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Investigation of Possible Behavioural and Molecular Effects of Mobile Phone Exposure on Rats

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***Abstract*—**The N-methyl-D-aspartate (NMDA)-dependent pathway is the major intracellular signaling pathway implemented in both short- and long-term memory formation in the hippocampus which is the most studied brain structure because of its well documented role in learning and memory. However, little is known about the effects of RF-EMR exposure on NMDA receptor signaling pathway including activation of protein kinases, notably Ca^{2+}/calmodulin-dependent protein kinase II alpha (CaMKIIα). The aim of the present study was to investigate the effects of acute and chronic 900 MHz RF-EMR exposure on both passive avoidance behaviour and hippocampal levels of CaMKIIα and its phosphorylated form (pCaMKIIα). Rats were divided into the following groups: Sham rats, and rats exposed to 900 MHz RF-EMR for 2 h/day for 1 week (acute group) or 10 weeks (chronic group), respectively. Passive avoidance task was used as a behavioural method. The hippocampal levels of selected kinases were measured using Western Blotting technique. The results of passive avoidance task showed that both acute and chronic exposure to 900 MHz RF-EMR can impair passive avoidance behaviour with minor effects on chronic group of rats. The analysis of western blot data of selected protein kinases demonstrated that hippocampal levels of CaMKIIα and pCaMKIIα were significantly higher in chronic group of rats as compared to acute groups. Taken together, these findings demonstrated that different duration times (1 week vs 10 weeks) of 900 MHz RF-EMR exposure have different effects on both passive avoidance behaviour of rats and hippocampal levels of selected protein kinases.

***Keywords*—**Hippocampus, protein kinase, rat, RF-EMR.

I. Introduction

THE brain is the organ that is responsible for what we call the mind. It is the anatomical basis for thinking, feeling, wanting, perceiving, curiosity, as well as learning and memory. In general terms, learning could be defined as the process in which the new information is acquired and memory could be defined as the process in which this knowledge is maintained [1]. In behavioral terms, we can define learning as a more or less permanent change in animal's or human's response to a repeated stimulus. Memory is a fundamental mental process, and without memory, we are only capable of simple reflexes and stereotyped behaviors [1]. Learning and memory play a very important role in both human and animal life. Therefore, elucidation of learning processes, anatomy of memory, and the molecular mechanisms by which brain codes, stores and retrieves memory traces are one of the most intriguing and challenging issues in the contemporary neuroscience.

In research of learning and memory, hippocampus is the most studied structure in the mammalian brain because of its well documented role in learning and memory [2]. Glutamate, the most prominent neurotransmitter associated with learning and memory functions in the hippocampus, exerts its signaling function by binding to hippocampal glutamate receptors, one of which is called as NMDA receptor [3]. The NMDA receptors (NMDARs) and the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPARs) are the major players for controlling synaptic plasticity and memory functions in the hippocampus [3]. 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 [3]. The NMDA-dependent pathway includes the activation of protein kinases, including Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) [3]-[5].

In mammals, CaMKII is encoded by four genes: α, β, γ, and δ. The γ and δ forms are mostly found in peripheral tissues, whereas the α and β forms are the predominant forms found in the central nervous system [4], [5]. The α form is mostly expressed postnatally in forebrain structures, whereas the β, another related kinase with similar properties, is expressed more uniformly throughout the brain and during embryonic development [4], [5]. The translated protein products, which are generally 50-60 kDa in size, contain a highly conserved N-terminal kinase (catalytic) domain (approximately 280 amino acids) followed by a autoinhibitory (regulatory) domain (approximately 40 amino acids) and a 150-220 amino acid C-terminal association domain [4], [5].

Activation-dependent molecular changes at synapses are mediated by a rise in intracellular calcium which enters the postsynaptic neuron through NMDA ion channels [3]. Calcium alone or conjugated with calmodulin acts as a powerful second messenger and leads to the activation of numerous synaptic proteins including CaMKII [4]. In the hippocampus, among many downstream signaling molecules of the NMDA receptor pathway, CaMKIIα has long been considered the key mediator in regulating the memory formation [5]. Studies have shown that pharmacological inhibition of CaMKII or genetically removing it leads to the blockage of long-term potentiation (LTP) induction which is

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thought to be the cellular mechanism in the hippocampus that contributes to learning and memory [6]. 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 [6].

