

Original Article

# Exposure to 835 MHz RF-EMF decreases the expression of calcium channels, inhibits apoptosis, but induces autophagy in the mouse hippocampus

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**ABSTRACT** The exponential increase in the use of mobile communication has triggered public concerns about the potential adverse effects of radiofrequency electromagnetic fields (RF-EMF) emitted by mobile phones on the central nervous system (CNS). In this study, we explored the relationship between calcium channels and apoptosis or autophagy in the hippocampus of C57BL/6 mice after RF-EMF exposure with a specific absorption rate (SAR) of 4.0 W/kg for 4 weeks. Firstly, the expression level of voltage-gated calcium channels (VGCCs), a key regulator of the entry of calcium ions into the cell, was confirmed by immunoblots. We investigated and confirmed that pan-calcium channel expression in hippocampal neurons were significantly decreased after exposure to RF-EMF. With the observed accumulation of autolysosomes in hippocampal neurons via TEM, the expressions of autophagy-related genes and proteins (e.g., LC3B-II) had significantly increased. However, down-regulation of the apoptotic pathway may contribute to the decrease in calcium channel expression, and thus lower levels of calcium in hippocampal neurons. These results suggested that exposure of RF-EMF could alter intracellular calcium homeostasis by decreasing calcium channel expression in the hippocampus; presumably by activating the autophagy pathway, while inhibiting apoptotic regulation as an adaptation process for 835 MHz RF-EMF exposure.

## INTRODUCTION

As the frequency and duration of mobile phone communication has increased, public concern about possible adverse biological health effects of exposure to radiofrequency electromagnetic fields (RF-EMF) have been growing. In 2011, the WHO's International Agency for Research on Cancer classified RF-EMF from cell phones as a 'possible human carcinogen (Class 2B). Many scientists have recognized RF-EMF as a potential cause to serious adverse risks to human health [1]. The main concern is the central nervous system (CNS), due to the close proximity between mobile phones and the brain during communication. Research on RF ra-

diation has demonstrated a negative impact on neurological factors; such as neurodevelopment, the blood-brain barrier, neurite outgrowth, neurotransmitter release, cognitive impairment, and ultimately, behavior [2-6]. Additional cellular effects of RF-EMF exposure included alterations of intracellular and molecular pathways; such as, the extracellular signal-regulated kinase pathway, apoptosis, and regulation of the cell cycle [5,7-9]. In addition, autophagy was induced in mammalian cells after exposures of 835 MHz and 1800 MHz RF-EMF [10,11]. Autophagy is a destructive mechanism in which unnecessary proteins or damaged organelles are encapsulated by an autophagosome, which then fuses with a lysosome [12]. Therefore, autophagy may play a protective



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role in various disease states and may aid in the maintenance of cellular homeostasis in the presence of various stressors [13].

The hippocampus belongs to the limbic system and plays a crucial role in the formation of new memories and spatial navigation, and is also related to learning and emotions [14-16]. Importantly, dysfunction of the hippocampus may lead to early Alzheimer's disease [17,18]. Shrinkage of the hippocampus is involved in severe mental disorders such as schizophrenia and major depressive disorders [19,20]. Exposure to RF-EMF leads to impairment of intracellular calcium homeostasis in the murine hippocampus [21]. A number of studies have highlighted the importance of calcium entry into the cell for neuronal survival. Alterations in intracellular calcium concentrations stimulate a variety of intracellular pathogenic events, including activation of apoptosis [22]. Therefore, calcium influx through voltage-gated calcium channels (VGCCs) is an important factor for the initial stages of apoptosis through the regulation of calcium ion entry across the plasma membrane [23]. A high level of calcium in mitochondria is essential to trigger apoptosis [22,24].

Previously, we reported that 12-week exposure to 835 MHz RF-EMF activates autophagy in the striatum and hypothalamus in mice brain; whereas, 4-week exposure to RF-EMF does not induce the autophagy pathways in these specific brain regions in mice [11]. However, we found that 4-week exposure to RF-EMF does lead to strong autophagic responses in the hippocampus of brain of mice [11]. In the present study, we aimed to investigate the molecular mechanisms (apoptosis and autophagy) that are affected by the calcium concentration in the mouse hippocampus after RF-EMF exposure. We detected the  $\alpha 1$  subunits of all types of voltage-gated calcium channels (VGCCs) to quantify their expression. In addition, expressions of Bcl2 and Bax were also quantified. Transmission electron microscopy (TEM) was used to examine the fine structure of morphologic changes after autophagy induction in hippocampal neurons following 4-week exposure of RF-EMF.

## METHODS

### Animals

6-Week-old mice (C57BL/6, male weighing 25-30 g) were purchased from Daehan Bio Link (DBL, Chungbuk, South Korea). The mice were maintained under specifically controlled conditions (ambient temperature  $23\pm 2^\circ\text{C}$ , 12-h light/dark cycle). Food pellets (DBL, Chungbuk, South Korea) and water were supplied *ad libitum*. After a week adaptation period, the mice were assigned to sham exposure or RF-EMF exposure for 4 weeks.

