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Research Report

Effects of prenatal exposure to a 900 MHz electromagnetic field on the dentate gyrus of rats: a stereological and histopathological study☆

Ersan Odaci[a,*], *Orhan Bas*[b], *Suleyman Kaplan*[c]

[a]*Department of Histology and Embryology, Karadeniz Technical University School of Medicine, Trabzon, Turkey*
[b]*Department of Anatomy, Afyon Kocatepe University School of Medicine, Afyonkarahisar, Turkey*
[c]*Department of Histology and Embryology, Ondokuz Mayis University School of Medicine, Samsun, Turkey*

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ABSTRACT

Electromagnetic fields (EMFs) inhibit the formation and differentiation of neural stem cells during embryonic development. In this study, the effects of prenatal exposure to EMF on the number of granule cells in the dentate gyrus of 4-week-old rats were investigated. This experiment used a control (Cont) group and an EMF exposed (EMF) group (three pregnant rats each group). The EMF group consisted of six offspring ($n=6$) of pregnant rats that were exposed to an EMF of up to 900 megahertz (MHz) for 60 min/day between the first and last days of gestation. The control group consisted of five offspring ($n=5$) of pregnant rats that were not treated at all. The offspring were sacrificed when they were 4 weeks old. The numbers of granule cells in the dentate gyrus were analyzed using the optical fractionator technique. The results showed that prenatal EMF exposure caused a decrease in the number of granule cells in the dentate gyrus of the rats ($P<0.01$). This suggests that prenatal exposure to a 900 MHz EMF affects the development of the dentate gyrus granule cells in the rat hippocampus. Cell loss might be caused by an inhibition of granule cell neurogenesis in the dentate gyrus.

1. Introduction

Neurons in most regions of the brain are formed during gestation and the process of neurogenesis is completed before birth (Guidi et al., 2005). Because of this developmental pattern, the adult brain is unable to replace nerve cells lost as a result of aging or pathological conditions, except for the dentate gyrus (DG) in the hippocampus and the subventricular zone of the lateral ventricle in several mammals' brains (Contestabile, 2002; Guidi et al., 2005). In these regions, neurogenesis begins during gestation, continues during the early postnatal period and, at a slower rate, through into adulthood. This is true for all species, including humans (Eriksson et al., 1998; Snyder et al., 2001; Magavi and Macklis, 2002; Guidi et al., 2005).The principle neuron type of the DG is granule cells, the production of which begins in the prenatal period and continues throughout postnatal life. However, many of these cells are formed by the third-week after birth (Rodier, 1980). Therefore, deleterious events during gestation may induce neurobiological or behavioral defects in offspring, including hippocampal formation, because this region is vulnerable to disruptive events (Lemaire et al., 2000).

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* *Corresponding author*. Fax: +90 462 325 2270.
E-mail address: eodaci@yahoo.com (E. Odaci).

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Electromagnetic fields (EMFs) can influence neuronal functions, including regulation of synaptic plasticity, neurotransmitter release, neuronal survival, learning and memory (Sakatani et al., 2002; Manikonda et al., 2007). It also induces cell death and inhibits the differentiation of neural stem cells into neurons during embryonic development (Salford et al., 2003). Therefore, it is claimed that prenatal exposure to EMFs could disturb granule cell neurogenesis in the DG, resulting in disturbed postnatal behavioral and cognitive functions related to the hippocampus (Hocking, 1998; Mausset et al., 2001). In the presented study, the effect of prenatal exposure to a 900 megahertz (MHz) EMF on the amount of granule cells in the DG of 4-week-old (4W-old) rats was investigated using the optical fractionator technique. Additionally, sections of the DGs obtained from both the control and experimental groups were histopathologically examined.

Table 1 – Mean values of total granule cell numbers, CV and CE of stereological analysis, mean dissector number, section thickness and number of steps for estimation of total neuron number in the DG of Cont and EMF groups of 4W-old rats

	Cont Group ($n=5$)	EMF Group ($n=6$)
Total granule cell number[a]	1,235,702±21,731	994,188±21,772[b]
CE	0.05	0.04
CV	0.04	0.05
Dissector particle number	428	351
Section thickness (μm)	28.72	28.11
Number of steps for counting	178	175
Number of sampled sections	14.6	15

[a] Values are as mean±SEM. DG, dentate gyrus; Cont, control group; EMF, electromagnetic field exposed group; MHz, megahertz; CE, coefficient of error; CV, coefficient of variation.
[b] $P<0.01$.

