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Research Report

Effect of 835 MHz radiofrequency radiation exposure on calcium binding proteins in the hippocampus of the mouse brain

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ABSTRACT

Worldwide expansion of mobile phones and electromagnetic field (EMF) exposure has raised question of their possible biological effects on the brain and nervous system. Radiofrequency (RF) radiation might alter intracellular signaling pathways through changes in calcium (Ca^{2+}) permeability across cell membranes. Changes in the expression of calcium binding proteins (CaBP) like calbindin D28-k (CB) and calretinin (CR) could indicate impaired Ca^{2+}homeostasis due to EMF exposure. CB and CR expression were measured with immunohistochemistry in the hippocampus of mice after EMF exposure at 835 MHz for different exposure times and absorption rates, 1 h/day for 5 days at a specific absorption rate (SAR) = 1.6 W/kg, 1 h/day for 5 days at SAR = 4.0 W/kg, 5 h/day for 1 day at SAR = 1.6 W/kg, 5 h/day for 1 day at SAR = 4.0 W/kg, daily exposure for 1 month at SAR = 1.6 W/kg. Body weights did not change significantly. CB immunoreactivity (IR) displayed moderate staining of cells in the cornu ammonis (CA) areas and prominently stained granule cells. CR IR revealed prominently stained pyramidal cells with dendrites running perpendicularly in the CA area. Exposure for 1 month produced almost complete loss of pyramidal cells in the CA1 area. CaBP differences could cause changes in cellular Ca^{2+}levels, which could have deleterious effect on normal hippocampal functions concerned with neuronal connectivity and integration.

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1. Introduction

The ubiquity of mobile phones has increased electromagnetic field (EMF) density and makes EMF exposure a growing concern (Dubreuil et al., 2002). EMF may have biological effects, especially on the central nervous system (CNS), given its close vicinity to the brain (Mausset et al., 2001). Although not sufficient to break covalent bonds, the energy level of radiofrequency (RF) radiation can still induce molecular responses, leading to cell proliferation or cell death (Moulder et al., 1999). Animal and human studies have shown EMF effects on neurotransmitters, blood–brain barrier (BBB) permeability, or behavior after exposure to a global system for mobile communication (GSM) (Dubreuil et al., 2002; Brillaud and de Seze, 2006; D'Andrea et al., 2003). Exposure to 900 MHz EMF decreases neuron number and causes neuronal damage in the cortex, cerebellum, hippocampus, and basal ganglia in animals (Mausset et al., 2001; Salford et al., 2003; Brillaud et al., 2007; Ammari et al., 2008).

There are strong correlations between brain tumors and RF radiation in epidemiological studies (Inskip et al., 2001). An increase in tumors ipsilateral to the side of the head with phone use was reported, but this was not substantiated by other studies (Hardell et al., 1999). RF exposure did not affect the incidence, malignancy, volume, multiplicity, latency, or fatality associated with any kind of neurogenic tumors (Zook and Simmens, 2006). Several electrophysiological studies on brain exposure to mobile phone frequency RF radiation show altered cognitive and physiological functions (Hamblin et al., 2004; Hinrichs and Heinze, 2004, Curcio et al., 2005; Huber et al., 2005).

Calcium binding proteins (CaBP), like calbindin D28-k (CB) and calretinin (CR), are important components in the regulation of calcium (Ca^{2+}) homeostasis by buffering intracellular Ca^{2+}(Blaustein, 1988). Ca^{2+}can control critical processes of the nervous system by virtue of its transmembrane flux, mitochondrial transport, and other actions within excitable cells (Fiskum and Lehninger, 1980; Racker, 1980). Weak electromagnetic fields can remove Ca^{2+}ions from cell membranes (Goldsworthy, 2008). Hence, EMF exposure could reduce CaBP expression and impair Ca^{2+}handling. Acute, high-level field exposure shows a detrimental effect from thermal injury. Amplitude modulated (AM) radio frequency fields can cause Ca^{2+}efflux from brain tissue *in vitro* (Blackman et al., 1979), while others have failed to confirm a similar effect of RF radiation on Ca^{2+}in brain tissue (Shelton and Merrit, 1980). These fields can decrease single channel formation and opening, as well as increase the rate of rapid burst like firing (D'Inzeo et al., 1988). AM radio radiation alters Ca^{2+}binding in the membrane, $Na^{+}K^{+}$-ATPase activity (Behari et al., 1998), Ca^{2+}-ATPase activity, cell permeability and central cholinergic activity (Kunjilwar and Behari, 1993).

RF radiation might alter intracellular signaling pathways through changes in ionic distribution and membrane fluidity (Hossmann and Hermann, 2003) or change Ca^{2+}permeability across cell membranes (Adey, 1981). RF could also alter the conformational energy of glycoproteins in the cell membrane to open Ca^{2+}channels (Thomas et al., 2000). These changes could cause pathophysiological changes in the brain such as tumorigenesis, neural degeneration, and cognitive deficits. Therefore, in the present study we investigated whether RF radiation at 835 MHz (CDMA type cellular phone) affected CB and CR in the mouse hippocampus at SAR value of 1.6 W/kg and 4.0 W/kg at different exposure durations.

2. Results

2.1. *Body weight changes*

We exposed mice to 835 MHz RF at two different specific absorption rates (SAR) low energy at different exposure durations, to study the expression of CaBP in the hippocampus. All mice gained weight normally, except Groups D and E (Supplementary data 1). No significant difference was noted between the groups ($p>0.05$).