The study was generated by 2 independent batches of RF-EMF exposure experiment under the same condition. Thus, we collected the samples of hippocampus of mice twice from two different batches of RF-EMF exposed experiments. In total, 13 indepen-

dent mice were used in each condition's group for qPCR, WB and TEM. On one pair of hippocampus, one was used for qRT-PCR (n=10) and the other was used for WB (n=10). The ultrastructure images by TEM were from the mice hippocampus of the first batch. We used 3 mice brain sample for TEM in both sham control and RF-EMF exposure group (n=3). Thus, 10 mice for qPCR/WB and 3 mice for TEM in each condition (total n=13). All procedures complied with National Institutes of Health guidelines of the NIH for animal research and were approved by Dankook University Institutional Animal Care and Use Committee (IACUC; DKU-15-001), which adheres to the guidelines issued by the Institution of Laboratory of Animal Resources (ILAR).

### Exposure system of RF-EMF signal

After the adaption week, mice were exposed to 835 MHz RF-EMF using our RF generator. The frequencies of the 800MHz band are technically advantageous in the area obstructed by buildings due to the long distances the wavelength can reach. Therefore, this bandwidth frequency is used intensively and broadly for telecommunication networks in many countries. Among these various bands, 829-839 MHz (835 MHz, middle point) are considered the main frequencies and are actively used by telecommunication companies. The everyday use and predominant exposure of 835 MHz RF-EMF, has made the finding of any biological effects to be of paramount importance. The whole body exposure was at a specific absorption rate (SAR) value of 4.0 W/kg for 5 h daily for 4 weeks. Additionally, in the safety level, 4.0 W/kg SAR value is the maximum permitted exposure to human limbs but whole body is allowed up to 0.08 W/kg SAR for general public person in accordance with the International Commission on Non-Ionizing Radiation Protection (ICNIRP) guidelines. However, we used 4.0 W/kg SAR value for our experimental purposes in our mice model. The SAR generated by our RF generator was evaluated by measuring E-field at the phantom position in the air; while simultaneously by considering the ratio of E-field in the liquid to E-field in the air at the same position in the same environment. Following calculation of SAR, the value is 4.14 W/kg which is proximity of setting up output power at SAR 4.0 W/kg of horn antenna of the exposure apparatus. As a result, measurement of RF signal and SAR value generated from our RF-EMF generator produce 835 MHz RF-EMF with 4.0 W/kg SAR. More information about the exposure system has been described in details in our previous reports [25]. The mice in control group received sham exposure in parallel for 4 weeks. Sham exposure group was maintained under same environmental conditions, except for the exposure of RF-EMF. Both groups were treated in the same circular pattern from housing cage to exposure cage throughout the experiments. The mice could move freely and were not restricted to access food and water during the exposure. The cage for RF-EMF exposure was placed in the RF-EMF generator. 835 MHz RF-EMF was delivered to the mice from a horn

antenna, installed above the mouse cage. The bottom and walls of the cage were covered by ceramic wave absorption material. All the animal experiments were completed under an automatic lighting system and air conditioning facility, which was maintained in constant temperature.

### Quantitative real-time PCR and semi-quantitative RT-PCR

In order to determine whether autophagy is also triggered in the hippocampus of mice brain following the 4 week exposure to RF-EMF, selected autophagy genes such as Atg4A/B, Atg5, Atg9A, Beclin1/2 and LC3A/B were examined. Expressional levels of these genes were found by using either quantitative real time-PCR or semi-quantitative RT-PCR, and changes were recorded. Mice were euthanized the day following the completion of the RF-EMF exposure for 4 weeks (28 days). After the mouse was euthanized by cervical dislocation and head was decapitated with scissors, the whole hippocampus was isolated from the brain. The dissected hippocampus was immediately frozen and stored at  $-80^{\circ}\text{C}$ . Total RNA was extracted from the whole hippocampus by using TRIzol reagent (Thermo Fisher Scientific, USA). Purified RNA was reverse transcribed to cDNA using MMLV Reverse-Transcriptase (Bioneer, South Korea) and an oligo-d(T)18 primer. Quantitative RT-PCR reactions were carried out with Rotor-gene SYBR Green supermix Kit (QIAGEN, Germany) and fluorescence was measured using Rotor Gene PCR Cycloer (QIAGEN, Germany). The expression levels of the genes were normalized to that of GAPDH, as a housekeeping gene. GAPDH primer was purchased from QIAGEN (Germany). The primers used for qRT-PCR and sqRT-PCR (Table 1) were synthesized from Bioneer or Cosmo-genetech (South Korea). The five biologically independently prepared samples by pooling were tested and each PCR reaction was done in triplicate. The relative levels of specific mRNA were calculated by normalizing to the expression of GAPDH by the  $2^{-\Delta\Delta\text{Ct}}$  method ( $n=10$ ). Also, the expression values of the RF-EMF exposed groups were normalized to those of the sham-exposed group. Semi-quantitative RT-PCR reactions were performed us-

ing PCR PreMix (Bioneer, South Korea). Subsequently, sqPCR product of each gene was electrophoresed in 1.5% agarose gel and signal intensity of PCR product was visualized the Syto 60 (Li-Cor, USA)-stained DNA using the Odyssey infrared imaging system (Li-Cor, USA).