2. Results

2.1. *Histopathological observations*

At the end of the 4th week, the histological appearance of the Cont and EMF groups' DGs were examined. The results are shown in Fig. 1. At the light microscopic level, the morphology of the granule cells was normal in the control rats. However, in the depths of the EMF group's DGs, darkly stained neurons were easily seen among the normal granule cells (Fig. 1).

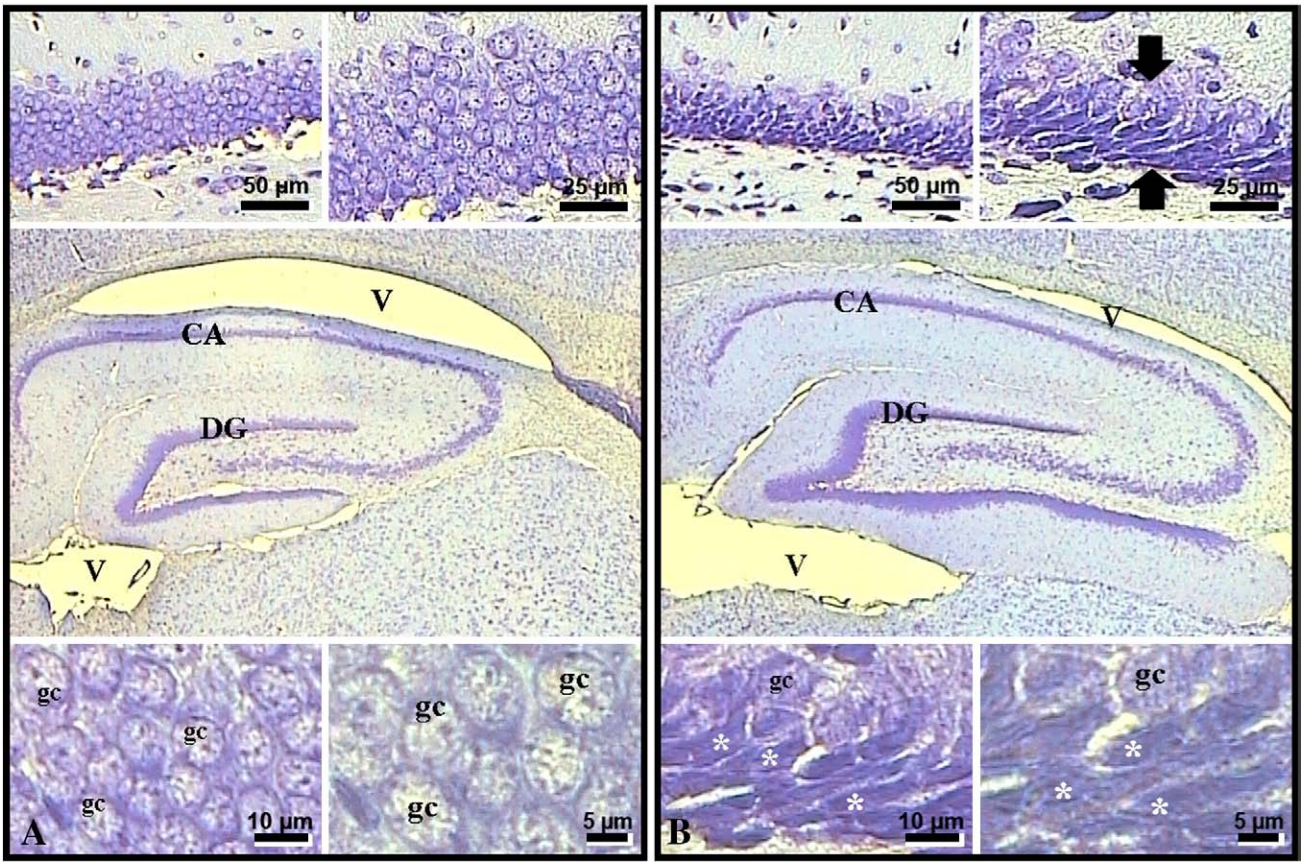

Fig. 1 – Representative photomicrographs of DGs belong to the Cont (A) and EMF (B) groups. Although granular cells of DG in the Cont group were normal in structures, but most of granular cells in the medial region of DG in the EMF group were condensed seen as dark-blue cells are interspersed among the normal nerve cells (*). Upper and bottom rows of each plate (A and B) show magnified the same area of DG. Arrows point to condensed cells. DG, dentate gyrus; Cont, control group; EMF, electromagnetic field group; V, ventricle; CA, cornu ammonis; DG, dentate gyrus; gc, granular cell; Cresyl fast violet staining.

