doi: 10.4149/gpb_2019020

Trafficking of synaptic vesicles is changed at the hypothalamus by exposure to an 835 MHz radiofrequency electromagnetic field

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Abstract. With the rapidly increasing use of mobile phones and their close-contact usage to the brain, there are some concerns about the possible neuronal effects induced by exposure to excessive electromagnetic radiation. Exposure to a radiofrequency electromagnetic field (RF-EMF) of 835 MHz (4.0 W/kg specific absorption rate (SAR) 5 h/day for 12 weeks) may affect hypothalamic presynaptic neurons in C57BL/6 mice. The number and size of the synaptic vesicles (SVs) in the hypothalamic presynaptic terminals were significantly decreased after RF-EMF exposure. Further, the density (SVs numbers/μm) of docking and fusing SVs in the active zones of the presynaptic terminal membrane was significantly decreased in hypothalamic neurons. The expression levels of synapsin I/II and synaptotagmin 1, two regulators of SV trafficking in neurons, were also significantly decreased in the hypothalamus. In parallel, the expression of calcium channel was significantly decreased. These changes in SVs in the active zones may directly decrease the release of neurotransmitters in hypothalamic presynaptic terminals. Therefore, we further studied the possible changes in hypothalamic function by testing the core body temperature and body weight and performed the buried pellet test. The trafficking of SVs was changed by RF-EMF; however, we could not find any significant phenotypical changes in our experimental condition.

Key words: RF-EMF — Synaptic vesicle — Active zone — Synapsin — Synaptotagmin — Hypothalamus

Introduction

With the ever-increasing use of mobile phones in modern life, humans are exposed to excessive levels of electromagnetic radiation from radiofrequency electromagnetic fields (RF-EMFs). Due to the proximity of mobiles phone to the head, there are public concerns about the potential adverse effects of RF-EMF exposure on the brain (Beard et al. 2006). In addition, in 2011, the International Agency for Research on Cancer (IARC) classified electromagnetic fields as group 2B carcinogens to inform mobile phone users of the potential harms associated with RF-EMF exposure (Baan et al. 2011). However, the direct correlation between cancer and EMF exposure remains controversial.

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However, the neurological effects of RF-EMF exposure have been reported by various researches using cellular or animal models, including activation of apoptosis and autophagy, alterations in the myelination of neurons and changes in ion channels expression in brain neurons. Therefore, RF-EMFs have been considered as an external stressor inducing various biological changes (Liu et al. 2012; Kim et al. 2016, 2017b, 2018).

Neurotransmission between neurons is carried out by the synapse, an essential structure for neuronal function (Robinson 2007). Generally, electrical activity in the presynaptic neuron is converted into the release of a neurotransmitter that binds to receptors located in the postsynaptic cell. Clusters of synapses are formed in specific regions of the brain. Their function is then determined by the presence of either excitatory or inhibitory postsynaptic neurons. Specific neurotransmitters are synthesized in presynaptic neurons and stored in synaptic vesicles (SVs) (Sudhof 2004).

SVs are mainly implicated in the storage, release, and secretion of neurotransmitters, which is accomplished by the cooperation of various synaptic vesicle-associated proteins such as synapsins, synaptotagmin, synaptophysin, synaptobrevin etc (Sudhof 2004; Brachya et al. 2006). In addition, the release of neurotransmitters in the synaptic cleft is regulated by activation of voltage-gated calcium channels. Calcium ions play a key role in the regulation of neurotransmitter release, excitability, and synaptic plasticity (Neher and Sakaba 2008). Therefore, alterations in calcium homeostasis in neurons may have significant effects on neurotransmitter release from synaptic terminals.

We previously reported that the number and size of the synaptic vesicles at presynaptic nerve terminals may be changed in the auditory brainstem, cerebral cortex and striatum of mice after exposure to RF-EMF (Kim et al. 2017a, 2019a, 2019b). These studies also suggested that alterations in SV trafficking are caused by changes in the expression levels of synapsins and calcium ion channels (Evergren et al. 2007; Cesca et al. 2010) . We also found that neurons in different regions of the brain are affected differently by RF-EMF exposure.

The hypothalamus, which is located between the thalamus and brainstem in the limbic system, is responsible for linking the nervous system to the endocrine system (Barron 2010). Body temperature, food intake, and circadian rhythms have been known to be controlled by the hypothalamus by regulation of specific metabolic processes and the autonomic nervous system (Humphries et al. 2008; Chughtai et al. 2009; Tyler and Allan 2014; Biran et al. 2015; Greenway 2015). Therefore, any changes in neurotransmission in the hypothalamic regions may affect various neurological functions. In the current study, we investigated the changes in SV trafficking in the hypothalamic regions induced by RF-EMF exposure (835 MHz, specific absorption rate: SAR at 4 W/kg and 5 h/day exposure for 12 weeks) and its possible neurological effects such as control of body temperature and food intake.

