The Effects of Prenatal Exposure to a 900 Megahertz Electromagnetic Field on Hippocampus Morphology and Learning Behavior in Rat Pups

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ABSTRACT

The purpose of this study was to examine the effect on hippocampus morphology and learning behavior in rat pups following prenatal exposure to a 900 megahertz (MHz) electromagnetic field (EMF). Female Sprague Dawley rats weighing 180-250 g were left to mate with males. The following day, pregnant rats identified as such by the vaginal smear test were divided into two groups, control (n=3) and EMF (n=3). No procedures were performed on the control group. The rats in the EMF group were exposed to 900 MHz EMF on days 13 to 21 of pregnancy, for 1 h a day. Female rat pups were removed from their mothers at 22 days old. We then established two newborn rat groups, a 13 member control group and a 10 member EMF group. Radial arm maze and passive avoidance tests were used to measure rat pups' learning and memory performance. All rats were decapitated on the postnatal 32nd day. Routine histological procedures were performed on the brain tissues, and sections were stained with Cresyl fast violet. The radial arm maze (p=0.007) and passive avoidance (p=0.032) tests were administered to both groups under identical conditions, and compromised learning behavior was determined in the EMF group rats. Morphological compromise was also determined in the EMF group sections. Our results show that the application of a 900 MHz EMF in the prenatal period adversely affected female pups' learning behavior and also resulted in histopathological changes appearing in the hippocampus.

Key Words: newborn female rats, hippocampus, electromagnetic field, learning behavior

NeuroQuantology 2013; 4:582-590

Introduction

The rapid growth of technology and the intensive use in daily life of devices that produce an electromagnetic field (EMF) effect pose a number of health problems. There is no doubt that due to their being used close to the head and being accessible to people of all ages, with the exception of babyhood, mobile phones head the list of such devices (Barcal and Vozeh, 2007; Odaci *et al.*, 2008; Wigle *et al.*, 2008). Mobile phones can also be described as the technological devices that most expose humans to the effect of EMF. However, these marvels of technology that produce considerable revenues for companies involved with mobile phones, are regarded as harmless due to the written and visual advertizing appearing in the media. Despite all the innocent and marvelous impressions given, research into the subject is increasingly raising doubts concerning the

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This study was given as an oral presentation at the Controversies on Electromagnetic Field in Medicine and Biology Symposium, Turkey, Samsun, June 17 2013.
Received: September 13, 2013; **Revised:** October 20, 2013;
Accepted: December 1, 2013
***e*ISSN** 1303-5150

effect of the EMF emitted by mobile phones (Odaci *et al.*, 2008; Bas *et al.*, 2009a; 2009b; Sonmez *et al.*, 2010; Hancı *et al.*, 2013). The mobile phone and all aspects of its probable effects on human health are therefore attracting considerable interest from researchers.

Studies investigating the effect of the EMF emitted by mobile phones using stereological methods (Kaplan *et al.*, 2012a; 2012b; İkinci *et al.*, 2013; Aktürk *et al.*, 2013) have shown that quantitative losses at the cellular level may develop in the brains and brain-related structures of rats exposed to the effect of 900 MHz EMF in both the prenatal (Bas *et al.*, 2009b; Odaci *et al.*, 2008) and postnatal (Bas *et al.*, 2009a; Sonmez *et al.*, 2010) periods. These studies encourage the investigation of the kind of effect exposure to EMF in the prenatal or postnatal terms will have on behavior. That is because an agent that causes losses at the cellular level will very probably also affects behaviors for which these cells are responsible.

Studies investigating the relationship between EMF and behavior have reported differing results. While some studies report that there may be problems with learning and memory in rats exposed to the effect of 900 MHz EMF (Dubreuil *et al.*, 2002; Hao *et al.*, 2013), others report the exact opposite (Dubreuil *et al.*, 2003; Kumlin *et al.*, 2007). For example, one study on rats using the eight-arm maze test showed that long-term EMF (916 MHz, 10 w/m^2) administered for approximately 10 weeks (5 times a week) for 6 h a day compromised spatial memory (Hao *et al.*, 2013). On the other hand, it has also been reported that neither spatial nor non-spatial memory were affected by EMF in rats exposed to 900 MHz EMF (*Specific absorption rate* (SAR) = 1 and 3 W/kg) (Dubreuil *et al.*, 2003). Another study on mice reported no adverse effect on spatial memory caused by 900 MHz EMF (SAR = 0.05 W/kg) (Sienkiewicz *et al.*, 2000).