2.2. *Histopathological observations in the hippocampus*

2.2.1. *Calbindin D28-k immunoreactivity*

CB IR was uniformly distributed in all six groups. The mean number of labeled cells per unit length did not vary significantly between the three types in the CA1 and CA3 area of the hippocampus (data not shown). The signal was weakest in the CA1 area of the sham group. In all groups, CB IR was more prominent in the dentate gyrus compared with all CA areas of the hippocampus (Fig. 1). CB IR was most prominent in the CA1 pyramidal cell layer of group E (Fig. 1). The CA1 and CA3 regions displayed some moderately stained CB neurons, primarily weakly labeled pyramidal cells and scattered multipolar neurons in the stratum pyramidale (Fig. 2). The single neurons in the CA1 area displayed intensely labeled soma with very faint or absent dendritic staining.

All three layers of the dentate gyrus were generally well differentiated by CB staining (Fig. 3). CB IR was more prominent in the granular layer and the polymorphous layer of the dentate gyrus than the molecular layer. Cell bodies and dendrites of granule cells displayed CB IR in the dentate gyrus, whereas mossy fibers could be traced projecting into the CA3 areas (Fig. 3).

2.2.2. *Calretinin immunoreactivity*

CR IR in the hippocampus displayed intense staining CR IR neurons were scattered throughout the CA1 area (Fig. 4). The majority of CR IR neurons in the CA1 area were pyramidal, bipolar, and multipolar neurons, but there were more neurons in the CA3 area. Pyramidal cells with axonal and dendritic arborization could be clearly seen in the stratum pyramidale of the CA1 area (Fig. 5). Their processes branched in three dimensions but often include one long neurite running perpendicularly across the striatum radiatum. Loss of pyramidal cells was observed in the CA1 area of group F (Fig. 5). The inner molecular layer and infragranular layer of the dentate gyrus displayed strong CR IR. CR IR in the inner molecular layer and granular layer highest in group D and lowest in group B (Fig. 6). The neuropil of the inner molecular layer displayed the most intense CR IR. The mossy fibers were weakly stained, whereas projections to the inner molecular layer were strongly stained (Fig. 6).

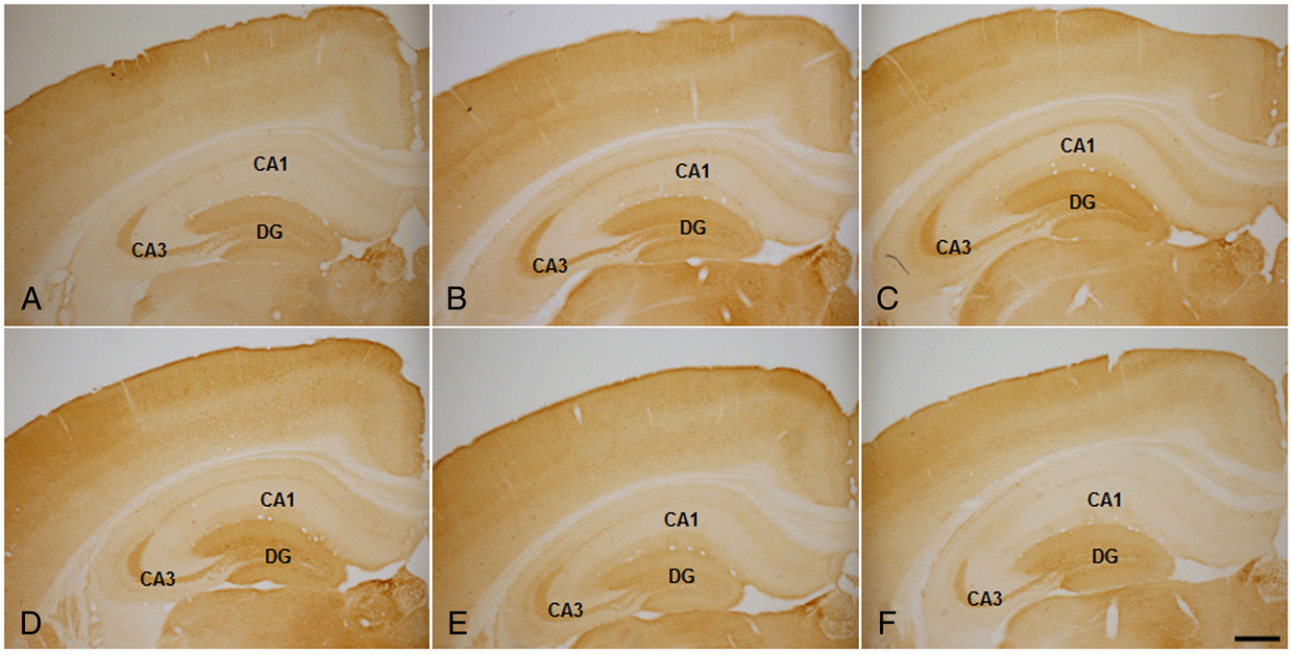

Fig. 1 – Photomicrographs of the Calbindin D28-k (CB) IR in the hippocampus sections belonging to Group A: Control group, Group B: 1 h at low energy for 5 days (1.6 W/kg SAR), Group C: 1 h daily at high energy (4.0 W/kg SAR) for 5 days, Group D: 5 h exposure for 1 day at low energy (1.6 W/kg SAR), Group E: 5 h exposure for 1 day at high energy (4.0 W/kg SAR), Group F: 1 month exposure at low energy (1.6 W/kg SAR). CB staining was uniform in all the groups, with dentate gyrus displaying more intense staining than CA areas. CA, cornu ammonis; DG, dentate gyrus. Scale bar = 100 μm.