Materials and Methods

Animals

C57BL/6 mice (6-week-old 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 ± 2°C, 12-h light/dark cycle). Food pellets (DBL, Chungbuk, South Korea) and water were supplied ad libitum. All mice had a week adaptation period and were afterward assigned to the sham exposure or the RF-EMF exposure group for 12 weeks. All procedures complied with the National Institutes of Health (NIH) guidelines of the NIH for Animal Research and were approved by the Dankook University Institutional Animal Care and Use Committee (IACUC; DKU-15-001), which adheres to the guidelines issued by the Institution of Laboratory Animal Resources.

RF-EMF exposure

Mice were exposed to 835 MHz RF-EMF using a Wave Exposer V20 RF generator, as previously described in detail (Kim et al. 2017b). The specific absorption rate (SAR) is a numerical expression of these absorbed waves. SAR refers to the amount of radio wave energy absorbed in unit mass of human body (1 kg or 1 g); units are W/kg or mW/g. National Radio Research Agency has released SAR standards of SAR-related international organizations and major countries with related matters. The SAR standard for limbs for normal user is 4 W/kg in general but the SAR standard for occupational user is higher such as 8 or 10 W/kg for head/trunk. In this study, the whole body of the mouse was exposed at an SAR of 4.0 W/kg for 5 h daily for a 12-week period. The other group also received a sham exposure for 5 days. The sham-exposed group was kept under the same environmental conditions and treated using the same circular pattern as the RF-EMF-exposed groups. The sham- and RF-EMF-exposed mice could move freely and had access to water in their exposure cage. The bottom and the walls of the cage were covered by a ceramic wave absorption material. All the experiments were conducted at our animal facility, which was maintained at a constant condition (23 ± 2°C; relative humidity 50 ± 10%; 12:12-h light dark cycle). The horn antenna for the RF-EMF exposure was located on the top of the mouse cage. After the 12-week exposure, the mice were sacrificed for ultrastructural or biochemical studies.

Transmission electron microscopy

The mice were euthanized by cervical dislocation, and the head was rapidly removed using scissors. The hypothalamus was then quickly dissected from each brain on ice. The hypothalamic samples dissected from mice of the different groups (n = 5) consisted of 300-μm-thick slices containing hypothalamic synapses cut using a vibratome (LEICA VT1000s, LEICA Microsystems, Heidelberger, Germany). The hypothalamic slices were immediately fixed in 2% glutaraldehyde and 2% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 2 h at 4°C. Following three washes in phosphate buffer, the brain tissues were post-fixed with 1% osmium tetroxide on ice for 2 h and washed three times in phosphate buffer. The tissues were then embedded in Epon 812 after dehydration in an ethanol and a propylene oxide series. Polymerization was conducted with pure resin at 70°C for 24 h. Ultrathin sections (~70 nm) were obtained with

a model MT-X ultramicrotome (RMC, Tucson, AZ) and collected on 100-mesh copper grids. After staining with uranyl acetate and lead citrate, the sections were visualized using a bio high-voltage electron microscope system (JEM-1400 Plus at 120 kV and JEM-1000BEF at 1,000 kV (JEOL, Japan)).

Measurement of the number and size of the SVs

The detailed methods for the measurement of the number and size of the SVs were previously described (Kim et al. 2017a). Briefly, the TEM (transmission electron microscopy) samples were immediately prepared from the sham control ($n = 5$) and the RF-EMF-exposed mice ($n = 5$) after a 12-week exposure to RF-EMF. We generated images of 4–5 synapses *per* mouse and counted the synaptic vesicles (SVs) in 20 and 22 synapses (control and RF-EMF-exposed group). In addition, the area of the synaptic vesicles (SVs) in all the pre-synapses used for counting SVs was measured. Only clearly distinguishable SV membranes were selected and the diameters of the SV membranes (control 1806/ RF-EMF 1134 SVs) were estimated without any prejudice.

Counting of the SVs at the active zone

To count the number of docking or fusing SVs at the active zones, enhanced magnification images of the hypothalamic excitatory presynaptic nerve terminals were used. The number of SVs was obtained following the instructions below. The number of pixels *per* 1-μm length was calculated by dividing the number of pixels of the acquired image by the length of the scale bar (0.2 μm) using the software ImageJ (NIH, Bethesda, MD). The active zone was defined as the region where the SV reached the membrane of the presynaptic terminal and where fusion between the SV and membrane occurred. Therefore, the membrane line appeared blurry. In addition, the average diameter of the 50 nm SVs located within 100 nm from the presynaptic terminal was counted. SV density was obtained by dividing the total number of SVs with the total length of the active zone.