### Western blot analysis

The hippocampus was quickly dissected from brain of mice and one side of hippocampus from each mouse ( $n=10$ ) was quickly lysed with RIPA lysis buffer (ATTO, Japan), which was supplemented with protease inhibitor and phosphate inhibitor cocktail (ATTO, Japan). The hippocampal lysates were then homogenized and sonicated briefly in ice. For Western blot, pooling samples were used depending on protein concentration. Protein concentration was determined by Lowry protein assay (Bio-Rad, USA). Lysated samples were separated by electrophoresis on 10-15% SDS-polyacrylamide gel and transferred to polyvinylidene difluoride (PVDF) membrane (ATTO, Japan). Membranes were blocked in Odyssey blocking buffer (Li-COR, Lincoln, NE, USA) for an hour at room temperature, then incubated in a specific primary antibody solution. Primary antibodies (Abs) were used: (i) anti-LC3B Ab (Cell Signaling Technology, USA), (ii) anti-Beclin2 Ab (Cell Signaling Technology, USA), (iii) anti-Bcl2 Ab (Santa Cruz Biotechnology, USA), (iv) anti-Bax Ab (Santa Cruz Biotechnology, USA), and (v) anti-Pan calcium channel Ab (Sigma-Aldrich, USA); binding all types'  $\alpha 1$  subunits of voltage-gated calcium channels. The goat anti-rabbit 800CW or goat anti-mouse 680RD (Li-Cor, USA), were used as secondary antibodies. The membrane was washed thoroughly with tris-buffed saline containing 0.1% Tween-20 (200 mM Tris, 137 mM NaCl, 0.1% Tween20, pH 7.4). Protein bands were quantitated by using Odyssey infrared imaging system (Li-Cor, USA) and normalized by using  $\alpha$ -tubulin band.

### Transmission electron microscopy (TEM)

The dissected hippocampus' were fixed immediately in 2% glu-

**Table 1. Sequences of oligonucleotide used for RT-PCR**

Genes	Forward (5'→3')	Reverse (5'→3')
Beclin1	CTGAAACTGGACACGAGCTTCAAG	TGTGGTAAGTAATGGAGCTGTGAGTT
Beclin2	ATTCAGATGAGGGTCCCTTG	CAAGGACTTGAGATAGGAATGG
Atg9A	TCATGCAGTTCCTCTTTGTGG	TCTGGCAGAGTGACCTTG
Atg4A	CCCTCACACAACCCAGACTT	CCCCTGTGGTTGCTACTTCT
Atg4B	ACGGAGGAAGACTTTAACGAC	AAACCTCTCCAGTCTCTCTAC
Atg5	GGAGAGAAGAGGAGCCAGGT	TGTTGCCTCCACTGAACTTG
LC3A	TGGTCAAGATCATCCGGC	CTCACCATGCTGTGCTGG
LC3B	TTCTTCTCTCTGGTGAATGG	GTGGGTGCCTACGTTCTCAT
Bcl2	ACCGTCGTGACTTCGCAGAG	GGTGTGCAGATGCCGGTTC
Bax	CGGCGAATTGGAGATGAACTG	GCAAAGTAGAAGAGG GCAACC

taraldehyde-2% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 2 h at 4°C. Following three washes in the phosphate buffer, the brain tissues were post-fixed with 1% osmium tetroxide on ice for 2 h and washed three times, all in the phosphate buffer. The tissues were then embedded in Epon 812 mixture after dehydration in an ethanol and propylene oxide series. Polymerization was conducted with pure resin at 70°C for 24 h. Ultrathin sections (~70 nm) were obtained with an ultramicrotome (MT-X, RMC, Tucson, AZ, USA) and then collected on 100 mesh copper grids. After staining with 2% uranyl acetate (15 min) and lead citrate (5 min), the sections were visualized using the transmission electron microscopy (TEM) (Technai G<sup>2</sup> Spirit Twin, FEI, Hillsboro, OR, USA) at 120 kV. The whole pair of hippocampus was used for TEM study (n=3).

### Statistical analysis

All data is presented as the mean±SEM. The significances of all data assessed by two-tailed, Student's *t*-test with probability values of  $p < 0.05$  considered statistically significant. We used GraphPad Prism 4 program (GraphPad Software, Inc., La Jolla, CA) for the statistical analysis.