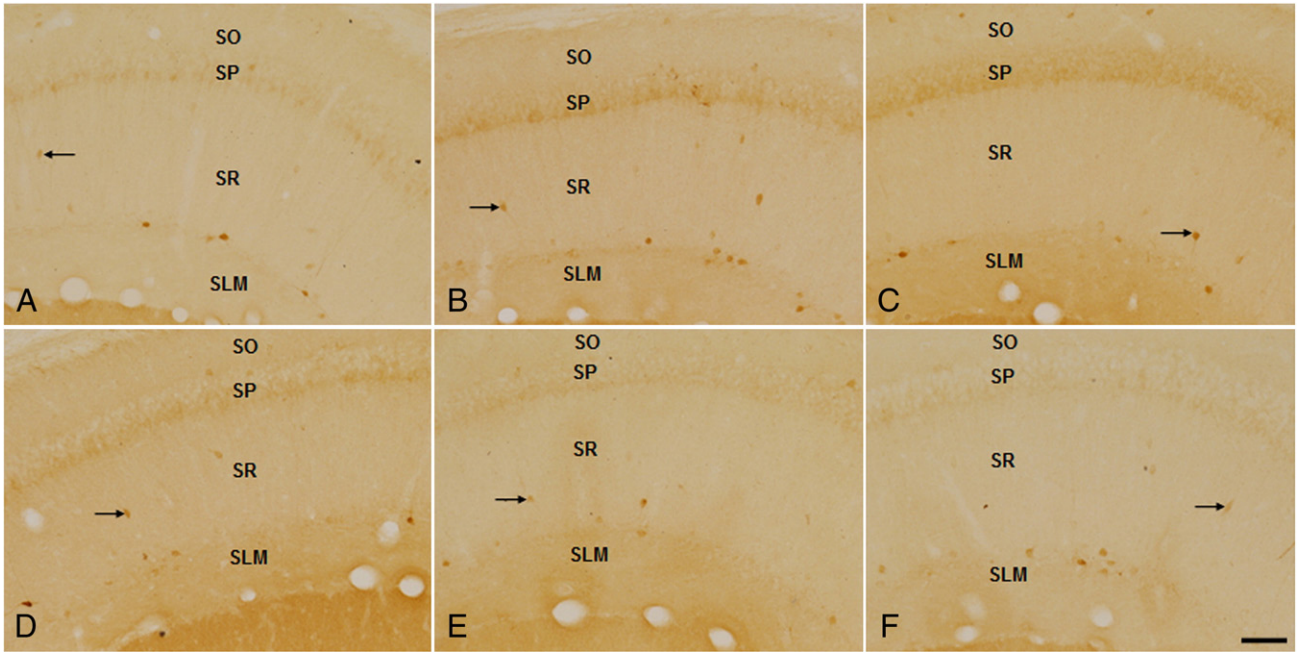

Fig. 2 – Photomicrographs of the Calbindin D28-k (CB) IR in the CA1 area of hippocamp us belonging to A Group A: Control group, Group B: 1 h at low energy for 5 days (1.6 W/kg SAR), Group C: 1 h daily at high energy (4.0 W/kg SAR) for 5 days, Group D: 5 h exposure for 1 day at low energy (1.6 W/kg SAR), Group E: 5 h exposure for 1 day at high energy (4.0 W/kg SAR), Group F: 1 month exposure at low energy (1.6 W/kg SAR). Moderately stained pyramidal cells (arrows) were noted in the CA1 areas of all the six groups. SO, stratum oriens; SP, stratum pyramidale; SR, stratum radiatum; SLM, stratum lacunosum moleculare; SL, stratum lucidum. Scale bar = 50 μm.

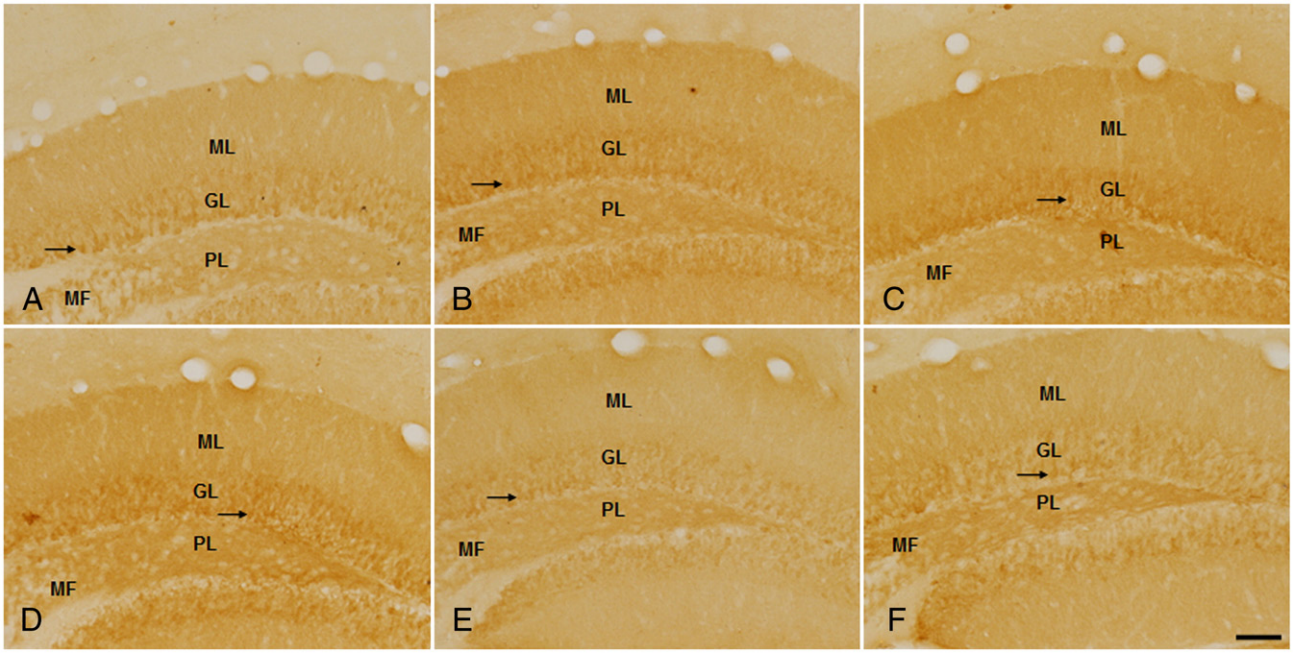

Fig. 3 – Photomicrographs of the Calbindin D28-k (CB) IR in the dentate gyrus belonging to Group A: Control group, Group B: 1 h at low energy for 5 days (1.6 W/kg SAR), Group C: 1 h daily at high energy (4.0 W/kg SAR) for 5 days, Group D: 5 h exposure for 1 day at low energy (1.6 W/kg SAR), Group E: 5 h exposure for 1 day at high energy (4.0 W/kg SAR), Group F: 1 month exposure at low energy (1.6 W/kg SAR). The three layers of the DG were well differentiated with the body and dendrites of the granule cell (arrows) displaying CR IR in the granular layer. ML, molecular layer; GL, granular layer; PL, polymorphous layer. Scale bar = 50 μm.