Quantitative real-time PCR

Total RNA was purified using a TRIzol reagent (Thermo Fisher Scientific, Pittsburgh, PA) from the hypothalamus ($n = 8$). RNA was reverse transcribed to cDNA using MMLV reverse transcriptase (Bioneer, Daejeon, South Korea) and an oligo-d (T)18 primer. Quantitative real time PCR (qRT-PCR) was carried out using the Rotor Gene SYBR Green supermix Kit (QIAgen, Hilden, Germany) and fluorescence was measured using a Rotor-gene PCR Cycler (QIAgen, Hilden, Germany). The primers were synthesized by Bioneer. The sequences for forward and reverse Syn primers were as follows: Syn I F: 5′- CAGGGTCAAGGCCGCCAGTC-3′and R: 5′-CACATCCTGGCTGGGTTTCTG-3′; Syn II F: 5′-AGGGGAGAAATTCCCAC-3′ and R: 5′-CCCAGAGCTTGTACCG-3′; Syn III F: 5′-CCAACAG-CGACTCTCG-3′ and R: 5′-GGTTGCGGATTGTCTC-3′ (Kim et al. 2017a), SYT1 F: 5′-GTGAGTGCCAGTCGTCCTGAG-3′ and R: 5′-TTCATGGTCTTCCCTAAGTC-3′(Peng et al. 2002). The glyceraldehyde 3-phosphate dehydrogenase (GAPDH) primer was purchased from QIAgen. Three biologically independent experiments were performed, and each PCR reaction was performed in triplicate. The relative levels of the specific mRNA were calculated by normalizing them to the expression of GAPDH using the 2-ΔΔCt method. Additionally, the expression values of the RF-EMF-exposed groups were normalized to those of the sham-exposed group.

Western blotting

The hypothalamus dissected from the mice brain of sham-exposed mice or RF-EMF-exposed mice was lysed with RIPA Lysis buffer (Thermo Scientific, Rockford, USA) supplemented with protease and phosphatase inhibitor cocktails (Thermo Scientific, Rockford, USA). Whole lysates were then homogenized in ice-cold buffer and briefly sonicated. Protein concentrations were measured using a BCA protein assay (Thermo Scientific, Rockford, USA), and total proteins (20–50 μg) were separated using electrophoresis in an 8–10% sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) and transferred using transfer buffer to a polyvinylidene difluoride (PVDF) transfer membrane (GE Healthcare, Buckinghamshire, UK). Syn I, Syn II, SYT1, and α-tubulin were detected in the membrane using anti-Synapsin I antibody (1:1000, Abcam #ab64581), anti-Synapsin II antibody (1:3000, Abcam #ab76494), anti-Synaptotagmin 1 antibody (1:500, Cell Signaling Technology #3347), anti-Calcium Channel (a1 subunit) (1:1000, Sigma-Aldrich #C1103), and anti-α-tubulin (1:5000, Santa Cruz #sc-23948). The protein bands were visualized using an Odyssey infrared imaging system (Li-Cor Biosciences, Lincoln, NE). The intensity of each band was quantified and normalized using α-tubulin as an internal loading control

Measurement of mice body temperature after RF-EMF exposure

The mice body temperature was measured by using a special temperature measuring instrument, testo 925 (Kalibrier-Protokoll, Germany). A mouse rectal probe was inserted to a depth of 1.5 cm of the mouse's anus in each group of mice (control; $n = 8$, RF-EMF; $n = 8$). We measured their body temperature three times (once a week for 3 weeks) right after a 5-h sham or RF-EMF exposure.

Buried pellet test

The buried pellet test was performed based on a protocol from a previous study (Lehmkuhl et al. 2014). Briefly, individually housed mice (control; $n = 8$, RF-EMF; $n = 8$) were food restricted for 1 day prior to and during testing. The test was carried out 1 day after the 12 weeks of exposure to RF-EMF. For the buried pellet test, a clean mouse cage (15 × 25 × 13 cm) was filled with 3 cm of clean bedding. One piece of food pellet purchased from DBL (Chungbuk, South Korea)