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 2nd generation (2G) mobile phones run on 900 MHz radiofrequency [7]. Mobile phones are known to be associated with potential adverse effects on human health, especially on the human brain [8]. 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, blood-brain barrier permeability, oxidant and antioxidant balance, neurotransmitter balance, nerve cell damage, genomic responses, short-term memory loss, and other cognitive functions have been studied [9].

One of the affected brain regions is the hippocampus. Experimental animal studies have shown that RF-EMR exposure can affect cognitive functions and the behaviour of animals [8], [10]-[12]. For example, the potentially devastating effects of 60 min 8 mT, 50 Hz electromagnetic field exposure on learning and information acquisition in passive avoidance learning task in both male and female mice have been demonstrated by Foroozandeh et al. [11]. It has been reported in another experimental study that mobile phone (900/1800 MHz) RF-EMR exposure leads to impaired spatial memory performance in the Morris water maze task in Wistar rats [12]. In another study of the same research group [8], they confirmed that mobile phone (0.9 GHz/1.8 GHz) RF-EMR exposure significantly altered the passive avoidance behaviour and hippocampal morphology in rats. However, much less attention has been paid so far to the molecular effects of RF-EMR exposure on NMDA receptor related signaling pathways on the hippocampus. In the literature, little is known about the effects of 900 MHz RF-EMR exposure on hippocampal level of CaMKIIα. Therefore, in the present study, an attempt was taken to address possible effects of both acute and chronic exposure to 900 MHz RF-EMR exposure on both passive avoidance behavior and hippocampal level of selected protein kinases.

II. Materials and Methods

A. Animals

Inbred young male albino Wistar rats were kept in home cages in groups of four under a constant temperature (23±1 °C) and a 12/12h light/dark cycle. They were given food and water ad libitum. The animal care procedures and all experimental manipulations were pursued in accordance with the Institutional Animal Care and Use Committee in Akdeniz University, Antalya, Turkey.

B. Electromagnetic Field Exposure

A RF generator which produces 900 MHz RF radiation was used to represent exposure of Universal Mobile Telecommunications System (UMTS).

Peak power of the generator was fixed at 5 W during exposure. The carrier frequency was 900 MHz, the modulation frequency was 217 Hz, pulse width was 577 ms, and the maximal peak power was 0-10 W. The exposure system was presented in Fig. 1.

The system was placed on a wooden table, and the monopole antenna of the generator was placed at the center of plexiglass carousels to provide equal exposure to the rats aligned around the antenna. The distance of the rats to the monopole antenna was 10 cm, and the generator terminal output was 1.5 W. The electric field strengths were measured by EMR300 m with appropriate probe (Narda, Germany).

The electric field background level in the shielded room was between 0.02-0.2 V/m. The average whole-body SAR was 5.284 mW/kg. The SAR for the brain was in average of 0.66 W/kg.

The numerical computation was performed using Finite Difference Time Domain (FDTD) Method [13]. Electrical properties, conductivity, and dielectric constant were taken from the literature [14].

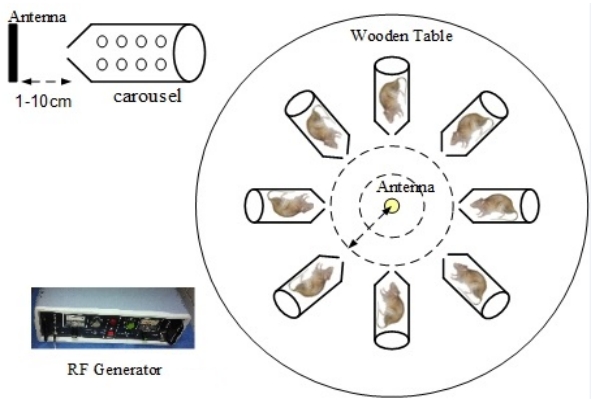

Fig. 1 Experimental setup

All rats were randomly divided into four groups after the adaptation process: Group 1: sham-exposed rats for 1 week (acute); Group 2: sham-exposed rats for 10 weeks (chronic); Group 3: rats exposed to 900 MHz RF-EMR for 1 week (acute); Group 4: rats exposed to 900 MHz RF-EMR for 10 weeks (chronic). The “acute” and “chronic” groups of rats were exposed to 900 MHz RF-EMR emitted from the signal generator for two hours per day.