Studies investigating the effect on the cognitive functions of rodents of EMF caused by mobile phones have particularly concentrated on different postnatal periods. To the best of our knowledge, however, there are insufficient studies investigating the effect of exposure to EMF emitted by mobile phones on cognitive functions in the prenatal period, the initial and most sensitive period of brain development (Rodier, 1980; Weinstock, 2001; Sakatani *et al.*, 2002; Tunc *et al.*, 2007). The purpose of this study was therefore to determine the probable changes that 900 MHz EMF applied between days 13 and 21 of the prenatal period would cause in female rats' behaviors and hippocampus morphology.

Materials and methods

Acquisition of newborn rats and establishment of newborn groups

At the beginning of the study, 14 female and 14 male rats (6-8 weeks old, weighing 180-250 g) were mated for the purpose of obtaining pregnant rats. These animals were obtained from the Karadeniz Technical University Surgery Research Center (KTUSRC), Turkey. They were kept in the KTUSRC in standard plastic cages on sawdust bedding in an air-conditioned room at 22 ± 1 ^{0}C under a 12-h light/12-h dark cycle. Ad libitum access to standard rat chow and tap water was allowed. Approval for the study was granted by the Karadeniz Technical University Faculty of Medicine Experimental Animal Ethical Committee. All Animal experiments and procedures were conducted in compliance with the U.S. National Institutes of Health Guide for the Care and Use of Laboratory Animals. On the evening of the first day, those of the 14 female rats exhibiting two regular cycles were placed in the same cages as male rats for mating purposes. The vaginal smear test was used on the following day to determine pregnancy. Rats whose smear specimens contained sperm were regarded as pregnant. That day was taken as day 0 of pregnancy. Six rats were identified as pregnant. These were then randomly divided into two groups containing three rats each. No procedures were performed on the first, control group (CG) during pregnancy. The other three rats were adopted as the EMF group (EMFG). These were placed inside a Plexiglas jar for 1 h at the same time every day, between days 13 and 21 of pregnancy, where they were exposed to EMF of 900-MHz. Apart from the application of EMF, no procedure was performed on the rats during the hour they spent in the Plexiglas jar, and they were allowed to move around freely.

Pregnant rats were placed in individual cages before giving birth. After birth, pups were allowed to feed naturally with their own mothers in the same cages. The newborn rats were not subjected to any procedure after birth. Thirteen female rat pups were obtained from the control group mother rats and 11 from the experimental group rats. The study proceeded with 24 female rat pups. Female pups obtained from control group mother rats were classified as the newborn control group (NCG) and female pups from EMF group mothers as the newborn EMF group (NEMFG). Female rat pups were kept in the same cages as their mothers until the 22nd day after birth, and were allowed to feed naturally with no procedure being performed. On the postnatal 22nd day, they were removed from their mothers and placed in a separate cage. The rat pups were kept for 4 days under the same laboratory conditions, with access to standard rat chow and tap water, in order for anxiety caused by separation from their mothers to subside (Savignac *et al*., 2011). One rat pup from the NEMFG died during this time. The study thus continued with a total of 23 newborn female rats, 13 in NCG and 10 in NEMFG. When the rats were 26 days old, learning and memory tests began. The eight arm radial maze test was applied to test rats' spatial memory (Hao *et al*., 2013), the passive avoidance test in order to investigate passive avoidance behavior (Yildirim and Marangoz, 2004) and the open field test to examine locomotor efficacy. The study was concluded on the 32nd day postnatally.