## RESULTS

### Decreased expression of VGCCs in the hippocampus

To investigate the effects of RF-EMF exposure on calcium homeostasis in the hippocampus, we focused on VGCCs, which controls intracellular calcium concentrations [26]. Total protein extracted from the hippocampus was used to perform western blots using an anti-Pan calcium channel antibody (Sigma, St. Louis, USA), which recognizes all types of  $\alpha 1$  subunits of VGCCs. The data showed that 4-week exposure to RF-EMF decreased the expression of VGCCs in the mouse hippocampus (Fig. 1). These data strongly suggested that exposure to RF-EMF could induce alterations to calcium homeostasis in the mouse hippocampus.

### The expression level of Bax is down but that of Bcl2 is up in the hippocampus after RF-EMF exposure

To elucidate the effects of 4-week RF-EMF exposure on apoptosis, the anti-apoptotic factor Bcl2 and the pro-apoptotic factor Bax were analyzed in the hippocampus; with both RT-PCR and western blotting. The RT-PCR results revealed that the transcript level of the pro-apoptotic gene Bax slightly declined in the hippocampus of RF-EMF-exposed mice (Fig. 2A). Similarly, immunoblotting analysis indicated that the level of anti-apoptotic Bcl2 protein was significantly increased; while Bax protein expression was significantly decreased, by nearly half in the hippocampus following RF-EMF exposure (Fig. 2B). These results indicated

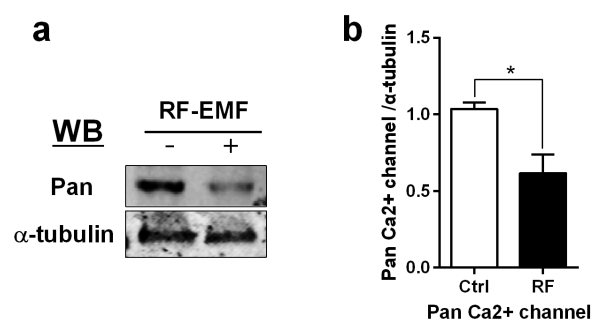
that the apoptotic pathway may be hindered following a 4-week exposure to RF-EMF.

### Autophagy-related genes are significantly up-regulated in the hippocampus following RF-EMF exposure

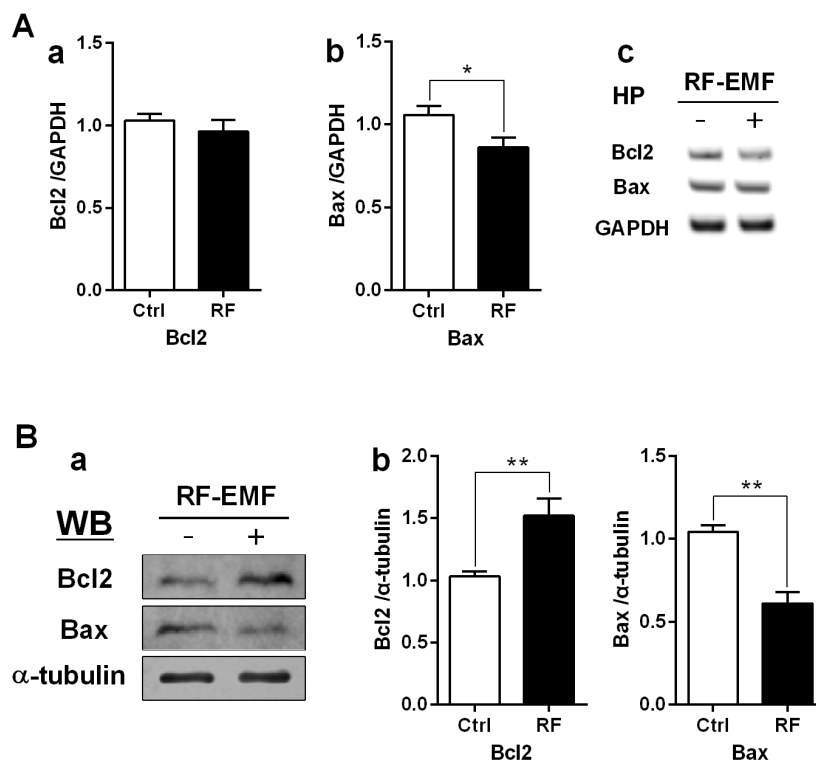
To determine whether autophagy is triggered in the mouse hippocampus following 4-week exposure to RF-EMF, we examined the expression of selected autophagy genes (Atg4A/B, Atg5, Atg9A, Beclin1/2, and LC3A/B) using quantitative real time-PCR (qRT-PCR) and semi-quantitative RT-PCR (sqRT-PCR). As shown in Fig. 3, the qRT-PCR results indicated that the expression of most of the genes related to autophagy (Atg4B, Atg5, Atg9A, Beclin2, and LC3B) were significantly increased at about 1.5-2.0 fold, as compared to controls in the hippocampus (Fig. 3). However, the expression of Atg4A, Beclin1 did not differ from that in control animals following RF-EMF exposure (Fig. 3). Of interest, the expression of LC3A was decreased in RF group (Fig. 3d). In addition, sqRT-PCR was carried out to further validate the changes in gene expression found with qRT-PCR. Generally, the results from sqPCR were consistent with the patterns seen with the qRT-PCR data, indicating that Atg4B, Atg5, Atg9A, Beclin2, and LC3A/B showed higher intensity in RF-EMF group compared to controls (Fig. 3i). Analysis of qRT-PCR indicated that there is upregulation of genes related to autophagy in the hippocampus in response to 4-week exposure of RF-EMF.