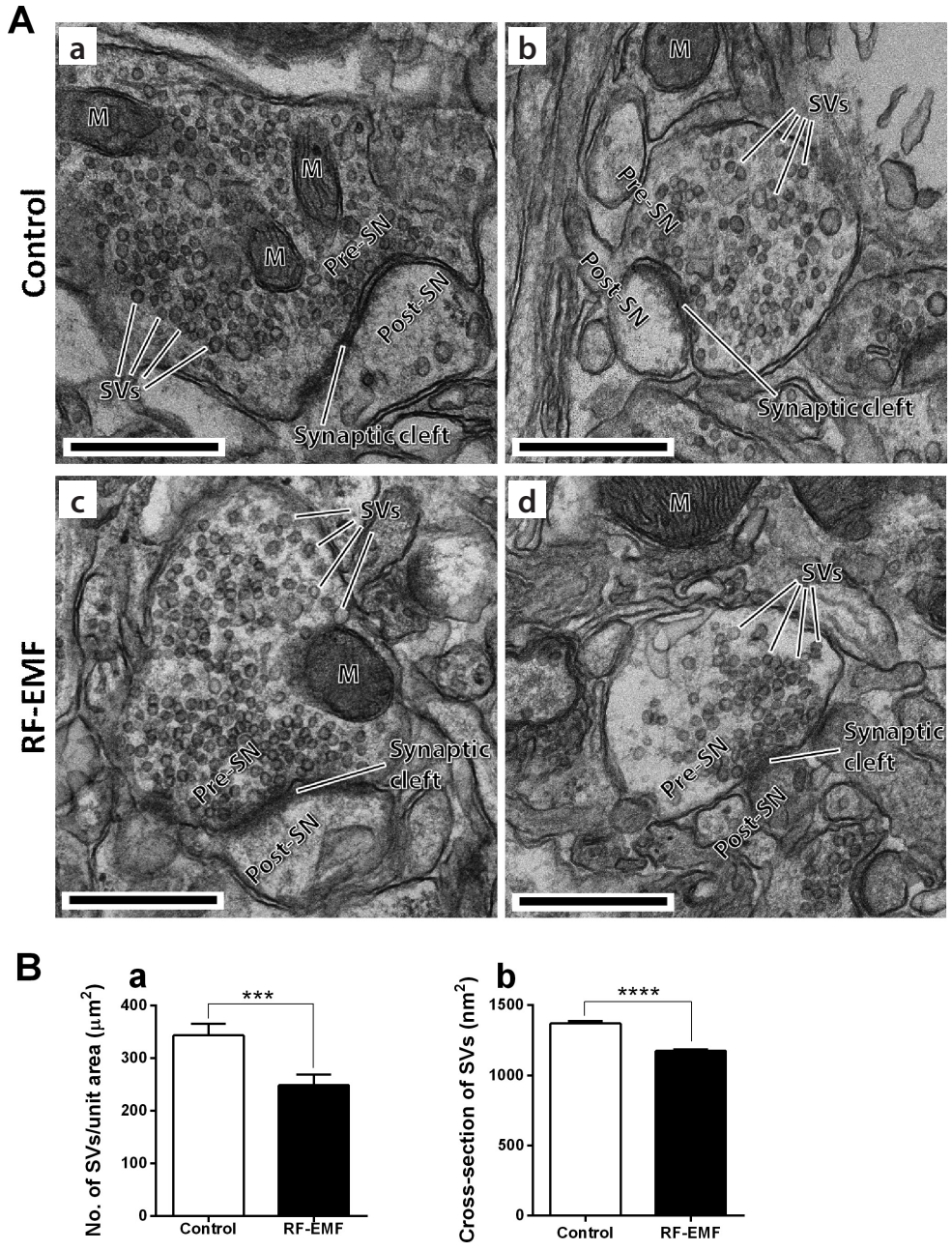

Figure 1. Number and size of the synaptic vesicles at the presynaptic boutons of the hypothalamic neurons. **A.** Representative TEM (transmission electron microscopy) micrographs of the synapse region in hypothalamic neurons of sham exposed (**a** and **b**) and RF-EMF exposed mice (**c** and **d**). M, mitochondria; Pre-SN, presynaptic neuron; Post-SN, post synaptic neuron; SVs, synaptic vesicles; scale bar size: 0.5 μm. **B.** Comparisons of the synaptic vesicle numbers (**a**; SVs /unit area (μm^2)) and size (**b**; the cross-section area (nm^2)) at the presynaptic nerve terminals of the hypothalamic neurons. Each bar represents the mean ± SEM. Statistical significance was evaluated by using a two-tailed unpaired Student's *t*-test. *** $p < 0.001$, **** $p < 0.0001$ ($n = 5$).

was buried along the perimeter of the cage approximately 1 cm below the bedding so that it was not visible. A mouse was then placed in the center of the cage and the latency to dig up and begin eating the cereal was measured. The exact time required to find the food pellet was observed with a CCD camera connected to a recording system.

Statistical analysis

Data are presented as mean ± SEM. The *n* values represent the number of animals used in the experiments. The statistical significance of the data was assessed using a Student's *t*-test with probability values. Significance was defined as follows: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. All statistical analyses were performed by using the GraphPad Prism 4 program (GraphPad Software, Inc, La Jolla, CA).

Results

Exposure to RF-EMF induced alterations in the size and number of the SVs in the presynaptic terminals of the hypothalamic neurons

To elucidate the possible effects of RF-EMF exposure on the hypothalamus, we studied the number and size of the SVs at the presynaptic terminal of hypothalamic neurons after 12 weeks of exposure to 835 MHz RF-EMF. The ultrastructural images of the synaptic terminals focusing on the SVs in the presynaptic terminals in hypothalamic neurons were collected randomly by TEM (Fig. 1A). We counted the number of SVs (*per* unit area) in the presynaptic terminals and measured the size of the SVs in 20 randomly selected synapses in both experimental groups. The data indicated that the density of the SVs (numbers/μm^2) was significantly reduced by approximately 30% in the RF-EMF-exposed group (248.5 ± 20.32/μm^2) compared to the control group (343.5 ± 22.08/μm^2) (Fig. 1Ba). Additionally, the size of the SVs (cross-section; nm^2) was also significantly decreased in the RF-EMF-exposed group (1174 ± 11.34/nm^2, from 1134 SVs) compared to the control group (1370 ± 15.69/nm^2, from 1806 SVs) (Fig. 1Bb).