EMF exposure system

A special EMF exposure room was prepared in the KTUSRC, compatible with normal laboratory conditions but containing only the EMF exposure system, solely for the application of 900 MHz EMF to NEMFG. NEMFG rats were only placed in this room during EMF application. Apart from during exposure to the 900 MHz EMF, the groups were kept in different cages but in the same room. EMFG rats were separated from CG animals for EMF exposure, which took place in the EMF exposure room. The EMF exposure system consisted of an ultra-high-frequency oscillator (1218-BV, Lockable Oscillator, 900–2000 MHz, General Radio Company, Concord, Massachusetts, USA, Serial No. 1483) with a constant power source (1267-B Regulated Power Supply, General Radio Company, Concord Massachusetts, USA, Serial No. 903) (with output power of approximately 300 mW and a frequency adjusted to 900-MHz) and a Plexiglas jar specifically produced for the study (30 cm X 42 cm X 52 cm). The oscillator was connected to a half-wave dipole antenna made from a 1 mm x 15 cm copper rod by means of a coaxial cable. The antenna was inserted into the central area of the jar, approximately 11 cm inside the open surface of a glass jar (Hancı *et al*., 2013). EMFG pregnant rats were placed inside the jar and exposed to 900-MHz EMF for 1 h (at the same time each day). Positional averaging of electrical field intensity was calculated using a wide-range measuring device with a measurement range of 100 kHz-2.5 GHz (Chauvin Arnoux CA43 Isotropic Electrical Field Intensity Meter).

Radial Arm Maze Test

The eight arm radial maze apparatus was made from wood. It was 50 cm from the ground, with dimensions of 50 x 12 x 24 cm and consisted of eight arms of the same size and a central area 40 cm in diameter at the junction of the arms. At the beginning of each arm was a rubberized flap, with a chamber containing chow at each end (Figure 1A). Before the experiment began, clues were sited around to make it easier for rats to learn their surroundings. The experiment was performed over three days, in three stages, habituation, acquisition and testing. In the habituation stage, two rats were placed in the equipment at the same time. Rats were allowed 10 min to familiarize themselves with and become accustomed to the apparatus and were able move around freely within the maze. The rats were then fasted for 24 h. The rats were placed in the apparatus 24 h later for the acquisition stage. All arms were closed, apart from one. Chow was placed in the open arm and rats were placed in the apparatus one by one. Rats were kept in the device for 10 min. The test itself was performed on the third day. In the test stage, all the arms were kept open. Chow was placed in the same arm in which it had been placed on the second day. Rats entered the apparatus from the same location. Time to entering the arm containing the chow and number of times arms with no chow were entered (error numbers) were recorded (Hao *et al*., 2013). All data were recorded on camera. After each procedure, the entire apparatus was wiped down with 30 % alcohol to eliminate scent clues.

Passive Avoidance Test

The passive avoidance test apparatus consisted of two parts, light and dark areas. The light section was 20 x 10 x 10 cm in size and the dark section 20 x 20 x 20 cm. A rubberized flap 5 x 7 cm divided the two sections. The light section was made of transparent material and was lit with a 60 W light source located 60 cm above the floor of the apparatus. The dark section was made out of wood painted black and contains a grill made out of stainless steel 2 x 3 cm in size on the floor. The grill was connected via a cable to an electroshock device capable of supplying 1.5 mA current (Figure 1B) (Yildirim *et al.*, 2013).

In the passive avoidance test, the aim was to produce a sensation of fear through administration of electroshock and for rats to learn and remember what they learned. The test was therefore performed in two days. On the first day, acquisition of passive avoidance behaviors (learning acquisition) and acquisition latencies were measured. For that purpose, the rats were first placed in the light area of the apparatus with the dark section closed. The rubberized flap to the dark section was opened 5 s later. We waited for rats to enter the dark area and lengths of time to entry were recorded. When rats entered the dark section, the flap was closed and a 1.5 mA electroshock was administered for 1.5 s. Rats were removed from the dark area and replaced in the light section, and the flap to the dark area was opened again. Rats were allowed 120 s to enter the dark section. Those that did not enter were regarded as successful. The procedure was repeated with those that did enter, and length of time to entry was recorded. The recall test was performed 24 h later. Rats were placed in the light section in the same apparatus. The rubberized flap of the dark area was opened. Lengths of time from passage from the light to the dark area (avoidance latencies) were recorded. The test was concluded for those rats that did not enter the dark area within 300 s, and the avoidance latency was recorded as 300 s. Lengths of time to entry for those rats entering earlier were recorded as avoidance latencies (Yildirim and Marangoz, 2004; Yildirim *et al.*, 2013).