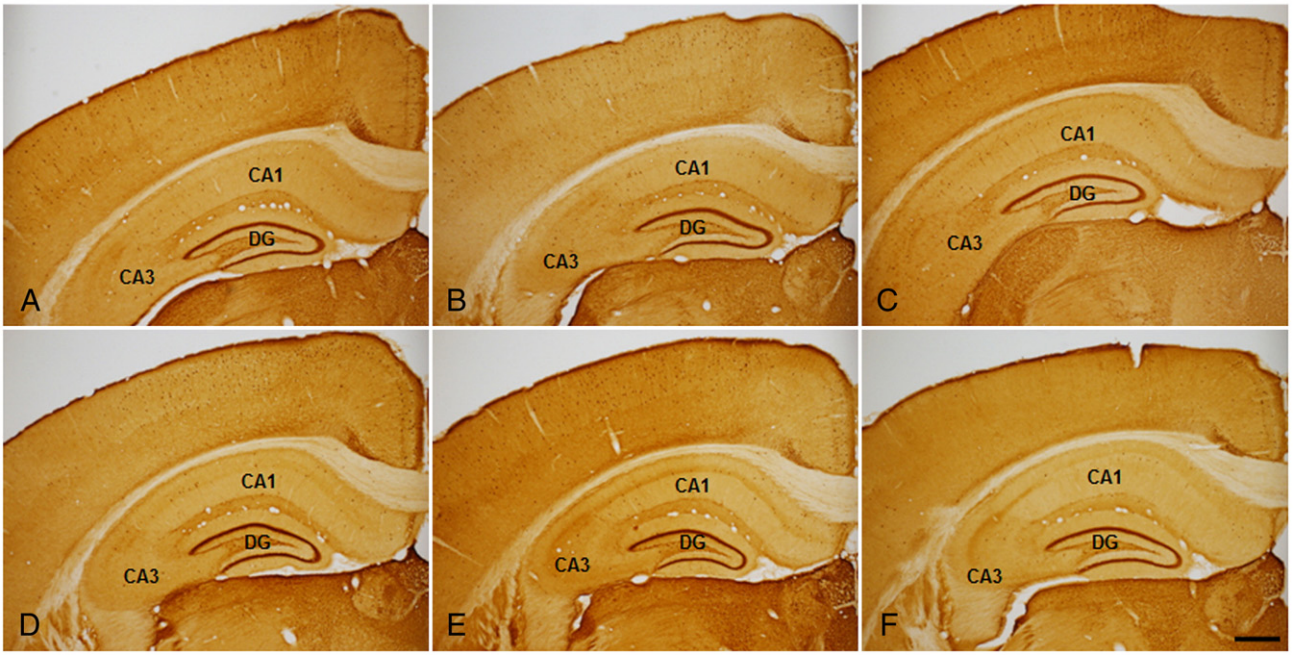

Fig. 4 – Photomicrographs of Calretinin (CR) IR in the hippocampus sections belonging to Group A: Control group, Group B: 1 h at low energy for 5 days (1.6 W/kg SAR), Group C: 1 h daily at high energy (4.0 W/kg SAR) for 5 days, Group D: 5 h exposure for 1 day at low energy (1.6 W/kg SAR), Group E: 5 h exposure for 1 day at high energy (4.0 W/kg SAR), Group F: 1 month exposure at low energy (1.6 W/kg SAR). Intense CR IR was noted in the hippocampal region of all the six groups. Numerous scattered CR IR neurons were noted in the CA1 areas. CA, cornu ammonis; DG, dentate gyrus. Scale bar = 100 μm.