Exposure to RF-EMF significantly decreased the number of SVs at the active zone in the hypothalamus

Neurotransmission is determined by the released neurotransmitters. Therefore, we further investigated the density (SVs numbers/μm) of the docking and fusing SVs at the active zones of the presynaptic terminal membrane. As a result of the docking and fusing of SVs with the membrane, the active zone forms blurry lines at the membrane of the presynaptic terminal (Fig. 2A). The number of SVs at the active zones was significantly decreased in the RF-EMF-exposed group (44.60 ± 2.597) compared with that in the control group (53.50 ± 3.911) (Fig. 2B). These results showed that the number of SVs at the active zones in the presynaptic terminal was significantly decreased and strongly suggested that hypothalamic neurotransmission is reduced by RF-EMF exposure.

Synapsin levels in the hypothalamus were significantly decreased by RF-EMF exposure

The synapsin I and II genes were analyzed using qRT-PCR to test whether RF-EMF exposure affects their expression level

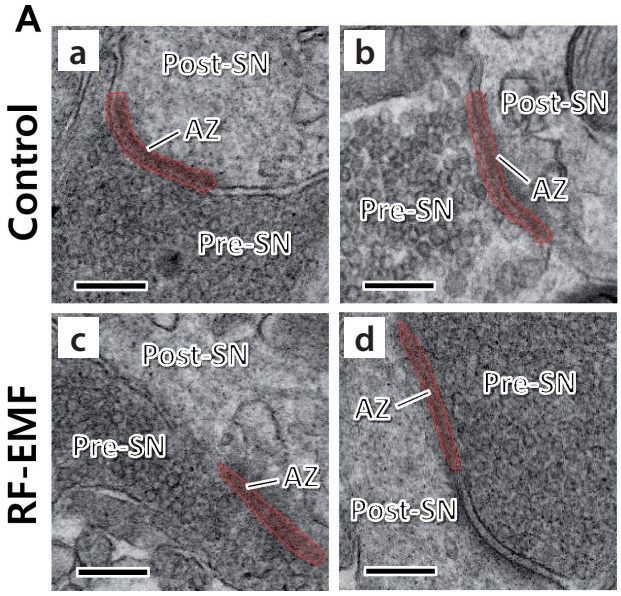

Figure 2. Number of synaptic vesicles (SVs) in the active zones of the presynaptic nerve terminals of the hypothalamic neurons. **A.** Electron microscopy images at an enhanced magnification in control mice (**a** and **b**) and RF-EMF-exposed mice (**c** and **d**) show the active zones, which were formed by docking SVs and fusion of the SVs to the membranes of the presynaptic terminals. The blurry lines (red; see online version for color figure) indicate the active zones in the presynaptic terminals. **B.** The number of SVs (SVs/unit area (μm^2)) in the active zone was calculated. AZ, active zone; Pre-SN, presynaptic neuron; Post-SN, post synaptic neuron (scale bar size: 200 nm). Statistical significance was evaluated by using a two-tailed unpaired Student's *t*-test. * $p < 0.05$ ($n = 5$).