### Autophagy structures are accumulated in the hippocampus

In parallel with the upregulation of mRNA level of autophagy in the hippocampus depending on RF-EMF exposure, we further explored the levels of autophagy-related proteins such as LC3B-



**Fig. 1. The expression of calcium channels in the mouse hippocampus following a 4-week exposure to RF-EMF.** Total hippocampus was isolated from sham-exposed mice (Ctrl) and RF-exposed mice (RF). (a) Representative immunoblot of pan-calcium channel antibody. (b) The expression level of calcium channels in the hippocampus is significantly decreased after 835 MHz RF-EMF exposure for 4 weeks. The bar represents the mean±SEM. \* $p < 0.05$  vs. Ctrl (n=10).



**Fig. 2. Regulation of apoptosis in the mouse hippocampus following RF-EMF exposure.** Hippocampal mRNA (A) and proteins (B) were analysed for the expression level of apoptotic genes/proteins. (Aa, Ab) Quantification of Bcl2 and Bax mRNA transcripts by qRT-PCR. (Ac) Representative gel images of Bcl2 and Bax by sqRT-PCR. The expression levels of the hippocampus of RF-EMF-exposed mice were normalized to those of the sham-exposed mice. The relative mRNA levels of each gene were calculated by normalizing to GAPDH expression by the  $2^{-\Delta\Delta Ct}$  method (n=10). (Ba) Representative immunoblots of hippocampus from sham-control and RF-EMF group. (Bb) The expression level of Bcl2 was significantly increased, but the expression level of Bax was significantly decreased after 835 MHz RF-EMF exposure for 4 weeks. \* $p < 0.05$  and \*\* $p < 0.01$ , vs. Ctrl. Average is the mean  $\pm$  SEM (n=10).

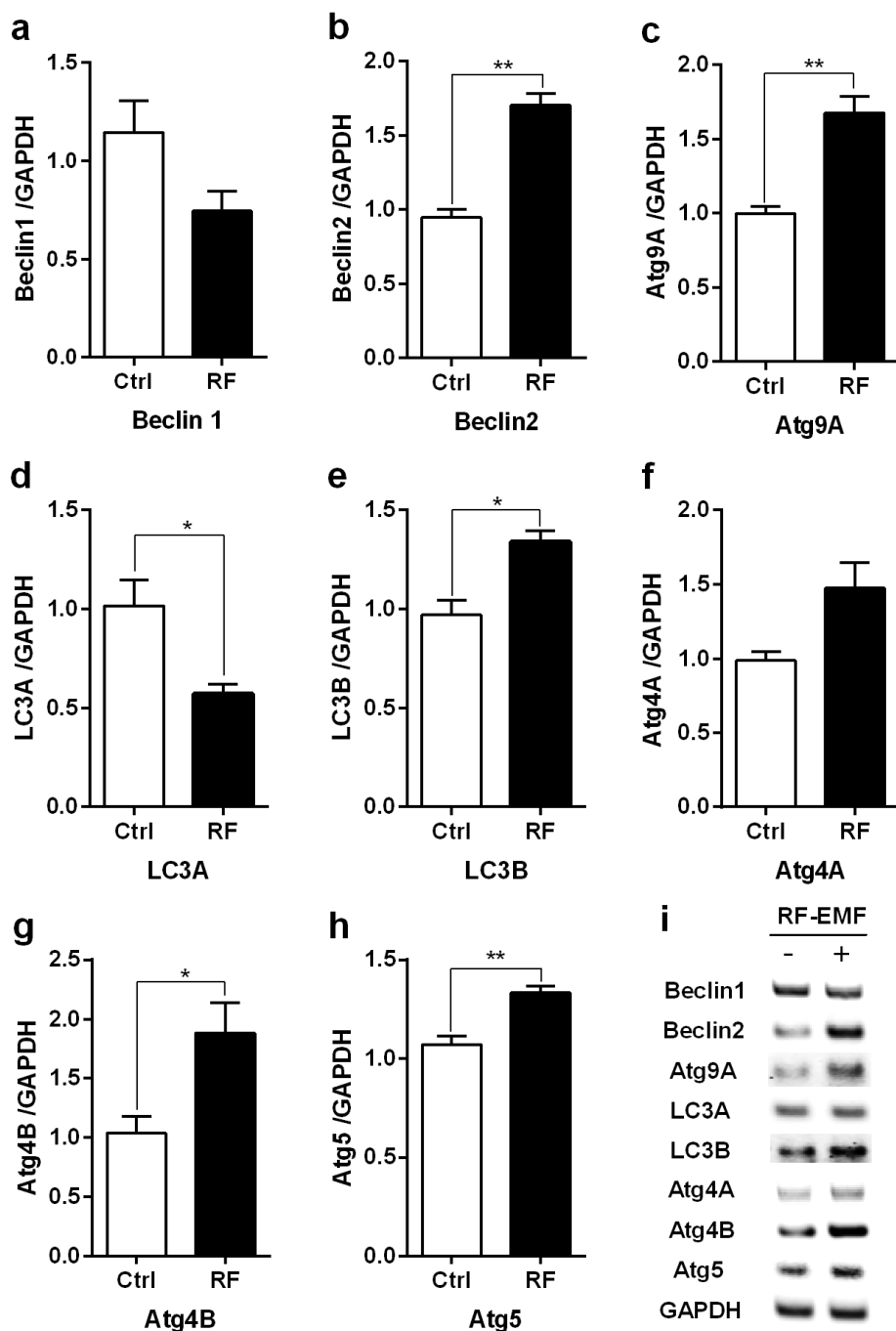
II and Beclin1; which play a key role in the formation of the autophagosome in response to environmental cellular stress [27-29]. The LC3B-II protein level was significantly increased by 1.7-fold compared to control in the hippocampus, but the expression of Beclin1 was not changed (Fig. 4A). Ultrastructural comparisons between sham and RF-EMF-exposed hippocampal neurons was conducted using TEM. The results indicated that the augmented number of autophagy structures, such as autophagosomes and autolysosomes, were observed in hippocampal neurons following RF-EMF exposure (Fig. 4B). These results indicated that 4-week exposure to RF-EMF could induce higher LC3B-II expression and accumulate more autophagy structures, in comparison to the sham control group in the hippocampus. The data strongly suggested that activation of the autophagy pathway is triggered in the mouse hippocampus by RF-EMF exposure.

