 2. The sequences of forward and reverse primers used for quantitative real-time PCR analysis.

Gene	Forward Primer 5'→3'	Reverse Primer 5'→3'	Annealing Temperature (°C)
AChE	ATACACCTCCCTGGCTTCT	AATCTACGTGAGCCACAGG	60
ChAT	AGTGCATGCAGCATCTGGTA	AGGAAGCCGGTAAGAGGAGA	60
VAcHT	GCGATGTGTTGCTTGATGA	TGGACAGTGCTCTTGACAGAA	60
GAPDH	TGATGACATCAAGAAGGTGGTGAAG	TCCTTGAGGCCATGTGGGCCAT	60

amplified in a reaction mixture containing the primers as given in Table 1. The qRT-PCR program consisted of the following temperature profile: initial denaturation at 95°C for 10 min, 40 cycles of melting at 95°C for 20 sec, annealing temperature varied depending on the gene as shown in Table 2, for 30 sec, and extension at 72°C for 30 sec. Primer 3 program was used to design primers. NCBI Blast tool was used to confirm the specificity of primers to rats. Each set of primer was carefully chosen to avoid primer dimerization and to ensure specificity of amplification. Specific annealing temperatures were verified by the use of a gradient thermal cycler.

Statistics

Data were presented as means $\pm$ SD and analyzed by One-Way Analysis of Variance (ANOVA). The Tukey's Honestly Significant Difference (HSD) test was used as a *post-hoc* test. Differences were considered as significant when *P* values less than 0.05. The SPSS software package 23.0 program was used for all analyses.

Results

Behavioral tasks

Object location test

The total time spent exploring the relocated object and the total number of touches to this object were evaluated by One-Way ANOVA. The results showed that both total exploration times ($p < .01$; $p < .05$, respectively)

and total touches to the newly located object ($p < .01$, respectively) were significantly lower in “2.1 GHz (65 V/m) RF-EMR” groups as compared to “sham-exposed” and “2.1 GHz (45 V/m) RF-EMR” groups (Figure 3).

Y-maze test

The total time spends exploring the novel arm and the entrance frequency to this arm were analyzed by One-Way ANOVA. Both total exploration times ($p \leq 0.001$, respectively) and total frequency of entrance to the novel arm ($p < .05$; $p < .01$, respectively) were significantly lower in “2.1 GHz (65 V/m) RF-EMR” groups as compared to “sham-exposed” and “2.1 GHz (45 V/m) RF-EMR” groups (Figure 4).

Biochemical assays

Western blotting

The immunoreactive protein bands for all proteins (AChE; 68 kDa, ChAT; 74 kDa, VAcHT; 56 kDa) obtained from hippocampal tissues of rat groups are represented in Figure 5a. Relative protein expressions were determined by comparing band intensities with that of internal standard control, β -actin (43 kDa). The differences in hippocampal levels of each protein (AChE, ChAT, and VAcHT) are given comparatively between the animal groups in Figure 5b-d, respectively.

The obtained quantitative immunoblot analysis results were examined with One-Way ANOVA along with the