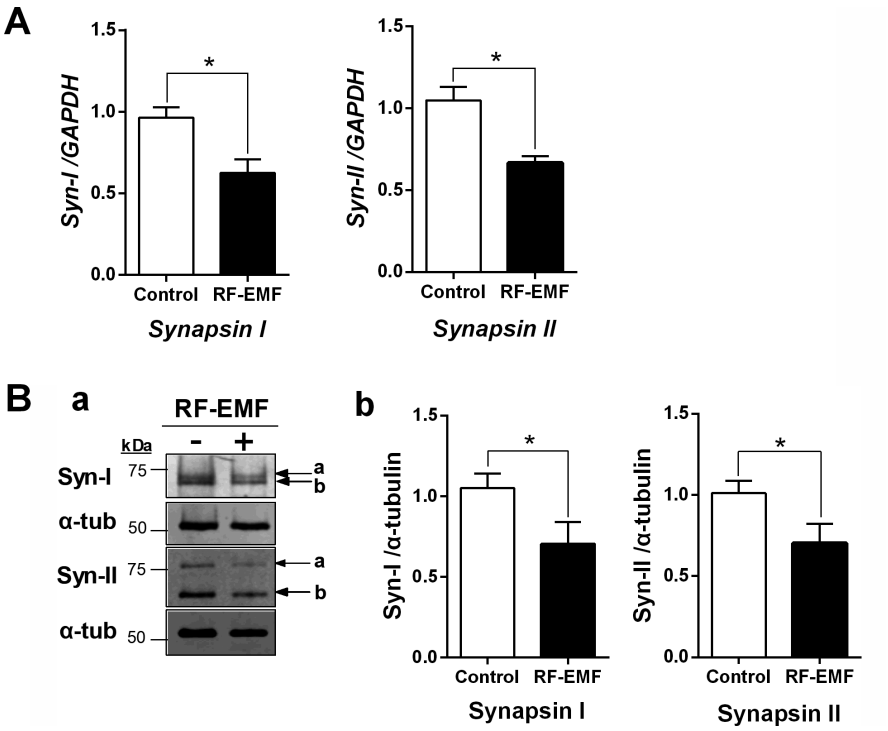

Figure 3. Expression changes of synapsin in the hypothalamus after exposure to RF-EMF for 12 weeks. **A.** Hypothalamic expression levels of synapsin I and synapsin II. Relative mRNA levels of synapsin I/II were significantly decreased by exposure to RF-EMF. **B.** Representative blotting images of synapsin I/II (**a**). Hypothalamic synapsin I and synapsin II protein levels were significantly reduced by RF-EMF exposure (**b**). Each bar represents the mean ± SEM. Statistical significance was evaluated by using a two-tailed unpaired Student's *t*-test. * $p < 0.05$ ($n = 5$).

in the hypothalamus. The mRNA levels of synapsin I and II in hypothalamus were significantly decreased after exposure to RF-EMF (Fig. 3A). The qRT-PCR results showed that the gene expression of synapsin I and II in the hypothalamus can be affected by 12 weeks of exposure to RF-EMF.

To validate the results of the qRT-PCR, we conducted an immunoblot using an anti-synapsin I/II antibody. Importantly, the anti-synapsin I/II antibody (Abcam) can detect both the mouse synapsin I/IIa and I/IIb subunits. The density of the synapsin bands were quantified *via* normalization with α-tubulin. The expression levels of both the synapsin I and II proteins were significantly decreased in the hypothalamus (Fig. 3B). Overall, these results indicated that synapsin I and II in hypothalamus were significantly decreased by 12 weeks of RF-EMF exposure.

The levels of synaptotagmin and voltage gated calcium channel in the hypothalamus were significantly decreased by RF-EMF exposure

The synaptotagmin 1 (SYT1) gene was analyzed using qRT-PCR to test whether RF-EMF exposure affects its expression level in the hypothalamus. The mRNA level of SYT1 in the hypothalamus was significantly decreased after exposure to RF-EMF (Fig. 4A). The qRT-PCR results showed that gene expression of SYT1 in the hypothalamus may be affected by 12 weeks of exposure to RF-EMF.

The expression changes in SYT1 and voltage gated calcium channels (VGCCs) were further studied. The expression level of SYT1 was significantly decreased in the hypothalamus following RF-EMF exposure (Fig. 4B). In parallel, the expressional level of voltage gated calcium channel was also significantly decreased by RF-EMF exposure (Fig. 4C).

Mice core temperature, body weight, and olfactory performance were not changed after RF-EMF exposure

To test whether RF-EMF exposure could influence core temperature, which is controlled by the hypothalamus, core body temperature was measured by inserting a mouse rectal probe. The core body temperatures were recorded three times (once a week for 3 weeks) right after a sham or RF-EMF exposure. The result showed that mice body temperature was not changed after RF-EMF exposure compared with the control group, suggesting that the core body temperature was not significantly affected by 835 MHz, 4.0 W/kg SAR for 5 h/day (Fig. 5A).

In addition, we measured body weight after sham or RF-EMF exposure for 12 weeks. The results indicated that the body weights of both groups were continually increasing during the experimentation period. However, we could not find any significant changes in body weight in the control vs the RF-EMF exposure group (Fig. 5B).