## DISCUSSION

This study evaluated the effects of RF-EMF exposure on calcium channel expression. Intracellular calcium concentrations are regulated by calcium channels and affect various neuronal processes [30]. Exposure to RF-EMF caused impairment of in-

tracellular calcium homeostasis in the murine hippocampus [21]. The calcium ion is an essential component for the regulation of a variety of cellular processes, including apoptosis [31]. The impairment of calcium homeostasis in a cell may lead to activation of apoptosis [23,31]. In this study, we showed that exposure to RF-EMF may alter the calcium homeostasis in the hippocampus of mice by decreasing the level of pan-calcium channels, which regulate calcium influx (Fig. 1). Calcium homeostasis can be regulated by several types of calcium channels, including VGCCs [32]. VGCCs are responsible for fast calcium influx into the cell, which controls the entry of calcium ions across the plasma membrane [32].

Intracellular calcium overload may activate pro-apoptotic genes, which cause the degeneration of neurons and ultimately cell death [31,33]. It has also been suggested that capacitive calcium influx through calcium release-activated calcium channels causes apoptosis [22]. However, inhibition of calcium channels in neurons, which causes a reduction in the level of intracellular calcium, may serve a neuroprotective function to promote neuronal survival [23]. This is consistent with our finding that, in response to RF-EMF exposure, apoptosis might be inhibited (Fig. 2) and the expression of VGCCs was also significantly reduced (Fig. 1). Further studies are required to gain more evidence on the direct



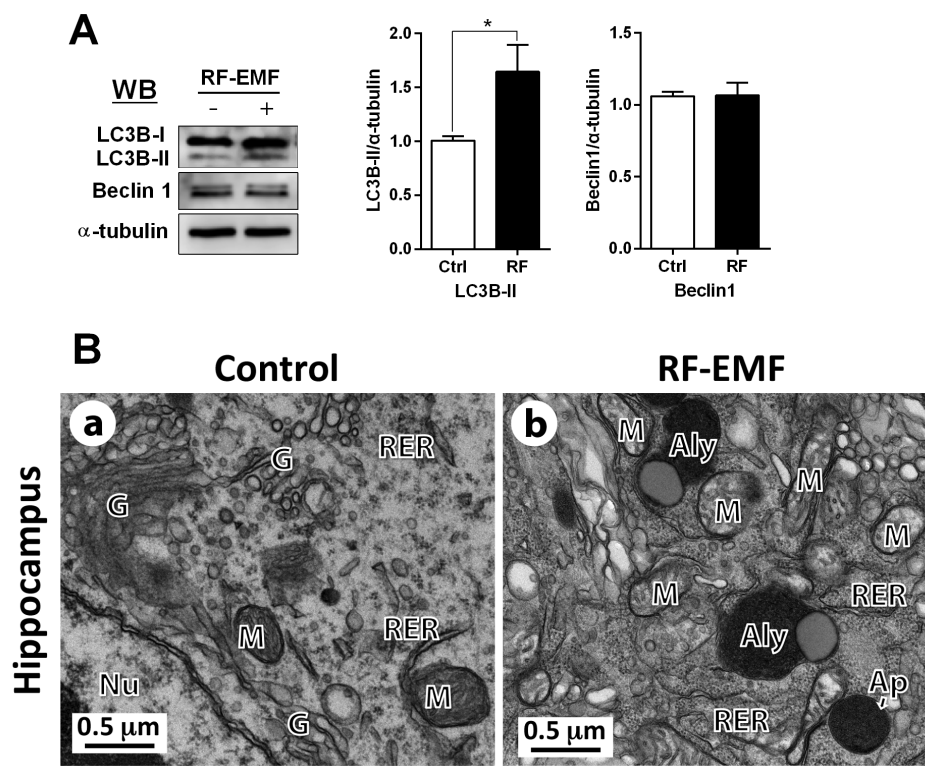
**Fig. 3. Expression levels of autophagy-related genes in mouse hippocampus.**