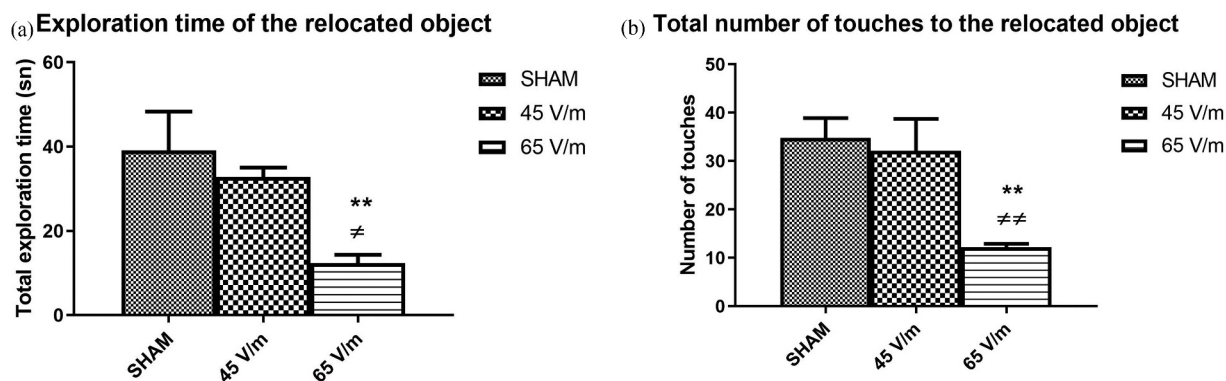


Figure 3. In the object localization test, comparison of (a) Total exploration time, and (b) Total number of touches to the relocated object among groups. Data are expressed as mean $\pm$ SD. The statistical difference of “2100 MHz (65 V/m)” group to “Sham-exposed” and to “2100 MHz (45 V/m)” groups is given by * and $\neq$, respectively. ** $p < .01$; $\neq p < .01$; $\neq p < .05$ indicates statistical difference.

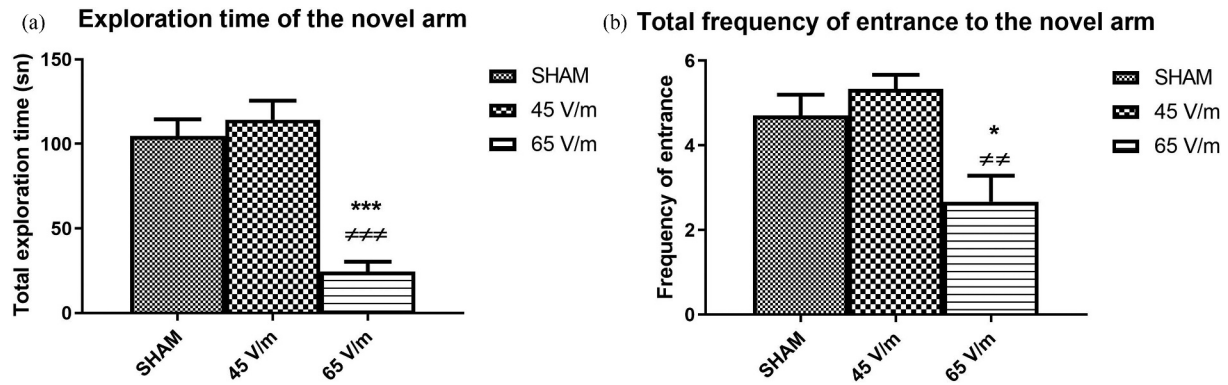


Figure 4. In the Y-maze test, comparison of (a) Total exploration time, and (b) Total frequency of entrance to the novel arm. Data are expressed as mean $\pm$ SD. The statistical difference of the “2100 MHz (65 V/m)” group to “Sham-exposed” and to “2100 MHz (45 V/m)” groups is expressed by * and $\neq$, respectively. *** $p \leq 0.001$; $\neq p \leq 0.001$; * $p < .05$; $\neq p < .01$ denotes statistical difference.