Histological procedures and histopathological examination

At the end of the study period (postnatal 32st day) (at the end of the learning, memory and passive avoidance tests), all newborn female rats were sacrificed on the same day by decapitation under deep anesthesia (Ketalar 50 mg/kg). The brains were extracted and placed in 10 % formaldehyde. After being kept in formaldehyde for 1 week, brains were placed under running tap water overnight. Brains were fixed in paraffin for routine histological tissue examinations. Brains embedded in paraffin were sliced into 30-micron sections with the aid of a microtome (Leica RM 2255, Leica Instruments, Nussloch, Germany) and stained with Cresyl violet. A research light microscope (Olympus, BX51, Japan) was used for histopathological examination of the stained sections, and images were produced using an Olympus DP 71 (Japan) camera microscope.

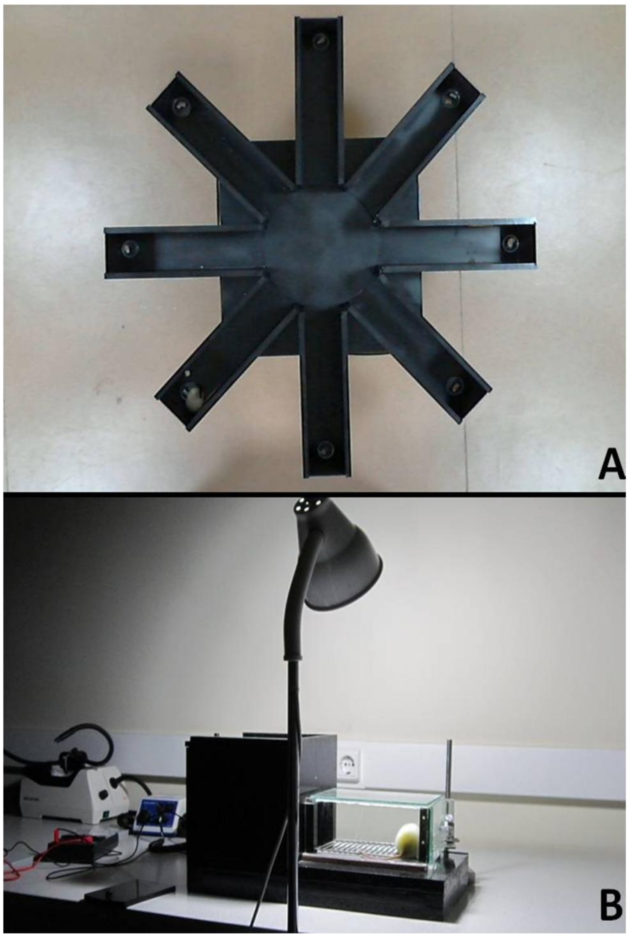

Figure 1. Radial arm maze test (A) and passive avoidance test apparatus (B).

Statistical analyses

The nonparametric Mann–Whitney U test (Tunc *et al.*, 2007) was used to compare results between the groups. Mean values are presented with their standard error means (SEM). Significance was set at $p<0.05$. All statistical analyses were performed on SPSS software (Statistical Package for the Social Sciences, version 15.0, SSPS Inc., Chicago, IL, USA).

Results and Discussion

Behavioral test results

Animals' eight arm radial maze test performances were assessed under two parameters; latency for finding the arm with the chow placed in it beforehand (correct arm selection), and number of incorrect choices before finding the right arm. Mean latency for finding the arm with chow left in it was 18±4 s in NCG and 63±27 s in NEMFG. Mean number of incorrect choices was 2.2±0.5 in NCG and 4.6±1.3 in NEMFG. Latency for finding the arm with chow left in it beforehand was significantly higher in NEMFG (p=0.007). Number of incorrect choices was also higher in NEMFG compared to NCG, though the difference was not statistically significant (p=0.086) (Figure 2).