2.2. *Granule cell numbers in the dentate gyrus*

Granule cell numbers in the DG were estimated in the Cont and EMF groups. The total number of granule cells in the EMF group was significantly lower than in the Cont group ($P<0.01$). Substantial granule cell loss was seen in the DG sections of the EMF group (Fig. 1). The coefficient of variation (CV) and the coefficient of error (CE) of the estimated number of granule cells for the Cont and EMF groups are shown in Table 1. These values were within acceptable ranges.

2.3. *Weight of pups*

There was no any statistical significant finding between EMF and Cont groups' weights, obtained at postnatal day 1 ($P>0.01$).

3. Discussion

The hippocampus is a part of the brain that controls some behavioral and cognitive functions, including spatial learning and working memory (Eichenbaum et al., 1992; McEwen, 1994; Lemaire et al., 2000; Miki et al., 2000; Eyre et al., 2003; Gokcimen et al., 2007). The production and plasticity of new neurons in the DG may have an important role in hippocampal functions, since the DG is the major source of afferent inputs into the hippocampus (Snyder et al., 2001). Although production of DG granule cells begins prenatally, they are formed at a high rate during the first twenty days after birth (Rodier, 1980; Guidi et al., 2005). Deleterious environmental conditions occurring prenatally affect profoundly neurogenesis in the brain. Such conditions may also induce neurobiological or behavioral defects in the offspring (Lemaire et al., 2000). We know that exposing rats to prenatal stress not only results in decreased granule cell numbers (Schmitz et al. 2002) but also in loss of DG neurogenesis. Consequently, hippocampal-related spatial tasks are impaired and learning-induced neurogenesis is blocked (Lemaire et al., 2000).

To date, there have been few studies that use high-precision design-based stereologic techniques to investigate the effect of EMFs on prenatal DG development. For this reason, we investigated whether prenatal exposure to a 900 MHz EMF during gestation affects the total number of DG granule cells in 4W-old rats. In this study, pregnant rats were exposed to a 900 MHz EMF. A 900 MHz EMF was chosen because most mobile phones in Europe generally work at a frequency of 900 MHz (in accordance with the Global System for Mobile communications, the most popular standard for mobile phones in the world) (Dubreuil et al., 2002, 2003; Koyu et al., 2005; Panagopoulos et al., 2007). Our results show that prenatal exposure to a 900 MHz EMF caused a progressive postnatal decline in the number of DG granule cells. This may be explained by the suggestion that 900 MHz EMF induces cell death and inhibits the differentiation of neural stem cells into neurons during the embryonic development (Salford et al., 2003), because it is well known that the production of rat DG granule cells begins from the prenatal period and continues in the postnatal life (Rodier, 1980). Histopathological observation showed an increased number of granule cells with dark-blue neurons in the EMF group of rats and the stereological results agreed with previously mentioned findings. Another study found that an acute exposure to GSM 900 MHz microwaves induces modifications in the rat brain, both at the molecular and cellular levels (Mausset-Bonnefont et al., 2004). Mausset–Bonnefont observed significant effects not only on the cortex (the superficial part of the brain) but also in deeper structures (striatum, hippocampus). Substantial neuronal loss after exposure to EMFs was also found in the cortex, hippocampus, and basal ganglia of the rat brain (Salford et al., 2003). Additionally, a study reported that perturbed neuronal functions may be caused by exposure to low frequency magnetic fields, since such exposure may alter Ca^{2+}signaling events, contributing to aberrant NMDA receptor activity in the hippocampus (Manikonda et al., 2007).

In the light of the literature and based on our observations, we would suggest that intrauterine exposure to EMFs during the critical period of organogenesis may damage normal development of the rat hippocampus and may also induce neurodevelopmental retardation. It is known that results of animal studies could not be directly translated to human development. However, if one wants to make a comment from that result for human, he/she should compare with that of the same stage of development in the human, regardless of whether it is tested during the fetal, prenatal or postnatal periods (Rodier, 1980; Jacobson, 1991; Odaci et al., 2004). For instance, the neonatal period of DG development in the rat corresponds to the third trimester of DG development in humans (Dobbing, 1970; Dobbing and Sands, 1973; Rodier, 1980; Jacobson, 1991).