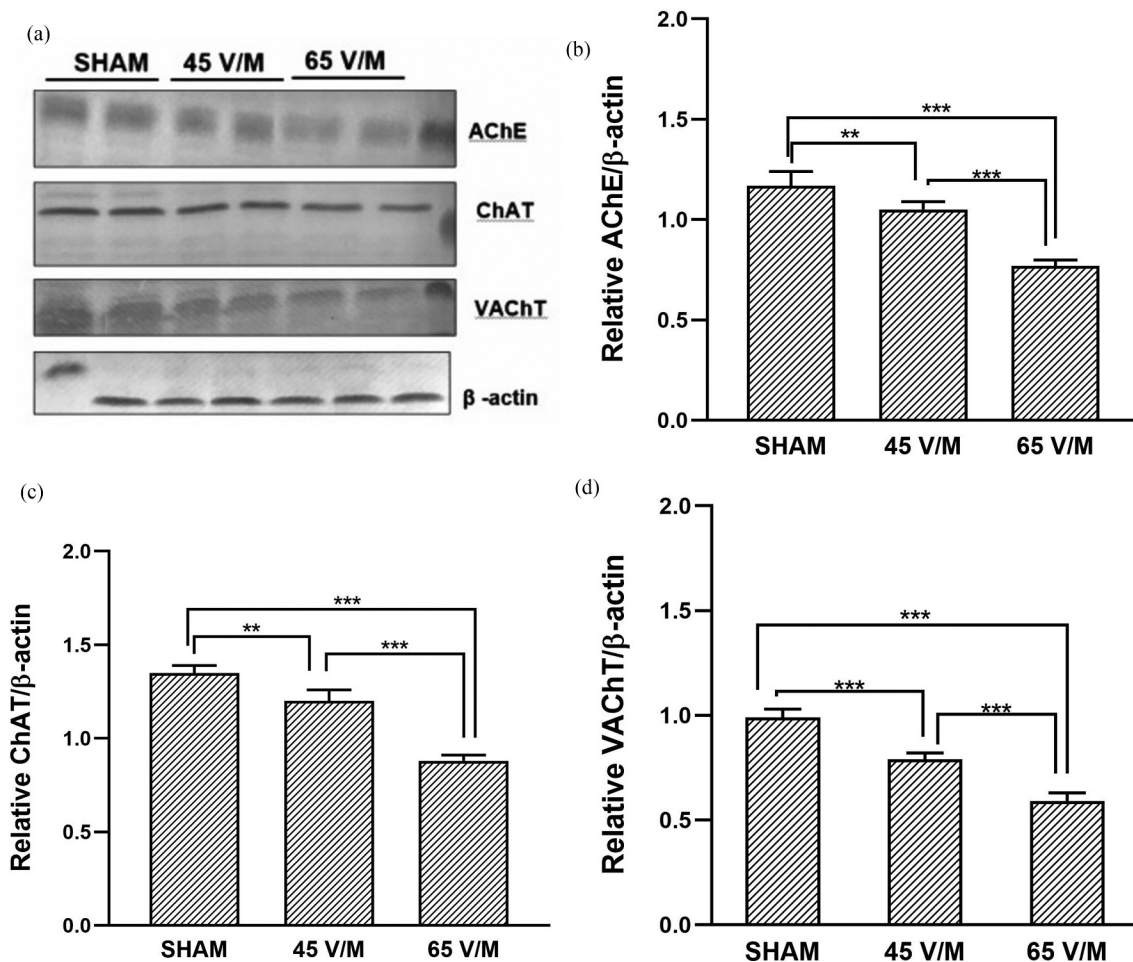


Figure 5. (a) Representative immunoreactive protein bands of AChE, ChAT, and VACHT from the hippocampus of “Sham-exposed”, “2.1 GHz (45 V/m) RF-EMR”, and “2.1 GHz (65 V/m) RF-EMR” rat groups. Comparison of the levels of (b) AChE; (c) ChAT; and (d) VACHT in the hippocampus of rat groups. Band quantifications are expressed as the mean $\pm$ SD of the relative intensity concerning that of β -actin, used as an internal control. Error bars denote $\pm$ SD. Asterisks denote the level of significance: ** $p < .01$; *** $p < .001$.

Tukey HSD Post-Hoc test for multiple comparisons. There were significantly higher expression levels of AChE, ChAT, and VACHT in “Sham-exposed” groups as compared to “2.1 GHz (45 V/m) RF-EMR” ($p = .011$;

$p = .002$; $p \leq 0.001$, respectively), and “2.1 GHz (65 V/m) RF-EMR” ($p \leq 0.001$, respectively) rat groups. Besides, the hippocampal expression levels of AChE, ChAT, and VACHT were significantly higher in “2.1 GHz (45 V/m)