C. Passive Avoidance Test

After RF-EMR exposure process, all rats were subjected to passive avoidance task which is a fear-aggravated test used to evaluate learning and memory in rat models. Rats naturally prefer dim spaces. As soon as they are placed in a brightly illuminated space, they swiftly pass the dark compartment where an aversive stimulus (foot shock) delivered. This leads to modification on their behaviour and consequently they

remain in the bright compartment. By this task, rats learn to avoid an environment in which an aversive stimulus was previously delivered.

The apparatus has two compartments, a rectangular light compartment and a dark compartment with a grid floor. The connection between the two compartments can be closed with a sliding door made of plexiglass. In the acquisition trial, each rat was placed individually in the light compartment and the time taken to enter the dark compartment was measured. As soon as the rat entered the dark compartment, the sliding door was closed and an electrofootshock (1 mA for 5 s) was delivered through the grid floor. The rat was then returned to its own cage waiting for the retention trial. The retention trial was carried out after 24h. The rat was placed in the light compartment and the latency of the step-through response (cut-off latency time 300 s) was recorded. Absence of entry into the dark compartment indicated positive memory retention.

D.Tissue Sample

After passive avoidance task, rats were anesthetized under urethane and brains were perfused transcardially with heparinized saline. Then, the perfused brains were rapidly removed, hippocampi was dissected and homogenized by ultrasonication in the presence of protease inhibitors. 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).

E. Western Blotting

For determination of protein expressions, hippocampal samples were run on 6-7.5% polyacrylamide gels. After electrophoresis was completed, gels were removed from set-up for western blotting. Gels were equilibrated in transfer buffer for 15 min with constant shaking to adjust the final size of gel and remove the buffer salts and SDS coming from SDS-PAGE step. Separated proteins were then transferred onto PVDF membrane. PVDF membrane, two filter papers and fiber pads of the transfer sandwich were placed in transfer buffer and saturated with this solution. Western blot sandwich was prepared as seen in Fig. 2.

A test tube was used to remove any air bubbles between the layers by gently rolling over the sandwich. This was the very critical step since any air bubbles formed between layers block the transfer of proteins. Then, the sandwich was put into the Mini Trans-Blot module 165-8033 (Bio-Rad Laboratories, Richmond, CA, USA) and module was filled with cold transfer buffer. Voltage and current were set to 90 V and 400 mA, respectively. Transfer process was carried for 90 min with Bio-Rad PowerPac basic power supply (Bio-Rad Laboratories, Richmond, CA, USA).

At the end of this process, the membrane carrying the transferred protein on it, i.e. “blot” was obtained and removed from the module. Then, membrane was transferred to a plastic dish as protein side facing upwards and washed with TBS-T for 10 min. This washing step removes the salts and buffers from transfer medium. Then, the blot was incubated with blocking solution (5% Non-Fat Dry-Milk in TBS-T) for 60 min so that empty spaces between transferred proteins were filled. This filling inhibits the non-specific binding of antibodies to the membrane. After that, primary and secondary antibodies were applied at suitable dilutions. A monoclonal antibody directed against β-actin was used as an internal standard control. After several washing steps, the final images were photographed and immunoreactive protein bands were then quantified by densitometric scanning method using an Image J software package.

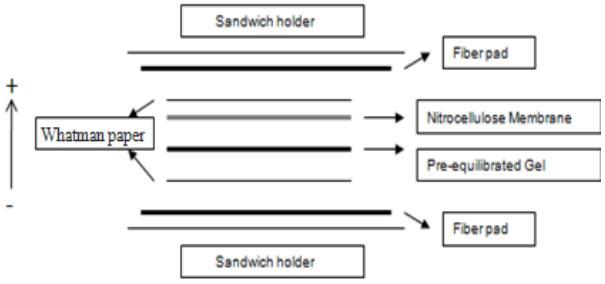

Fig. 2 Preparation of western blot sandwich

F. Statistical Analyses

The statistical analysis of the obtained data was evaluated by SPSS v.20 (SPSS, Chicago, IL, USA) software of Windows. Statistical analyses were performed by One-way ANOVA with all pairwise multiple comparison procedure done by Tukey’s HSD post hoc test. A p value less than or equal to 0.05 was considered as statistically significant.