In respect of the body weight of offspring we did not find a significant difference between two groups as previously reported by Berman and coworkers (Berman and Carter, 1984; Berman et al., 1984, 1985).

In conclusion, the results presented here show for the first time a cell loss in the DG due to prenatal EMF exposure using state art of stereological methods. This cell loss can be seen as a result of chronic prenatal exposure of the rat DG granule cells to EMFs. This may encourage researchers to evaluate the human cerebrum. However, further developmental studies are required to confirm this and to refine EMF administration in studying the development of granule cells in the DG.

4. Experimental procedures

4.1. *Animals*

Male and female Wistar albino rats weighing between 250–280 g, were used in this study. Rats were obtained from the Experiment Animals Research and Application Center of Afyon Kocatepe University. Firstly, male and female rats were housed separately in plastic cages where they rested for two days. During the experiments, rats were kept on a 12:12-h day/night cycle in a temperature-controlled animal room (22±1 °C) in the laboratory, and were allowed access to food and water *ad libitum*. After two days, they were mated overnight and then separated when a vaginal plug was found, the female rats were accepted as pregnant and the day was

designated as gestational day (GD) 1. There were six pregnant female rats that were housed in individual cages during the gestation stage. Pregnant rats were randomly divided into two equal groups (three pregnant rats each group), the Cont and EMF groups. The Cont group pregnant rats were kept under the same laboratory conditions as the EMF group of pregnant rats, but were not subjected to stress or EMFs. The EMF group was exposed to a 900 MHz EMF during gestation, as described below.

4.2. *Electromagnetic field exposure system*

A special device consisting of a round plastic tube cage (diameter: 5.5 cm, length: 12 cm) and a dipole exposure antenna (Koyu et al., 2005; Yildiz et al., 2006), was used in this study. A 900 MHz continuous modulated wave electromagnetic energy generator [the peak specific absorption rate (SAR) was 2 W/kg, average power density 1±0.4 mW/cm^2] was manufactured at the Electromagnetic Compatibility Laboratory of Suleyman Demirel University (Koyu et al., 2005; Ozguner et al., 2005; Yildiz et al., 2006). The Peak SAR value was obtained by model calculations. An EMF meter was used for the power density measurements (Holaday Industry Inc., Adapazarı, Turkey).

4.3. *Exposing pregnant rats to the electromagnetic field*

To expose pregnant rats to the EMF, entire rats were positioned in close contact above the dipole antenna. The tube was ventilated while the rats were inside, in order to decrease stress (Koyu et al., 2005; Ozguner et al., 2005; Yildiz et al., 2006). They were exposed for 60 min/day to a 900 MHz EMF between GD 1 and the end of gestation. The exposure period was from 13:30–14:30 a.m. each day. The rats were positioned with a distance 1 cm each other and perpendicularly dipole antenna inside the tube. Their heads were positioned in the direction of antenna. Antenna was fixed with 1 cm distance around the rats' heads. The long axis of the antenna was the perpendicular to the long axis of the rats in order to all rats equally was exposed to EMF between two ends of antenna. Everyday, their situations were changed among them during the exposure period.

4.4. *Perfusion and fixation*

The 14 pups were obtained from control pregnant rats (4, 5 and 5 pups from Cont 1, 2 and 3 pregnant rats, respectively) and the 15 pups were obtained EMF exposed pregnant rats (4, 5 and 6 pups from EMF 1, 2 and 3 pregnant rats, respectively) after spontaneously delivery. They were weight postnatal day 1 and fed for 4 weeks. At the end of the 4th week, totally 11 pups were randomly selected for experimentation, without regard to their sex. They were divided into the Cont group (n=5), obtained randomly from the control group of pregnant rats, and the EMF group (n=6), obtained randomly from the EMF group of pregnant rats. They were anaesthetized intraperitoneally with urethane (1.25 g/kg) and neutral formalin was perfused intracardially. After that, the brains were processed in graded alcohols and xylene, and embedded in paraffin for sectioning. Paraffin sections of the hippocampus were taken using a rotary microtome (Leica RM 2135, Leica Instruments, Nussloch, Germany). Disposable metal microtome blades (Type N35, Feather Company, Osaka, Japan) with a cutting (knife bevel) angle of about 5° were used to obtain 40-μm thick serial sections in a sagittal plane from the blocked tissues. Each selected sections through the hippocampus was collected on gelatin-formaldehyde coated slides and stained with cresyl fast violet dye (Bancroft et al., 1994). All experiment procedures were conducted according to institutional guidelines. The Animal Ethic Committee of Afyon Kocatepe University approved the protocol and appropriate measures were taken to minimize pain or discomfort.