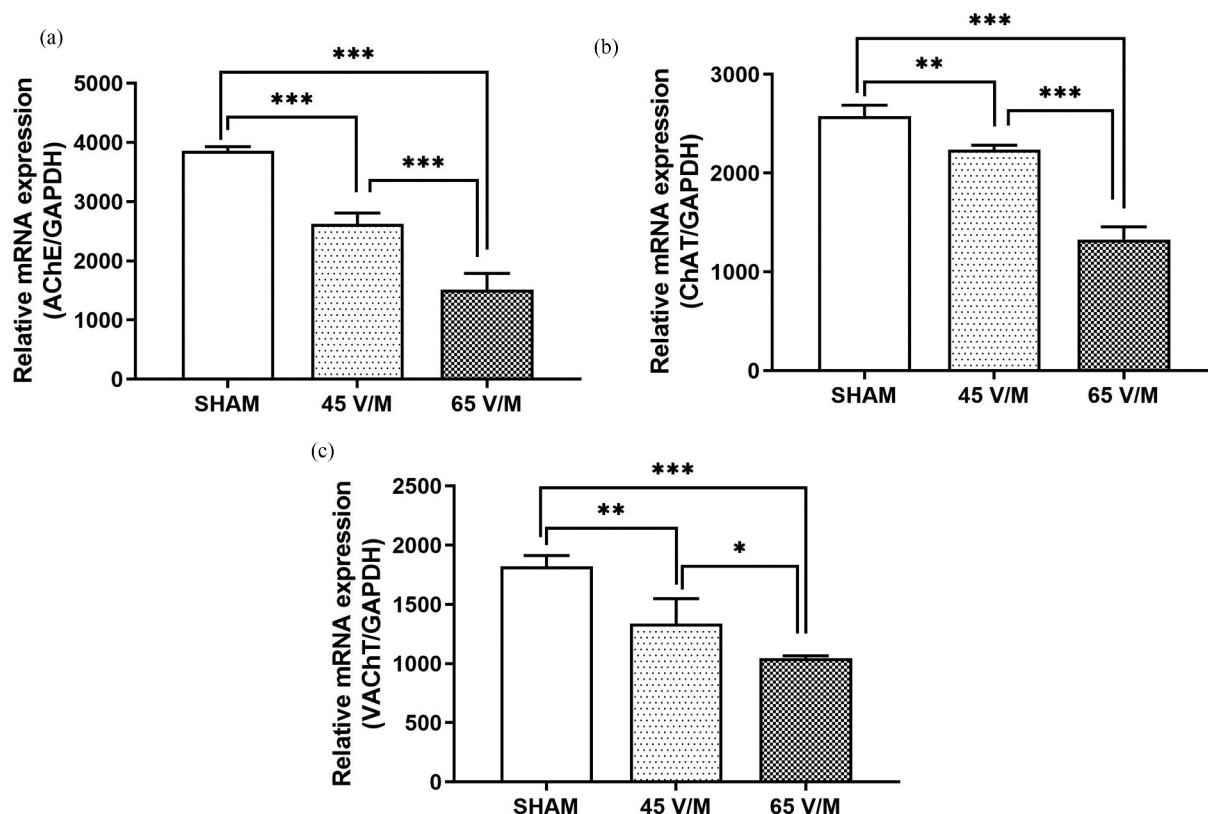


Figure 6. Alterations in mRNA expressions of (a) AChE; (b) ChAT; and (c) VACHT genes in the hippocampus of “Sham-exposed”, “2.1 GHz (45 V/m) RF-EMR”, and “2.1 GHz (65 V/m) RF-EMR” rat groups. Results were presented as the mean from three independent experiments ($n = 6$ /per group) and expressed as relative mean $\pm$ SD. mRNA expressions of tested genes were normalized to housekeeping GAPDH mRNA. Error bars denote $\pm$ SD. Asterisks denote the level of significance: * $p < .05$; ** $p < .01$; *** $p \leq 0.001$.

RF-EMR” groups as compared to “2.1 GHz (65 V/m) RF-EMR” ($p \leq 0.001$, respectively) rat groups (Figure 5).