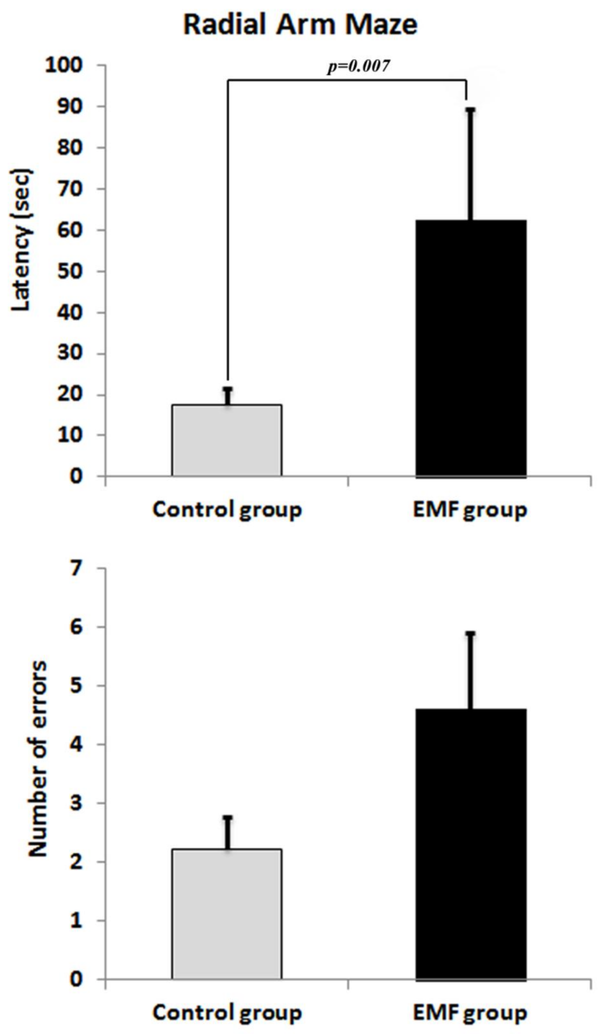

Figure 2. Radial arm maze test results from the newborn control and newborn electromagnetic field (EMF) groups. Statistical analysis revealed that newborn EMF group rats had a significantly higher latency in terms of finding the maze arm containing food (p=0.007), while the number of false selections was not significant (p=0.086).

In the passive avoidance test, mean acquisition latency before application of electric shock was 12±4 s for NCG and 12±3 s for NEMFG. Statistical analysis revealed no significant difference between the groups. Mean avoidance latency, recorded on the second day of the test and regarded as the primary finding in the learning of passive avoidance behavior, was 262±26 s in NCG and 151±48 s in NEMFG. Avoidance latency was significantly lower in NEMFG compared to NCG (p=0.032) (Figure 3).

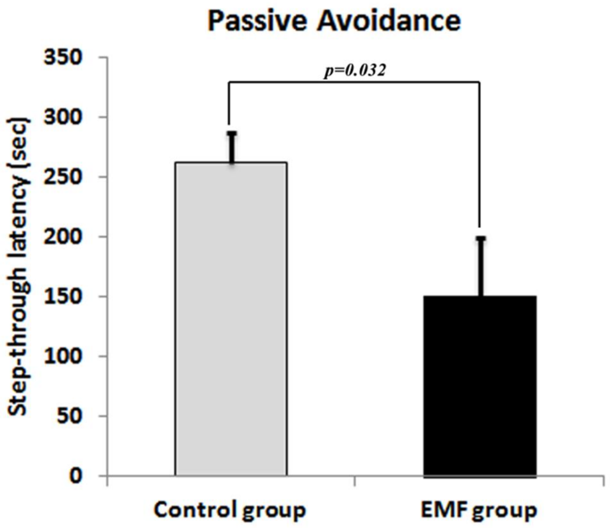

Figure 3. Passive avoidance test results from the newborn control and newborn electromagnetic field (EMF) groups. Statistical analysis revealed significantly lower avoidance latency in the newborn EMF group rats than in newborn control group (p=0.032).

Physical examination and histopathological observation

Histopathological examination of the cornu ammonis sections revealed no pathology in the NCG (Figure 4 A, B and C). However, both neuronal and morphological compromise was determined in the NEMFG (Figure 4 D, E and F). Physical examination revealed no skeletal anomalies or unexpected findings in either group.