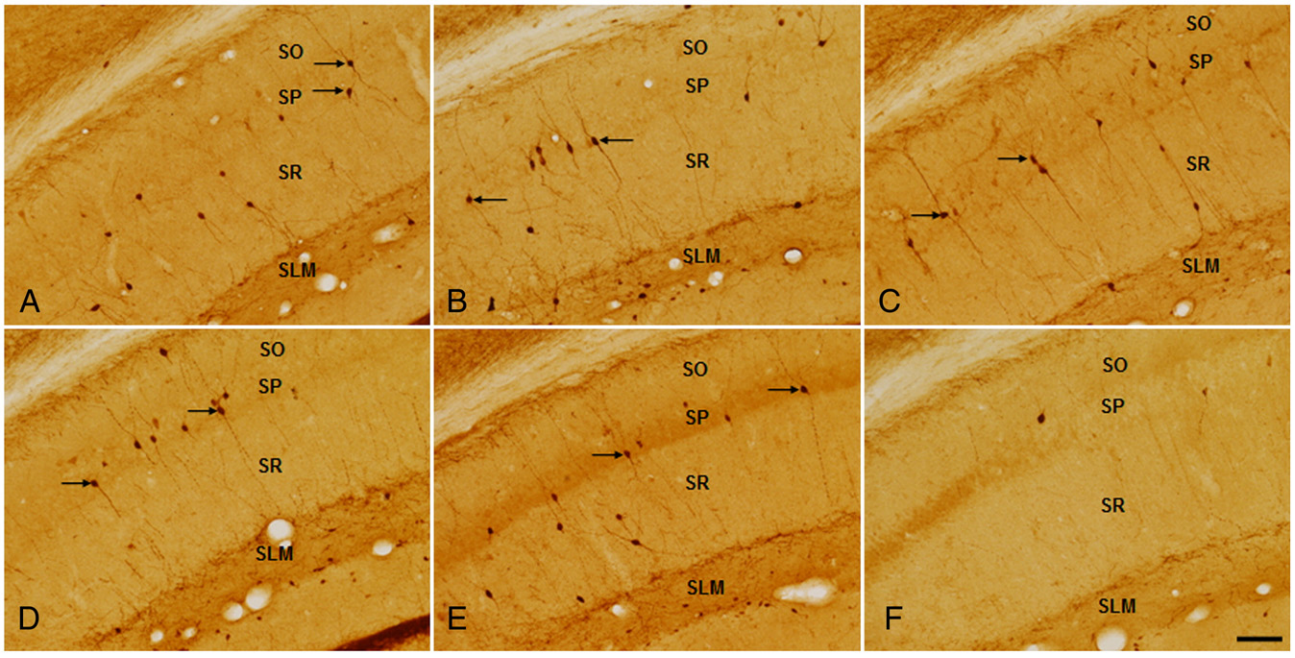

Fig. 5 – Photomicrographs of the Calretinin (CR) IR in the CA1 area of hippocampus belonging to Group A: Control group, Group B: 1 h at low energy for 5 days (1.6 W/kg SAR), Group C: 1 h daily at high energy (4.0 W/kg SAR) for 5 days, Group D: 5 h exposure for 1 day at low energy (1.6 W/kg SAR), Group E: 5 h exposure for 1 day at high energy (4.0 W/kg SAR), Group F: 1 month exposure at low energy (1.6 W/kg SAR). Numerous stained pyramidal cells (arrows) were located in the stratum pyramidale of CA1 areas of all groups except group F, where almost complete loss of the pyramidale cell was noted. The pyramidal cells displayed long axons running perpendicularly in the CA1 areas. SO, stratum oriens; SP, stratum pyramidale; SR, stratum radiatum; SLM, stratum lacunosum moleculare; SL, stratum lucidum. Scale bar = 50 μm.

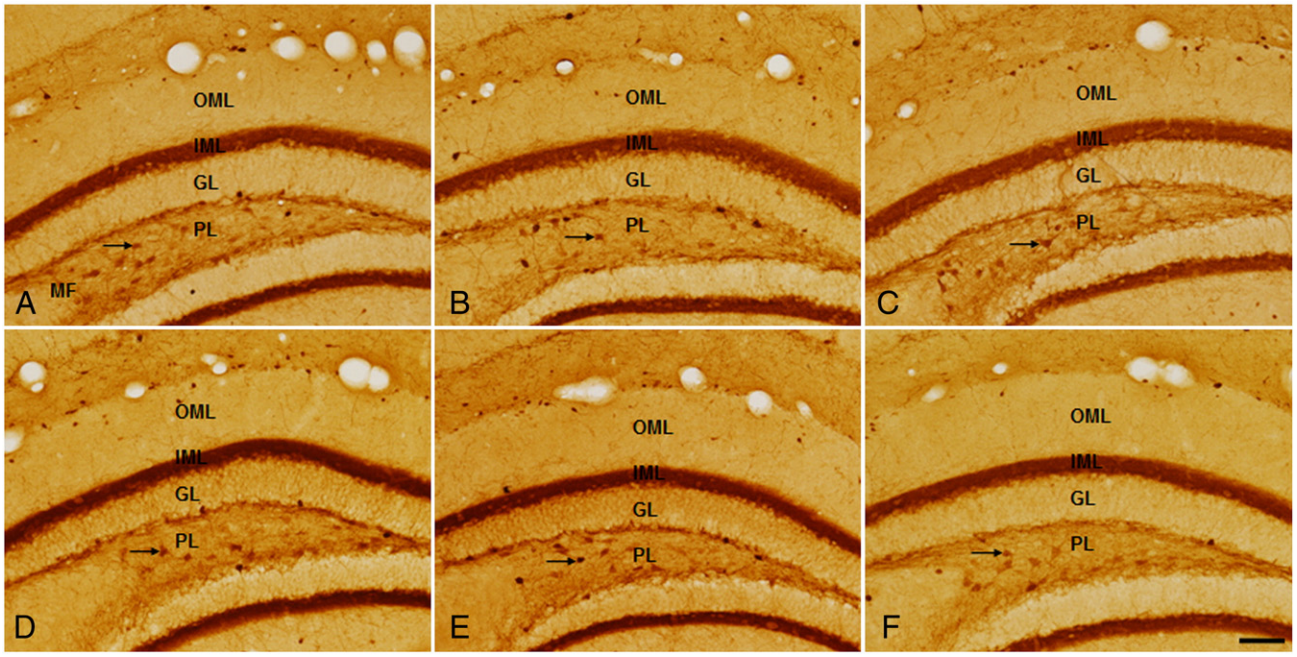

Fig. 6 – Photomicrographs of the Calretinin (CR) IR in the dentate gyrus belonging to Group A: Control group, Group B: 1 h at low energy for 5 days (1.6 W/kg SAR), Group C: 1 h daily at high energy (4.0 W/kg SAR) for 5 days, Group D: 5 h exposure for 1 day at low energy (1.6 W/kg SAR), Group E: 5 h exposure for 1 day at high energy (4.0 W/kg SAR), Group F: 1 month exposure at low energy (1.6 W/kg SAR). Strong CR IR was observed in the inner molecular layer and infra granular layer of the DG. Darkly stained neurons (arrows) were observed in the polymorphous layer of the dentate gyrus. OML, outer molecular layer; IML, inner molecular layer; GL, granular layer; PL, polymorphous layer. Scale bar = 50 μm.