Quantitative mRNA expression by real-time PCR

Quantitative real-time PCR technique was used to analyze mRNA expressions of AChE, ChAT, and VACHT genes using specific primers. The obtained results were normalized with GAPDH (housekeeping gene). The data were evaluated by One-Way ANOVA along with the Tukey HSD Post-Hoc test for multiple comparisons. As seen in Figure 6., mRNA expressions of AChE, ChAT, and VACHT enzymes were significantly higher in “Sham-exposed” groups as compared to “2.1 GHz (45 V/m) RF-EMR” ($p \leq 0.001$; $p < .01$; $p < .01$, respectively), and “2.1 GHz (65 V/m) RF-EMR” ($p \leq 0.001$; $p < .01$; $p \leq 0.001$, respectively) rat groups. Besides, mRNA expressions of AChE, ChAT, and VACHT enzymes were significantly higher in “2.1 GHz (45 V/m) RF-EMR” groups as compared to “2.1 GHz (65 V/m) RF-EMR” groups ($p \leq 0.001$; $p \leq 0.001$; $p < .05$, respectively).

Discussion

Given the widespread use of cellular phones in contemporary life, whether RF-EMR exposure from cellular phones affects the brain functions is a major interest for researchers. Hippocampus is the most studied limbic structure of the mammalian brain because of its well-documented role in learning and memory functions as well as its simple, tri-synaptic circuitry (Leuner and Gould 2010; Voss et al. 2017). Experimental animal studies reported that RF-EMR exposure from cellular phones detrimentally affects the hippocampal activity, morphology, chemistry, as well as animal behavioral performances in hippocampal-dependent tasks (Hao et al. 2013; Narayanan et al. 2010). In the literature, there are many behavioral studies documenting that exposure to different band frequencies of RF-EMR on different duration times may have different effects on rats’ performances on hippocampal-dependent tasks (Hao et al. 2013; Narayanan et al. 2010; 2009; Wang and Lai

2000). In such studies, the rats are generally exposed to a certain dose of RF-EMR, however, till date there is no report on the effects of different doses of RF-EMR exposure at a certain frequency on rat's behavioral performances. Therefore, in the present study effects of short-term 2.1 GHz RF-EMR exposure at different doses (45 V/m and 65 V/m), which obtained by positioning the rat's head at 4 cm and 8 cm away from the antenna, on rat's object location and Y-maze hippocampal-dependent task's performances were evaluated.

The Y-maze task is one of the hippocampal-dependent tasks which generally used for the assessment of short-term and spatial memory. The collected data showed both total exploration times and total frequency of entrance to the novel arm were significantly lower in "2.1 GHz (65 V/m) RF-EMR" groups as compared to "sham-exposed" and "2.1 GHz (45 V/m) RF-EMR" groups. A similar result was also obtained in the object location test where short-term and spatial memory were evaluated together. Analysis of variance revealed both total exploration times and total touches to the relocated object were significantly lower in "2.1 GHz (65 V/m) RF-EMR" groups as compared to "sham-exposed" and "2.1 GHz (45 V/m) RF-EMR" groups. The reason why "2.1 GHz (65 V/m) RF-EMR" group rats' performances in both Y-maze and object location tasks is worse than the other experimental groups may be due to the stronger effect of "65 V/m" dose of 2.1 GHz RF-EMR where rats' snouts were located at the nearest distance (4 cm) from the monopole antenna. The impaired object location task performance of rats exposed to "2.1 GHz (65 V/m) RF-EMR" is consistent with the study showing impaired memory for objects and their temporal order of presentation in the episodic-like memory test in rats exposed long-term (55 weeks) to GSM-900 MHz radiation (Nittby et al. 2008). Besides, the result of the the Y-maze task in the current study is consistent with the study in which the impairment effects of long-term (25 days) extremely low-frequency (ELF) magnetic fields (50 Hz) exposure on the recognition of the novel arm in the Y-maze test was reported in mice (Fu et al. 2008). In addition, the significant cognitive deficits in Y-maze paradigm were observed in rats after long-term (28 days) exposure of EMR-2450 MHz while no significant cognitive dysfunction was observed with exposure of EMR-900 and EMR-1800 which indicates the critical role of duration and frequency of exposure of EMR in cognitive deficiency (Gupta et al. 2018). In contrast to earlier reports where impairment cognitive effects of long-term exposure to different frequencies of electromagnetic radiation were shown by different experimental methods, in the present report, by taking a different

approach, dose-dependent (45 V/m and 65 V/m) effects of short-term (1 week) exposure to 2.1 GHz radiation on behavioral performances of rats on the hippocampus-dependent tasks have been demonstrated.