Controversy still surrounds the potential side-effects of EMFs emitted by mobile phones on the human central nervous system (Hietanen, 2006; Corle *et al.*, 2012). However, effects have been reported in animal brain tissue morphology and physiological activities

(Salford *et al.*, 2003; Odaci *et al.*, 2008; Bas *et al.*, 2009a; 2009b; Maskey *et al.*, 2010). One particularly controversial suggestion is that the use of mobile phones may be able to contribute to malignant pathologies such as brain tumors (Hardell *et al.*, 1999; 2006; 2007) and developmental anomalies (Bas *et al.*, 2009a; 2009b; Sonmez *et al.*, 2010). We therefore think that this study will represent a significant contribution to the literature. Our study examined the cognitive functions of 32-day-old female rats exposed during the prenatal term to the effect of EMF emitted by mobile phones. Behavioral tests were performed and the hippocampuses were assessed histopathologically. Negative effects on learning and memory were determined in the NEMFG rats in the radial arm maze and passive avoidance tests. Additionally, neuronal and morphological compromise was observed in the EMF group. While both the behavioral and morphological results bear similarities to studies by some previous authors, they conflict with those reported by others.

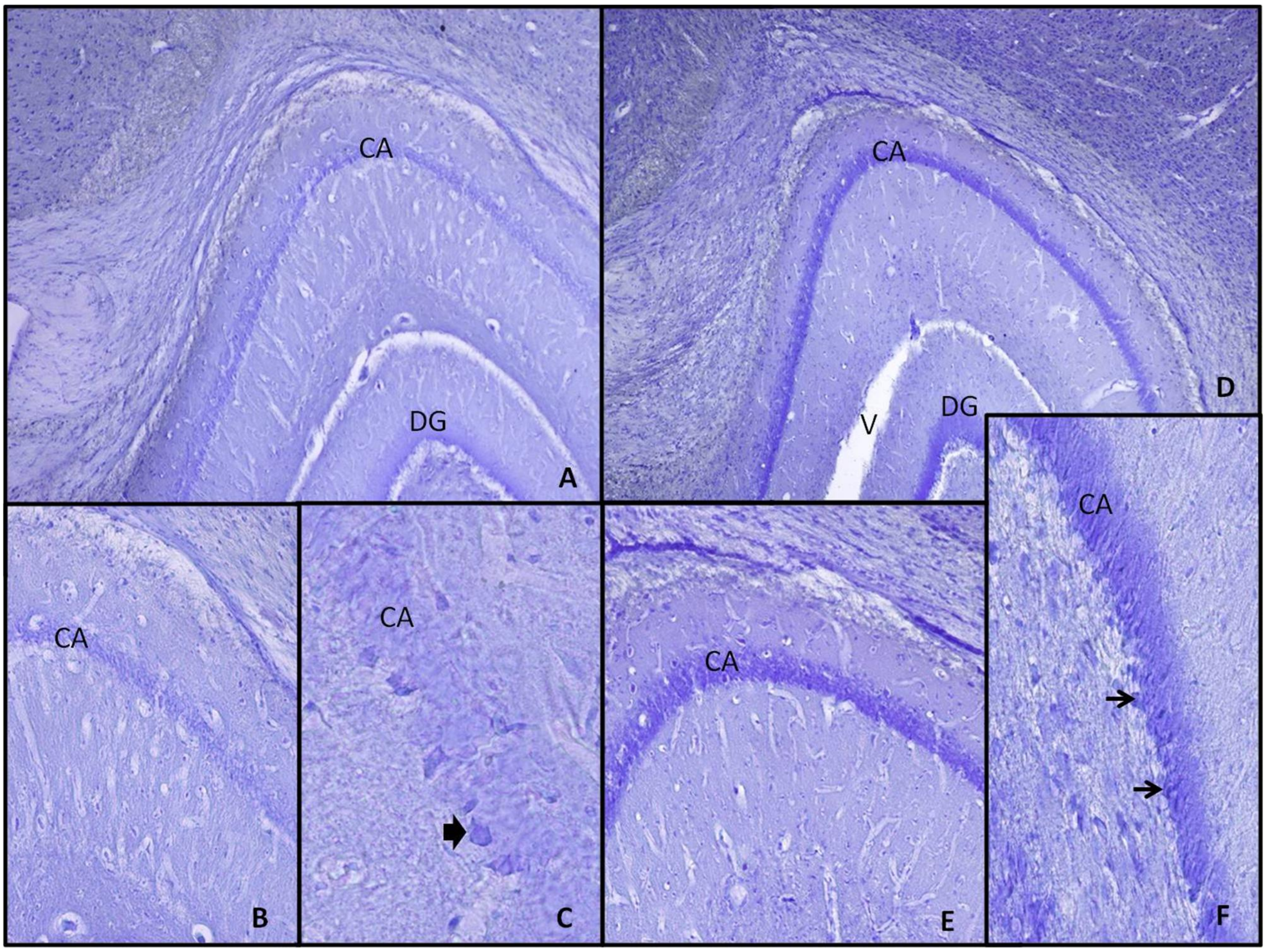

Figure 4. Histological views of the NCG (A, B and C), and NEMFG (D, E and F) groups. No distinct morphological difference can be seen between the groups in images A and B for NCG, and D and E for NCG. Morphological differences in pyramidal cells can be seen between the groups in the magnified equivalent views (image C for NCG and F for NCG). As described in the results section, prenatal exposure to EMF led to compromise of the pyramidal cell in the NEMFG cornu ammonis (arrow) (F) compared with NCG (arrow head) (C). NCG, newborn control group; NEMFG, newborn electromagnetic group; EMF, electromagnetic field; CA, cornu ammonis; DG, dentate gyrus; V, ventricle, (Cresyl fast violet staining), (A and D, X 40; B and E, X 100; C and F, X 200).

Contradictory results have been reported in studies examining the effect on cognitive functions of the 900 MHz EMF emitted by mobile phones. In one study, rats exposed subchronically (SAR=1.5 W/kg, 45 min/day, 8 weeks) and chronically (SAR=6 W/kg, 15 min/day, 24 weeks) to 900 MHz EMF from the head only were tested in a radial arm maze apparatus. Neither application affected spatial memory (Ammari *et al.*, 2008). The effect of EMF (900 MHz, 1 and 3.5 W/kg, 45 min) applied to the head only in rats was examined using classic radial maze and open field tests; no difference was determined between the experimental and control groups in terms of cognitive performance (Dubreuil *et al.