2.3. *Density of immunoreactivity in the hippocampus*

2.3.1. *Density of calbindin D28-k immunoreactivity*

Maximum CB IR density was detected in the granular layer, followed by polymorphous, molecular, CA3, and CA1 areas. Control values in the molecular layer were different than Group B, Group C, Group D, Group E, and Group F ($p<0.0001$, 0.05, 0.0001, 0.01, 0.01 respectively), as were values in the polymorphous layer Group B, Group C, Group D, Group E, and Group F ($p<0.001$, 0.001, 0.001, 0.05, 0.05, respectively) (Fig. 7A). The CA1 area mean density was different in controls and Group B, Group C, Group D, and Group E ($p<0.01$, 0.05, 0.0001, 0.05 respectively) (Fig. 7A), but not between controls and Group F in the CA1 and CA3 areas.

2.3.2. *Density of calretinin immunoreactivity*

Maximum CR IR occurred in the inner molecular layer in all the six groups, followed by the polymorphous layer, granular layer, and outer molecular layer (Fig. 7B). CR IR was higher in the CA3 than CA1. Staining was significantly higher ($p<0.01$) in group B, C, D, and E in the inner molecular layer and group D and E of the granular layer and group E and F in CA1 areas (Fig. 7B). Relative density was not different in the outer molecular layer, polymorphous layer of the dentate gyrus, or the CA3 areas of group D and E, suggesting CR IR changes in the same manner in both groups. Significant differences were observed in Group B ($p<0.01$) and group F ($p<0.001$) compared with group A. CA3 and the outer molecular layer of Group C also was significantly different ($p<0.05$) than group A (Fig. 7B).

3. Discussion

The rapid increase in mobile communications has contributed to the debate on the harmful effects of EMFs, especially given the close proximity to the brain. Animal and human studies indicate that even extremely low frequencies can alter the activity of the central and peripheral nervous systems. These changes can influence neuronal functions, including regulation of synaptic plasticity, neurotransmitter release, neuronal survival, learning, and memory (Mausset et al., 2001, 2004; Salford et al., 2003; Manikonda et al., 2007). The present study examined the effect of 935 MHz radiation in the hippocampal region of CA1, CA3 and dentate gyrus of the mice by immunostaining with CB and CR. Resulting data indicate layer specific distribution of both CB and CR in the CA areas and the dentate gyrus.

The hippocampus is involved in the control of behavioral and cognitive function, including spatial learning and memory, via changes in calcium concentration (Eichenbaum et al., 1992; McEwen, 1994; Lemaire et al., 2000; Miki et al., 2000; Eyre

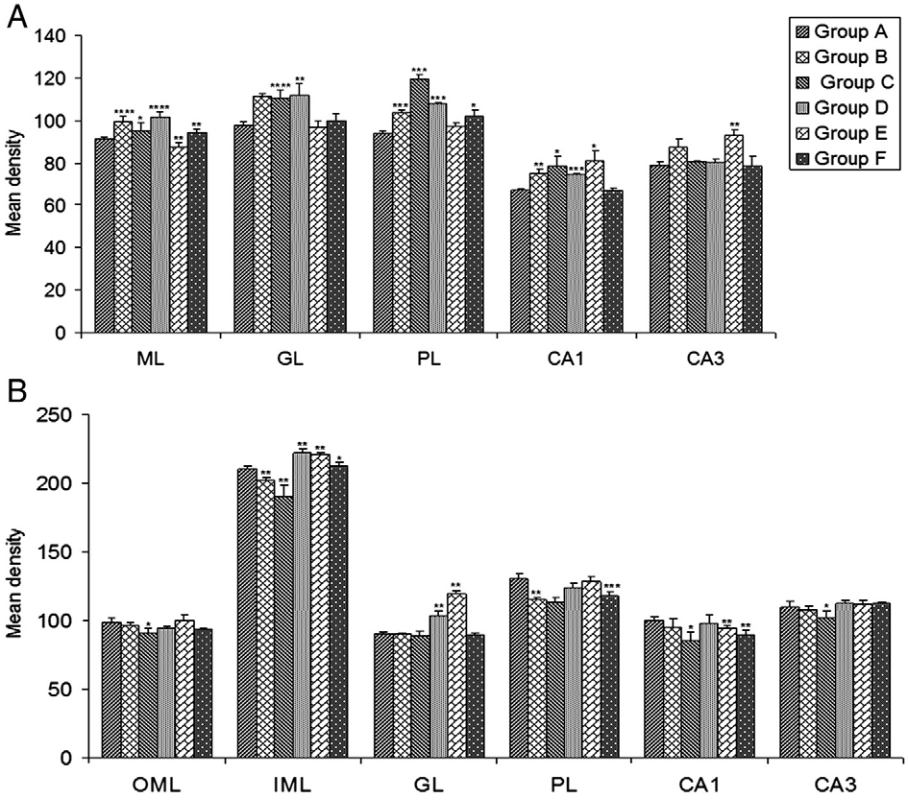

Fig. 7 – Changes in CB (A) and CR (B) IR presented by image analysis after radiofrequency exposure on the mean density in the CA1, CA3, and dentate gyrus of mice Group A: Control group, Group B: 1 h at low energy for 5 days (1.6 W/kg SAR), Group C: 1 h daily at high energy (4.0 W/kg SAR) for 5 days, Group D: 5 h exposure for 1 day at low energy (1.6 W/kg SAR), Group E: 5 h exposure for 1 day at high energy (4.0 W/kg SAR), Group F: 1 month exposure at low energy (1.6 W/kg SAR). CA, cornu ammonis; OML, outer molecular layer; IML, inner molecular layer; ML, molecular layer; GL, granular layer; PL, polymorphous layer. ($^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$, $^{****}p<0.0001$).