Total RNA was extracted from the hippocampus of sham-exposed mice (Ctrl) and RF-EMF exposed mice (RF). (a-h) Quantification of Beclin1/2, Atg9A, LC3A/B, Atg4A/B, Atg5 mRNA was analysed by qRT-PCR. (i) Representative gel images of autophagy related genes, amplified by sqRT-PCR. The relative expression levels of each gene were calculated by normalizing GAPDH expression by the  $2^{-\Delta\Delta Ct}$  method. Each bar represents the mean  $\pm$  SEM. \* $p < 0.05$  and \*\* $p < 0.01$  vs. Ctrl (n=10).

relationship between calcium signaling and apoptosis in neurons in response to RF-EMF exposure.

In the present study, we found significant augmentation of both autophagy-related genes and proteins in the hippocampus following a 4-week exposure to 835 MHz RF-EMF with 4.0 W/kg SAR (Figs. 3 and 4A). Several studies previously suggested that intracellular calcium is an important regulator for initiation of autophagy processes [34,35]. Additionally, the autophagic flux was attenuated by increased cytosolic calcium concentration, due to prevent the fusion between autophagosomes and lysosomes in hepatocytes [36]. Prevention of cytosolic calcium influx by calcium

channel blockers effectively restored the autophagic flux, presenting by accumulation of autophagosomes and increase in LC3-II level in the obesity-induced autophagy arrest model [36]. As calcium channel blockers can prevent the cytosolic calcium influx, inhibition of calcium channels could be a molecular mechanism for initiation of the autophagic processes in autophagy defects. In this study, we found that the expression of calcium channels was decreased and the processes of autophagy were induced by exposure of RF-EMF. These results suggested that decreased level of calcium channel expression could interfere with the intracellular calcium influx, therefore, autophagy processes can be activated in



**Fig. 4. LC3B-II and beclin1 expression and ultrastructure of autophagy in the hippocampal neurons.** (A) The expression level of LC3B was significantly increased by RF-EMF exposure, but beclin1 was not changed. Average is mean $\pm$ SEM. \* $p$ <0.05 vs Ctrl. (n=10). (B) Representative TEM micrographs were acquired from the hippocampal neurons of sham exposed control (Ba) and RF-EMF exposed mice (n=3) (Bb). In the hippocampus of RF-EMF-exposed mice (Bb), numerous autophagosome (Ap) and autolysosome (Aly) were observed compared to sham control mice. Abbreviations are: Ap, autophagosome; Aly, autolysosome; G, Golgi apparatus; M, mitochondria; Nu, nucleus; RER, rough endoplasmic reticulum. Size bars: 0.5  $\mu$ m.

hippocampus of mice.

Further evidence for autophagy induction after RF-EMF exposure is that although hippocampal structures (CA1-CA4 and DG) were not distinguished in our situation. Due to the difficulty of hippocampal staining, autophagic structures; such as autophagosome and autolysosomes were accumulated in hippocampal neurons of brain of mice exposed to RF-EMF as a whole. Additionally, we obtained continuously consistent results in randomly cut all area of hippocampus by TEM experiment. Previously, we have reported that 12-week exposure to RF-EMF activates autophagy in the striatum and hypothalamus in mice brain, whereas 4-week exposure to RF-EMF does not induce the autophagy pathways in these brain regions in mice [11]. However, we found that 4-week exposure to RF-EMF leads to strong autophagic responses in the hippocampus of mice brain. Our results showed that autophagy can be triggered in the hippocampus following RF-EMF exposure, as a physiologic means of adaptation to cellular stress to 835 MHz radiofrequency at 4.0 W/kg SAR for 4 weeks. However, the processes of autophagy induced by RF-EMF exposure were not observed at hypothalamus in previous study [11]. It is not clear yet why different region of brain response differentially to RF-EMF. We speculated that the structure or function of the hippocampus are more sensitive than hypothalamus to the stress from radiofrequency electromagnetic field. The hippocampus belongs to the limbic system and plays important roles in the consolidation of information from memory; but the hypothalamus is also responsible for the regulation of certain metabolic processes and other activities to link the nervous system to the endocrine system. In

addition, the location of brain regions to RF-EMF exposure or the different tissue configurations could contribute the differential responses. Further researches will be required to investigate the differential responses among brain regions.