*, 2002). In another study, adult male Wistar rats were exposed to a GSM mobile telephone (900/1800 MHz) in vibration mode for 3 weeks (50 unanswered calls per day). The Morris water maze test was used in that study, and examination of rats' spatial memory performances revealed that exposure to mobile phone resulted in memory compromise (Narayanan *et al.*, 2009). In another study rats were exposed to 900 MHz EMF (0.6 and 60 mW/kg) for 2 h every week for 55 weeks. No difference was determined in terms of inquisitive behavior in the open field test, but memory was impaired in rats exposed to EMF in the episodic-like memory test (Nittby *et al.*, 2008). In our study, too, learning and memory performances of rats exposed to 900 MHz on prenatal days 13-21, every day for 1 h per day, was seen to be compromised in the passive avoidance and radial arm maze tests.

In one study in which Wistar rats were exposed to low level EMF (17.5-75 mW/kg) during pregnancy in order to investigate brain development and the effect of EMF emitted by mobile phones, prenatal exposure to EMF resulted in no loss of cognitive performance (Bornhausen and Scheingraber, 2000). In our study, however, prenatal exposure compromised both spatial memory and learning. We think that the contradictory findings from the two studies may be attributed to methodological differences involving intensity, duration and frequency of EMF application. On the other hand, another study applied mobile phone signals to young male Wistar rats for 5 weeks (2 h/day, 5 days/week) (mean SAR value=0.3 or 3.0 W/kg). Behavioral analysis using the open field test, plus maze test and acoustic startle response test determined no significant difference between the groups. Interestingly, however, learning and memory increased significantly in the water maze test in rats exposed to EMF (Kumlin *et al.*, 2007). On the basis of the findings, that study Kumlin et al., (2007) concluded that mobile phone radiation at the level to which humans are exposed does not represent a threat to health. Additionally, no degenerative changes involving neuron death and permeability of the blood-brain barrier were observed at morphological examination in that study.

However, Kumlin et al.'s (2007) study involves significant differences from both our and other studies on the subject. Our histopathological analysis revealed both neuronal and morphological compromise in the EMF group. Several previous studies have also reported neuronal damage in the cortex, cerebellum, hippocampus, and basal ganglia in animals exposed to 900 MHz EMF (Mausset *et al.*, 2001; 2004; Salford *et al.*, 2003). For example, one study reported that 900 MHz EMF can induce cell death, and that this can inhibit differentiation of neuronal stem cells into neurons in the embryonic period (Salford *et al.*, 2003). These studies naturally strengthen the probability that the effect of the EMF emitted by mobile phones can create irreversible health problems, such as brain tumor, in humans.