et al., 2003; Gokcimen et al., 2007). Several studies have reported decreases in neuron number and neuronal damage in the cortex, cerebellum, hippocampus, and basal ganglia in the brains of animals exposed to 900 MHz EMF (Mausset et al., 2001; Salford et al., 2003). We found almost complete loss of pyramidal cells in the CA1 area in group F. Pyramidal cell loss occurs in the CA areas in newborn rats with prenatal exposure to 900 MHz (Bas et al., 2009a), and in female rats (Bas et al., 2009b). Rats treated with EMF show scattered and grouped dark neurons, which were often shrunken and darkly stained, homogenized with loss of discernible internal cell structures (Salford et al., 2003). Prenatal exposure of 900 MHz produced cell loss in the dentate gyrus of 4-week-old rats, indicating disruption of prenatal and postnatal neuronal development (Odaci et al., 2008, Unal et al., 2004). We did not see cell loss in the dentate gyrus. The loss in the pyramidal as well as the interneurons of the CA1 area could have been due to neuronal cell death which was probably induced by the radiation exposure. The loss of the interneurons in the CA1 area could change $GABA_A$ and $GABA_B$ receptor responses in the hippocampus due to alteration of inhibitory control of excitability and neuronal synchrony with behavioral states (Shetty and Turner, 1998). The pyramidal cells are an important source of output from the hippocampus mainly projecting to the medial temporal cortex as well as to the orbital and medial frontal cortex (Cavada et al., 2000; Zhong et al., 2006). Hence the loss of pyramidal cells will severe the CA1 connections with other cortical areas and might be related to the behavioral manifestations. Deficits such as the neuronal loss in the CA1 area would certainly affect the hippocampal trisynaptic circuit which might lead to potential learning and memory deficit.

CB and CR are useful markers of specific cell and fiber populations, as in the cerebral cortex where each CaBP is in different GABAergic interneurons (Van Brederode et al., 1990). CaBP can maintain intracellular Ca^{2+}homeostasis and is involved in important neuronal activities. CaBP changes may lead to pathological conditions and neurogenerative conditions by changing Ca^{2+} buffering (Roberts, 1993, 1994; Lenzi and Roberts, 1994). Ca^{2+}mediates most physiological effects triggered by EMF, such as neurotransmitter release. Disruption leading to Ca^{2+}leakage would increase background levels, making synapses hypersensitive, which could cloud normal mental activity, trigger random thoughts, and cause concentration loss (Goldsworthy, 2008).

Chronic MW radiation may alter brain enzymes related to growth and lead to brain malfunction (Paulraj et al., 1999). RF radiation changes Ca^{2+}efflux from both bird and cat brain, and from human neuroblastoma cells even at low SAR values (0.05 and 0.005 W/kg at 147 MHz with amplitude modulation of 16 Hz) (Dutta et al., 1989). Further, enhanced Ca^{2+}efflux at 0.05 W/kg peaked at 13–16 Hz and at the 57.5–60 Hz modulation ranges (Dutta et al., 1989). RF radiation thus induces responses in widely different animal species, including humans. Other studies did not conclude that Ca^{2+}exchange in living nervous tissue is affected (Blackwell and Saunders, 1986).

EMFs emitted by mobile phones could affect neurons at the cellular level (Mausset et al., 2001, 2004; Salford et al., 2003; Manikonda et al., 2007). Exposure to extremely low-frequency magnetic fields changes Ca^{2+}signaling events and N-methyl-D-aspartate receptor activity in the rat hippocampus (Manikonda et al., 2007). RF exposure did not cause significant changes in body weight, as reported previously (Bas et al., 2009b; Kim et al., 2008, Odaci et al., 2008).

In conclusion, the results presented here show for the first time an almost complete pyramidal cell loss in the CA1 area of mice due to EMF exposure for 1 month at SAR = 1.6 W/kg. An RF at 900 MHz also causes pyramidal cell loss in the CA areas in newborn and adult rats (Bas et al., 2009a,b). Our study indicates that 835 MHz may also be harmful, and the effects on Ca^{2+}in human cerebrum should be tested given its role in signal transduction. Further studies with varied SAR exposure and duration can further elucidate the interaction between $Ca^{2+}$and EMF in the brain.

4. Experimental procedures

4.1. *Animal experimentations*

Six-week-old male ICR mice, 20–30 g (Orientbio Inc.) (n = 60) were used for the experiment. Following their arrival in the laboratory, the mice were kept under controlled conditions (ambient temperature of 20 to 25 °C, 12-h light/dark cycle). Food (Samtako Bio Korea, Osan, South Korea) and water were supplied ad libitum. All animal procedures were performed according to the NIH guidelines of animal research and were approved by "Dankook University Institutional Animal Care and Use Committee" (DUIAC), which adheres to the guidelines issued by the Institution of Laboratory of Animal Resources (ILAR).