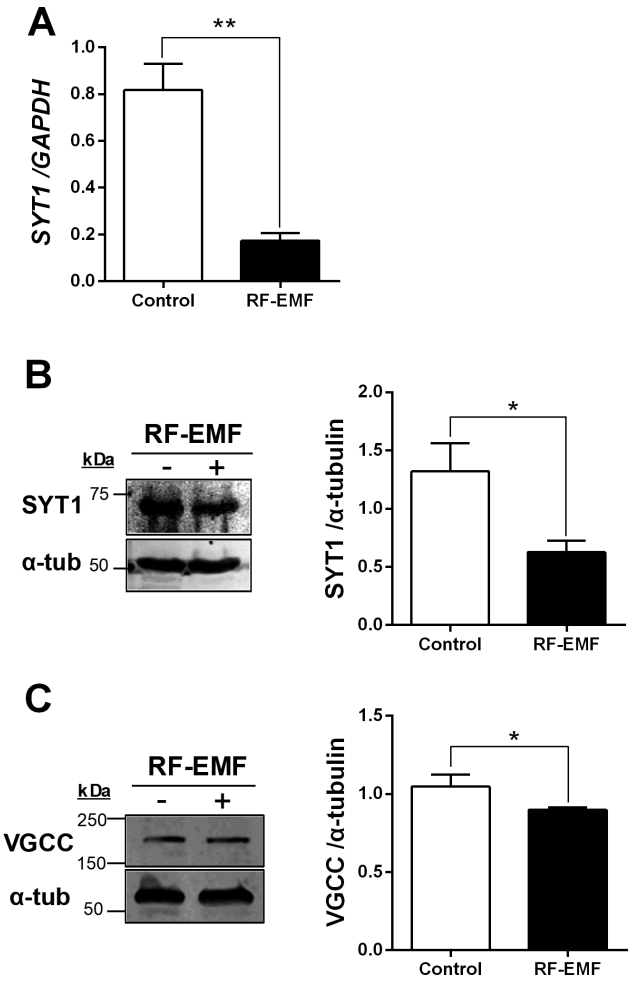

Figure 4. Expression changes of synaptotagmin 1 and voltage gated calcium channel in the hypothalamus after exposure to RF-EMF for 12 weeks. **A.** Hypothalamic expression levels of synaptotagmin I (SYT1). Relative mRNA level of SYT1 was significantly decreased by exposure to RF-EMF. **B.** Representative blotting images of SYT1. The expression levels of SYT1 protein in the hypothalamus were significantly decreased by RF-EMF exposure. **C.** Representative blotting images of voltage gated calcium channels (VGCC). The expression levels of VGCC in the hypothalamus were significantly decreased by RF-EMF exposure. Each bar represents the mean ± SEM. Statistical significance was evaluated by using a two-tailed unpaired Student's *t*-test. * $p < 0.05$, ** $p < 0.01$ ($n = 5$).

It is known that multi-synaptic neuronal pathways from the olfactory system transmit odor information to the hypothalamus (Klein et al. 2015). A buried pellet test was conducted to assess olfactory performance and the function of the hypothalamus. The buried pellet test revealed that there was no significant difference in the time to find the buried food pellets between control and RF-EMF-exposed mice (Fig. 5C).

Discussion

In this study, we found that the number and size of the synaptic vesicles of the hypothalamic neurons were significantly decreased in presynaptic terminals. Further, the number of docking synaptic vesicles at the active zone was also decreased by RF-EMF exposure. The present findings strongly suggest that hypothalamic neurotransmission could be reduced by 835-MHz, RF-EMF at 4.0 W/kg SAR for 5 h daily for 12 weeks. We further investigated the possible changes in core body temperature, body weight, and olfactory function, regulated by the hypothalamus but none of those phenotypic functions were not significantly changed.

Synapses are the connections between neurons that provide a mechanism for transferring information from one neuron to another by releasing neurotransmitters from presynaptic terminals. Neurotransmitters are stored in synaptic vesicles (SVs) in the pre-synapse and released by the docking and fusing of SVs with the membrane of the presynaptic bouton. The trafficking of SVs is regulated by various proteins such as synapsins, synaptotagmin, synaptophysin, synaptobrevin, VAMP, SNARE, etc (Brachya et al. 2006).

Synapsins have been suggested as key regulators of SV dynamics in presynaptic terminals (Vasileva et al. 2012). Therefore, any changes in these proteins may contribute to changes in neuronal SVs. It is known that mammals have three kinds of synapsin, each with at least two isoforms. Synapsins have been used as synaptic-vesicle markers in neurons (Hilfiker et al. 1999). The best-known function of synapsin proteins is to regulate synaptic transmission by controlling the storage and mobilization of SVs (Vasileva et al. 2012). We previously reported that RF-EMF exposure could lead to alteration in the number and size of SVs located in the cortex (Kim et al. 2017a), striatum (Kim et al. 2019b), and medial nucleus of the trapezoid body (MNTB) neurons in the auditory system (Kim et al. 2019a). The number of SVs was significantly decreased but the size of the SVs was significantly increased in cortical neurons and dopaminergic neurons of the striatum after RF-EMF exposure (Kim et al. 2019b). However, the number of SVs in MNTB neurons in the auditory system was increased by RF-EMF exposure (Kim et al. 2019a). These data indicated that different regions of the brain are differentially respondent to RF-EMF exposure.

The number and size of SVs were decreased significantly with synaptic I/II transcripts and proteins by RF-EMF exposure in hypothalamic neurons (Fig. 1 and Fig. 3). These data are consistent with previous studies with regards to the number of SVs but is inconsistent with previous studies in terms of the size of the SVs (Kim et al. 2017a, 2019a, 2019b). It has been previously reported that overexpression of synapsin Ia leads to a decrease in the size of SVs and active zones in the rat calyx of Held, thus mediating SV distribu-

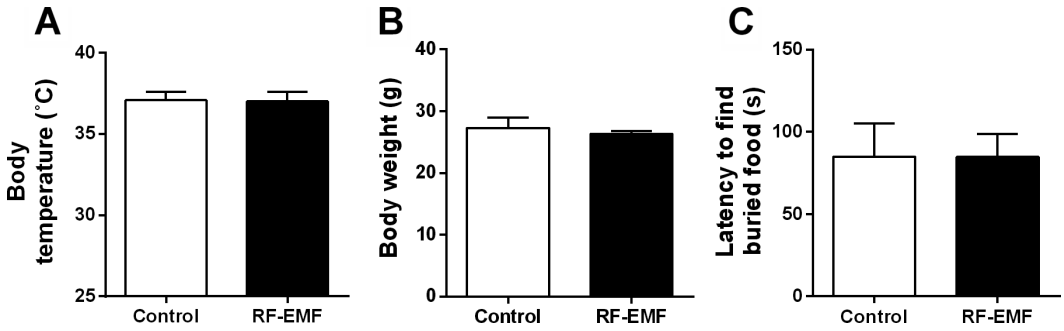

Figure 5. Core body temperature (**A**), body weight (**B**) and behavioral test of food finding (**C**) after 12 weeks 835 MHz RF-EMF exposure. No significant changes were seen between controls and the RF-EMF group (n = 8).