The central cholinergic system plays an important role in performing hippocampus-dependent behavioral tasks in which learning and memory performances are evaluated (Himmelheber et al. 2000). Acetylcholine (ACh), the first neurotransmitter discovered, is thought to be the most crucial neurotransmitter involved in the mediation of cholinergic neurotransmission as well as regulation of cognitive functions (Oda 1999). There are three major central cholinergic biomarkers that are responsible for the biosynthesis, transportation, and degradation of ACh, which are called as ChAT, VACHT, and AChE, respectively (Oda 1999). Our results showed that both protein and mRNA levels of AChE, ChAT, and VACHT, were significantly lower in "2.1 GHz (65 V/m) RF-EMR" groups as compared to "sham-exposed" and "2.1 GHz (45 V/m) RF-EMR" groups. Earlier, a study has reported that long-term (one hour of per day for 28 consecutive days) exposure to EMR 2450 MHz leads to a decrease in the level of ACh that estimated in the hippocampus using spectrofluorometer as well as leading to cognitive deficiency in terms of novel arm entries in Y-maze task in male rats, but these effects have not been detected in EMR 900, and 1800 MHz subjected rats (Gupta et al. 2018). In that study, rats were positioned at a distance of 8, 11 and 15 cm away from the pyramidal horn antenna for long-term exposure to continuous modulated electromagnetic radiation of 900, 1800 and 2450 MHz, and the average whole-body SAR values were found to be 0.0227, 0.030, and 0.0616 W/kg for EMR 900, 1800 and 2450 MHz, respectively. In another study (Obajuluwa et al. 2017), a significant increase in the anxiety-linked behavior in the rotarod test and a significant decrease in the locomotor activity in the open field test have been found in rat's subjected to long-term (8 weeks) 2.5 GHz band radiofrequency waves from a WiFi device by positioning 50 cm away from the antenna. Furthermore, there was a significant decrease in cerebral cortex AChE activity and an increase in mRNA expression this in turn points to the adverse effects of WiFi exposure on both neurobehavioral parameters and cerebral cortex AChE activity. Moreover, Kunjilwar and Behari (1993) did not record any significant effect of short-term exposure of amplitude-modulated microwave radiation (147 MHz) on AChE activity, while long-term exposure caused a decrease in enzyme activity.

Unlike the above studies where the effects of different band frequencies and different durations of radiation exposure from WiFi, microwave, EMR source, etc.

were tested with different experimental setups, in the current study, it has been demonstrated that short-term exposure to different doses of (45 V/m and 65 V/m) RF-EMR at a constant band frequency (2.1 GHz) may have different effects on both hippocampus-dependent behavioural parameters and hippocampal levels of main cholinergic biomarkers. The stronger effect of “65 V/m” dose of 2.1 GHz RF-EMR, where rats’ snouts were located at the nearest distance (4 cm) away from the monopole antenna, has been observed on both rat’s behavioral performances and hippocampal levels of AChE, ChAT, and VACHT. Besides, by simulating the specific energy absorption rate (SAR) distributions on a voxel rat model in which each tissue has its own electrical and thermal features, the dosimetric effects of different doses of electromagnetic fields from the generator which create signals similar to a 3 G cell phone (2.1 GHz) have also been evaluated. The simulated SAR values were found higher at 65 V/m electric field strength where both behavioural and molecular effects were observed more.

For the first time, we report the potential dose-dependent effects of short-term exposure to 2.1 GHz RF-EMR on rat’s object location and Y-maze hippocampal-dependent task’s performances as well as hippocampal protein and mRNA levels of main cholinergic biomarkers including AChE, ChAT, and VACHT in male Wistar rats. To understand the mechanisms by which RF-EMR from cellular phones influence the function of the central cholinergic system in the mammalian brain, further researches are needed to get solid conclusions.

Author contributions

ÇGS, and ND, designed the study; ÇGS, GA, and ŞÖ, performed RF-EMR exposure; KA performed simulations; ÇGS, and GA, performed behavioural tests; ÇGS, GA, and SK, performed biochemical assays; ÇGS analysed the data; ÇGS wrote the manuscript.

Disclosure statement

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