It has been suggested, at examination of studies of the relation between mobile phone use and brain cancer, that this probability, if it exists, may be greater in children and young people (Christ *et al.*, 2010). One of the main reasons for this is that children and young people start using mobile phones for communication at early ages, even though other communication tools are available (Lenhart *et al.*, 2010). They will therefore be exposed to greater effects of EMF during their lives. Second, the skull and structures inside the skull have not yet completed their morphological development in childhood. For example, the skull bone has not yet attained the thickness it has in adulthood, and comparing an adult and a child, the EMF emitted by mobile phones can reach the brain and structures inside the brain much easier through the child skull (Christ *et al.*, 2010). Another factor is that children and young people are more interested in mobile phones than adults. Lenhart et al., (2010) reported that more than four out of five children/teenagers aged 12 or more in America sleep with a cell phone beside them, or often under the pillow. They also reported that one in three teenagers sends more than 100 text messages a day, or 3000 texts a month. Such calls represent a central function of mobile phones for teenagers. Additionally, voice is the primary mode of communicating with parents for many teenagers (Lenhart *et al.*, 2010). Besides, Christ et al., (2010) compared the SAR in various regions of the brain cortex for various MRI-based head phantoms in adults and children exposed to various makes of mobile phone. They reported that children exposed to mobile phone EMF of 1.800 MHz experience greater exposures to such areas of the brain cortical regions, the hippocampus and the hypothalamus, as well as the eye, than adults. They also reported that tissues such as the pineal gland exhibit no increase since the distanced between them and mobile phones are not age-dependent (Christ *et al.*, 2010). Some authors have therefore strongly advocated that a change in brain tumors incidences in younger age groups may ensue as exposure to cell phones reaches long-term status and exceeds 10 years or longer (Christ *et al.*, 2010; Corle *et al.*, 2012).

The results of an experimental animal study cannot be applied directly to humans. In the same way, developmental animal studies cannot be directly compared with studies involving human development. However, animal study results should be compared with those from the developmental stage in humans, irrespective of whether testing takes place during the fetal, prenatal, or postnatal periods (Rodier, 1980; Jacobson, 1991; Odaci *et al.*, 2004). For example, the development of the rat hippocampus in the prenatal period can be compared with development of the hippocampus in the third trimester in humans (Dobbing, 1970; Dobbing and Sands, 1973; Rodier, 1980; Jacobson, 1991). Therefore, the results of this and previous studies of ours (Odaci *et al.*, 2008; Bas *et al.*, 2009b) may be interpreted as meaning that long-term (1 h per day) mobile phone use in pregnancy can damage the development of the hippocampus in the human fetus. Since girls use mobile phones more in general in comparison with males (Lenhart *et al.*, 2010), we used female rats in the present study. It would therefore be more accurate to interpret our study results as meaning that mobile phone use in pregnancy may affect hippocampus development in the female fetus. Since most mobile phones operate at a frequency of 900 MHz in Europe (Koyu *et al.*, 2005; Panagopoulos *et al.*, 2007), we used 900 MHz EMF in our studies.

Conclusion and outlook

The purpose of this study was to investigate how the effects of the 900 MHz EMF emitted by mobile phones on the development of nervous system structures in the prenatal period might affect both behavior and hippocampus morphology in the postnatal period. The literature on the subject contains different findings. We encountered no studies assessing the behavior and hippocampus development of female rat pups exposed to the effect of 900 MHz EMF on days 13-21 of pregnancy. We therefore think that this study can add a new dimension to the debate. Our results show that exposure to a 900 MHz EMF in the prenatal period had an adverse effect on female pups' learning behavior and also caused histopathological changes in the hippocampus. The fact that rats' learning behavior was affected, in addition to the histopathological changes seen in the hippocampus, suggests that there may be an association between pathological changes and behavior. However, further electromicroscopic, immunohistochemical and autoradiographic studies are needed in order to reveal this relationship. It should also be borne in mind when planning these studies that our results involve female rats.

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