4.5. *Stereological analysis*

Estimates of total numbers of granule cells in the DG were carried out using the optical fractionator (West et al., 1991; Hausdorf et al., 2008) and a stereological workstation, consisting of a modified light microscope (type BX50; Olympus, Tokyo, Japan), a motorized specimen stage for automatic sampling (Prior, Rockland, MA, USA), an electronic microcator (Heidenhain, Traunreut, Germany), a CCD colour video camera (JVC, Tokyo, Japan), a personal computer with frame grabber board (type FlashPoint 3D, Integral Technologies, Indianapolis, IN, USA), stereology software (CAST; Olympus, Glostrup, Denmark) and a 17″ television screen monitor (Hyundai, South Korea). Analysis was carried out at a final magnification of ×1440 (i.e., by using a 100×Leica HCX Plan Apo objective; NA=1.35).

4.5.1. *The sampling and counting schedule*

The sampling and counting schedule were determined on the basis of an earlier pilot study. The first section in the series to be analyzed was chosen at random from the first 5 sections and every successive 5th section was collected from the series, giving a 1/5 section sampling fraction (*ssf*). About 15–20 sections from each brain were known to be adequate to estimate the total neuron number when using the optical fractionator method for cell counting (Gundersen and Jensen, 1987; West et al., 1991; Tunc et al., 2006; Ragbetli et al., 2007). Collecting each series from the outside of the dentate gyrus, we examined them to find sections that contained dentate gyrus. After finding the first section in the series, section sampling was begun from a random point between 1 and 5, and alternate sections were collected as reserves. The counting frame size was 100 μm^2 and the area sampling fraction (*asf*) was 100 μm^2/596.72 μm^2 were found to be optimal. Dissector height was 10 μm and a 5-μm zone at the uppermost part of the section was excluded from the analysis at every step as the upper guard zone. Therefore, a thickness sampling fraction (*tsf*) of 10 μm/t was used, where t represents the mean section thickness.

4.5.2. *Counting procedure and estimation of coefficients*

The granule cells in the GD were counted if the widest profile of nucleus comes into focus within optic dissector volume. Estimated total numbers of granule cells were calculated from the number of counted granule cells and the sampling probability (Gundersen, 1986). Details of the counting procedure

are summarized in Table 1. The total granule cell number of a GD was estimated using the following formula:

$$N = \sum Q \cdot \frac{1}{ssf} \cdot \frac{1}{asf} \cdot \frac{1}{tsf}$$

where N is the total granule cell number, ΣQ is the total dissector granule cell number, *ssf* is the section sampling fraction, *asf* is the area sampling fraction and *tsf* is the thickness sampling fraction.

The efficiency of sampling for estimation of total neuron number for each individual and each group were checked by estimation of coefficient of error (CE) and coefficient of variation (CV) as previously described, respectively (Gundersen and Jensen, 1987; West et al., 1991; Odaci et al., 2003, 2004; Unal et al., 2004; Tunc et al., 2007). The coefficient of error (CE) of the sampling schedule of the hippocampus was validated from a pilot study, as stated previously (CE should be $\leq$10%). It was also possible to estimate the coefficient of variation (CV) within the hippocampus in each group. This is valuable data to see whether the number of subjects in each group is enough. In this study, the mean CV for each group and the mean CE for stereological estimation of the number of neurons, and other stereological parameters are given in Table 1.

4.6. *Statistical analysis*

The mean cell number of the Cont and EMF groups was compared using the Mann–Whitney *U* test. Results have been expressed as Mean ± SEM. A *P* value less than 0.05 was considered statistically significant. Statistical analyses were performed using SPSS software (Statistical Package for the Social Sciences, version 13.0, SSPS Inc., Chicago, IL, USA).

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