tion within the presynaptic terminal (Vasileva et al. 2013). Interestingly, the opposite phenotype was reported by the same group after deletion of all synapsin isoforms (Vasileva et al. 2012). These data strongly indicate that the expression levels of the synapsin proteins can regulate both SV and active zone size in presynaptic terminals.

Although synapsins are considered one of the key regulators of SV dynamics in presynaptic neurons, other regulatory proteins involved in SV trafficking are involved in the regulation of the size of SVs in hypothalamic neurons. Calcium ions play a key role in the regulation of neurotransmitter release, excitability, and synaptic plasticity (Neher and Sakaba 2008). The synaptic vesicle protein SYT1 acts as a key Ca^{2+} sensor for fast synchronous synaptic vesicle exocytosis (Geppert et al. 1994; Li et al. 2017). The calcium homeostasis in neurons can be regulated by several types of calcium channels, including VGCCs. VGCCs are responsible for fast calcium influx into the cell, which controls the entry of calcium ions across the plasma membrane. The expression of VGCCs was also significantly reduced at the hypothalamus in response to RF-EMF exposure (Figure 4C). Further, SYT1 interacts with the clathrin adaptor protein AP-2, thus suggesting a role in clathrin-mediated endocytosis and coupling synaptic vesicle fusion to retrieval (Li et al. 2017). The rate of endocytosis was slowed in constitutive SYT1 knockout neurons (Nicholson-Tomishima and Ryan 2004). The expression of SYT1 transcripts and proteins was decreased by RF-EMF exposure, suggesting that the retrieval of endocytosis is changed in the hypothalamus (Fig. 4). Together, the decreased expression of synapsin I/II, SYT1, and VGCCs by RF-EMF exposure may contribute to a decrease in the number and size of the SVs in hypothalamic neurons.

In this study, we further tested the possible changes in hypothalamic function because the trafficking of SVs were changed by RF-EMF exposure. The hypothalamus is part of the limbic system and is important for linking the nervous system to the endocrine system (Barron 2010). The hypothalamus has been known to regulate critical functions such as thermoregulation, appetite, thirst, fatigue, sleep and circadian rhythms (Humphries et al. 2008; Chughtai et al. 2009; Tyler and Allan 2014; Biran et al. 2015; Greenway 2015). Additionally, it is well-known that the hypothalamus is an important central regulator of the endocrine system, receiving chemosensory information which modulates distinct endocrine responses and neurodegenerative diseases, often accompanied by olfactory deficits (Meyer et al. 2018). We investigated the possible changes in core body temperature, body weight, and olfactory function, regulated by the hypothalamus. The core body temperature measured with the rectal probe was not significantly different in the RF-EMF-exposed group compared to controls (Fig. 5A). Also, we did not observe any difference in body weight increasing between the control and the RF-EMF-exposed group (Fig. 5B). The buried pellet test was performed to study olfactory performance via hypothalamic function. However, there was no significant difference between the control and the RF-EMF-exposed mice (Fig. 5C). Therefore, the effect of changes in SV trafficking caused by exposure to RF-EMF on specific behavioral phenotypes are currently not known. However, it is possible that the phenotypic changes could be observed in a hypothalamic disease model because of disturbing the hypothalamic neurotransmission of disease. Future studies are needed to address this question.

In summary, the number and size of SVs were significantly decreased by exposure to 835 MHz RF-EMF (SAR of 4.0 W/kg for 5 h/day for 12 weeks). Moreover, the number of SVs in the active zone was decreased, suggesting that trafficking of SVs in hypothalamic neurons was affected by RF-EMF exposure. In parallel, synapsin I/II and SYT1, two regulatory factors of SV trafficking, were significantly decreased in hypothalamic presynaptic neurons. The expression of VGCCs was also significantly reduced at the hypothalamus, suggesting hypothalamic calcium levels could be affected in response to RF-EMF exposure. Although, trafficking of SVs was altered by exposure to RF-EMF, it was not enough to induce any phenotypical changes in our

experiments (835 MHz RF-EMF, SAR of 4.0 W/kg for 5 h/day for 12 weeks). However, the trafficking of SVs at hypothalamus was affected significantly by RF-EMF exposure, the possible biological effects of hypothalamic neurons after exposure to RF-EMF should be further studied in various exposure condition.

Acknowledgements. This study was supported by the research fund of Dankook University in 2017 (R201601332).

Conflict of interest. The authors declare that there are no conflicts of interest.

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Received: April 9, 2019
Final version accepted: May 15, 2019
First published online: August 14, 2019