III. Results

During the passive avoidance test, the entrance latency to the dark compartment was significantly less for RF-EMR exposed rats when compared with the sham exposed rats. In addition, the mean value of entrance latency of acute groups is shorter than the mean value of entrance latency of chronic groups. This demonstrated that acute groups of rats did not remember the passive avoidance task to some extent on the following day which is an indicator of the impairment of the memory. These findings are in agreement with the results showing the impairing effects of short term RF-EMR exposure on cognitive functions by using different behavioural tasks [10], [11].

Quantitative immunoblot analysis of CaMKIIα and pCaMKIIα levels in the hippocampus of rats revealed significantly higher expression of both CaMKIIα and pCaMKIIα in chronic groups as compared to acute groups ($p \leq 0.001$, respectively). In addition, in both acute and chronic groups, mean numbers of pCaMKIIα level were significantly ($p \leq 0.001$) higher than mean numbers of CaMKIIα level. The mean±SEM values of CaMKIIα and pCaMKIIα obtained from western blot protein expression assay from all groups are listed in Table I.

IV. Discussion

The passive avoidance task is a fear-aggravated test used to

evaluate learning and memory in rat models. Based on our results, it can be concluded that both acute and chronic exposure to 900 MHz RF-EMR impairs the passive avoidance behaviour with minor effect on behaviour of chronic group of rats. In the memory retention test (24 h after the aversive stimulus), acute groups showed shorter entrance latency (mean=39.62) than chronic groups (mean=125.88). This demonstrated that they did not remember the passive avoidance task to some extent on the following day which is an indicator of the impairment of the memory. These findings are in agreement with the results showing the impairing effects of short term RF-EMR exposure on cognitive functions by using different behavioural tasks [10], [11]. For example, impairment on the consolidation of spatial memory in a water maze after 20 min exposure to an 8 mT, 50 Hz magnetic fields has been provided by Jadidi et al. [10]. In addition, the devastating effects of 60 min 8 mT electromagnetic fields exposure on learning and information acquisition in passive avoidance learning task in both male and female mice have been demonstrated by Foroozandeh et al. [11].

TABLE I
THE MEAN±SEM VALUES OF CAMKIIA AND PCAMKIIA IN EACH GROUP

Groups	Duration of exposure	Protein expression (mean±SEM) of CaMKIIα and pCaMKIIα, respectively
Sham	1 week (acute)	1.02±0.01 ; 1.05±0.01
Sham	10 weeks (chronic)	1.33±0.02 ; 1.45±0.03
900 MHz	1 week (acute)	2.09±0.05 ; 2.25±0.07
900 MHz	10 weeks (chronic)	2.75±0.06 ; 3.08±0.03

In the present study, the comparison of hippocampal level of CaMKIIα and pCaMKIIα between acute and chronic groups of 900 MHz RF-EMR was revealed that hippocampal level of these kinases was significantly higher in chronic groups as compared to acute groups. In the study of Manikonda et al. [15], long term exposure (90 days) of rats to 50 Hz extremely low frequency (ELF) caused increased intracellular Ca^{2+} levels together with increased activities of protein kinase A (PKA) and calcineurin in hippocampal regions as 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 Ca^{2+} influx signal through postsynaptic NMDARs that arise from following 30 mW/cm² microwave exposure [16].

The increased level of the CaMKIIα and pCaMKIIα found in the present study is consistent with the results of above studies and might be related with the increased intracellular Ca^{2+} and calcineurin levels [15], [16].

V. CONCLUSION

To summarize, findings indicate that both acute and chronic exposure to a 900 MHz RF-EMR can impair passive avoidance behaviour of rats and have different effects on hippocampal levels of selected protein kinases.

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