In this study, we also found that the apoptosis pathway was inhibited, while autophagy was increased in the hippocampus after 4 weeks exposure of RF-EMF (Fig. 3). RF-EMF exposure was reported to upregulate the caspase-3-dependent apoptotic pathway in primary cultured rodent brain neurons [8,37]. Similar cellular stressors can cause either autophagy or apoptosis in a cell; the method triggered depends on the threshold of sensitivity to the specific stressor [38]. Both mechanisms are tightly regulated by the inhibition of mediator proteins Bcl2 and Beclin1 [39]. Activation of autophagy blocks the apoptosis pathway through inhibition of the Bax protein [38]. Anti-apoptotic Bcl-2 protein was upregulated, thereby inhibiting the pro-apoptotic Bax protein in the hippocampus after RF-EMF exposure (Fig. 2).

Autophagy is an essential cellular mechanism that processes selective degradation of organelles or unusual protein aggregates [40]. Importantly, dysregulation of autophagy may cause various neurodegenerative diseases [41-43]. The analysis of the expression of autophagy-related genes in this study suggests that they may play a crucial role for autophagosomal formation [44]. Activity of endoplasmic reticulum (ER)-associated class III PI3K (also known as Vps34) drives vesicle nucleation by the formation of a multi-complex with Beclin1/2, which initiates autophagosomal formation [40]. The trans-membrane protein Atg9A/B may recruit lipids to the phagophore [38,45]. During the autophagy

process, LC3 is cleaved by cysteine protease Atg4A/B and undergoes post-translational modifications [46]. A lipidated form of LC3, microtubule-associated protein 1 light chain 3 (MAP1LC3)-II (LC3-II) is associated with autophagic vesicles [27,47]. Importantly, a complex of an E3 ubiquitin ligase-like enzyme Atg5 with Atg12-Atg16L1 regulates autophagosomal elongation and this complex is essential for LC3-I combination with phosphatidylethanolamine (PE) to form LC3-II (LC3-PE) [27,38,46,48]. Eventually, mature autophagosomes that engulfed intracellular organelles fused with lysosome generate autolysosome [27,38]. Therefore, the level of LC3B-II might reflect the relative amount of autophagosomes in hippocampal neurons [29]. RF-EMF exposure significantly increased the protein level of LC3B-II, a protein that is used for autophagy markers as well as represents overall autophagic flux [29], in the hippocampus (Fig. 3A). As shown in Fig. 3B, exposure of RF-EMF resulted in the accumulation of autophagic vesicles; such as autophagosomes and autolysosomes, which might also represent active autophagy in the hippocampus. Thus, RF-EMF exposure likely induces autophagy in mouse hippocampal neurons.

The hippocampus is crucial for its role in the formation of new memories and spatial navigation, and is also involved in learning and emotions [14-16]. Importantly, dysfunction of the hippocampus may lead to early Alzheimer's disease (AD) [17,18]. AD is mainly caused by extracellular amyloid beta (A $\beta$ ) deposits, but autophagy could provide neuroprotection through the disruption of A $\beta$  aggregates [49,50]. This study may provide evidence that autophagy offers neuroprotection in the hippocampus in response to RF-EMF exposure. Moreover, autophagy may prevent neuronal cell death through the down-regulation of apoptosis, which can lead to neurodegenerative diseases.

Calcium signaling plays a key role in the regulation of neurotransmitter release, as well as cell death [51,52]. Changes in calcium homeostasis may have significant effects on neurotransmitter release from synaptic terminals. Therefore, RF-EMF exposure may cause alterations to neurotransmission in the hippocampus, leading to abnormal behavior and/or disease states, such as learning impairments, anxiety, and depression [53,54]. Our previous study demonstrated that RF-EMF exposure to mice may lead to hyperactivity-like behavior by generation of demyelination in the cerebral cortical neurons [25]. However, more detailed studies are necessary to elucidate the scope of impact of RF-EMF on the brain.

In summary, we found that the level of calcium channels, which play a key role in calcium homeostasis, were decreased in the mouse hippocampus following a 4-week exposure to 835 MHz RF-EMF at 4.0 W/kg SAR, 5 h per day. RF-EMF exposure likely alters intracellular calcium by decreasing the expression of calcium channels, activating the autophagy pathway and suppressing the apoptotic pathway. The expression of genes and proteins related to autophagy were significantly increased, in parallel with the accumulation of autolysosomes in hippocampal

neurons. Our results suggest that RF-EMF is acting as a stressor to the central nervous system and causing increases in autophagy and down-regulation of apoptosis.

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## CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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