4.2. *Exposure system*

The apparatus, a Wave Exposer V20, was designed by the Division of Information Technology Engineering, Soonchunhyang University, to study electromagnetic fields and radiation effects on mice (Fig. 8). The Korean CDMA mobile phone frequency, 835 MHz, was applied and radiation powers were

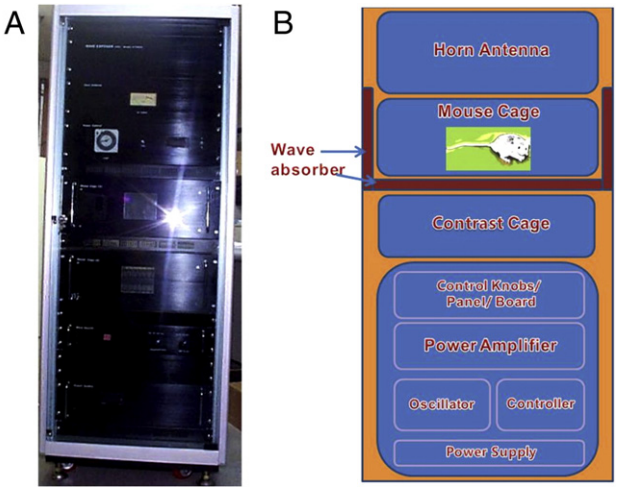

Fig. 8 – Radiofrequency exposure instrument, Wave Exposer V20 is used for radiation exposure of mouse to 835 MHz at SAR value of each 1.6 W/kg and 4.0 W/kg. The overall view (A) and the schematic design (B) for Wave Exposer V20 can be visualized.

controlled from 1.6 to 4.0 W/kg SAR (specific absorption rate) by the selection switch on the front panel. Applied SAR 1.6 and 4.0 W/kg are as same value as electric field intensity 59.56 and 94.18 V/m for the muscle (=0.92, =57 and=1020 kg/m^3) on 835 MHz CDMA frequency. Waves were generated and amplified in an electronic unit, and eventually were radiated by a pyramidal rectangular horn antenna connected by waveguide to coaxial transition. The 22 in. of a standard mouse cage was used for the apparatus. Output powers of horn antenna of the exposure apparatus are 2.5 W for SAR 1.6 W/kg and 6.3 W for SAR 4.0 W/kg. Electric field intensities due to SAR values can be calculated, and we obtained the value of power by the computer simulation for which HFSS (High Frequency Structure Simulator) manufactured by Ansoft Co. (Pittsburgh, PA) was used. For simulation, 5 cylinder-shape mice model apart from horn aperture were used. The simulation variable was both the mice location and the distance form horn aperture. The circumstance of simulation was focused by the real action of mice in the cage. We obtained the power by the averaging of simulated peak electric field intensities on each mouse body. The wave exposes from horn antenna to the mouse cage, and bottom and wall of cage is provided by wave absorption material (TDK ceramic absorber) mimicking the radiation exposure in the open environment which excludes the possibility of the influence the number of mice might have on exposure. Exposure apparatus provides automatic light system and air condition system with water feeder, with no restriction in movement in the cage during the exposure duration eliminating the risk of stress to mice during the exposure duration.

4.3. *Experimental design*

For the RF exposure experiment, the entire body of mice was exposed to 835 MHz radiation with an average SAR of 1.6 W/kg and 4.0 W/kg using Wave Exposer V20 (Fig. 8). The mice were divided randomly into 6 groups (n=10) as follows. Group A: Control group, Group B: 1 h at low energy for 5 days (1.6 W/kg SAR), Group C: 1 h daily at high energy (4.0 W/kg SAR) for 5 days, Group D: 5 h exposure for 1 day at low energy (1.6 W/kg SAR), Group E: 5 h exposure for 1 day at high energy (4.0 W/kg SAR), Group F: 1 month exposure at low energy (1.6 W/kg SAR). The control group was kept under the same laboratory conditions as the exposed groups but not subjected to radiation exposure. Animal weight was recorded before and after the final exposure. Three hours after the final exposure, animals were anesthetized with diethyl ether and their brains were collected using perfusion and fixation with phosphate buffer saline (PBS) and 4% paraformaldehyde (PFA) solution. Anesthesia was also used to avoid animal stress and to lower the augmentation of blood pressure during perfusion and fixation.

4.4. *Immunohistochemical analysis*

The brain was dissected and post-fixed overnight in 4% PFA followed by a sucrose series (10%, 20% and 30%) solution at 4 °C for cryoprotection. Serial coronal sections, 40 μm, were cut with a freezing, sliding microtome and collected in wells. We selected at least 5 slides of each cortical area from each mouse and measured all specifically stained cells in corresponding areas. The nomenclature of the brain areas involved in this study was based on the atlas of the mouse brain by Paxinos and Franklin (2001). Immunohistochemistry was performed with the free floating method, as described earlier (Hong et al., 2008). Briefly, coronal sections of hippocampal region were incubated for 48 h at 4 °C with primary antibodies, polyclonal anti-rabbit calbindin D28-k (1:5,000; AB1778; Millipore, CA, USA) and polyclonal anti-goat calretinin (1:15,000; AB1550; Millipore, CA, USA) in phosphate buffer saline based blocking buffer containing 1% bovine serum albumin, 0.3% Triton X-100, and 1% normal horse serum. After three washes with PBS, sections were incubated with the biotinylated secondary antibodies at 1:250 for 1.5 h at room temperature. Sections were then treated with an avidin–biotin–peroxidase complex (Vectastain ABC mouse Elite kit; Vector Laboratories, Burlingame, CA, USA) following the manufacturer's manual. The reaction was visualized using a solution containing 0.0125% diaminobenzidine (DAB) and 0.0005% hydrogen peroxide. Sections from each group were stained together to minimize variability. Following additional washes, sections were mounted on gelatin coated slides, dehydrated in ethanol, cleared in xylene and cover slipped with DPX.

4.5. *Image analysis*

Analysis was performed with an Olympus BX 51 microscope in each hippocampal region of CA1, CA3 and dentate gyrus (molecular, granular and polymorphous layer) and pictures of the sections were taken by a digital camera system (DP50, Olympus, Japan). The NIH image program (Scion Image) was used to determine staining densities. The sum of the gray values of all pixels in each corresponding regions (hippocampus CA1, CA3, dentate gyrus) was divided by the total number of pixels in the region to determine the mean density of immunoreactivity (IR) per unit area (mm^{-2}).

4.6. *Statistical analysis*

For statistical analysis, data were expressed in terms of mean ±SD and were compared by unpaired Student's t-test. Differences were considered significant when $p<0.05$.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.brainres.2009.11.079.

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