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Influence of Whole Body Exposure of 914 MHz Radiofrequency Identification on Endocrine system

by

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Major in neuroscience
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A Dissertation Submitted to The Graduate School of Ajou University in Partial Fulfillment of The Requirements for The Degree of Ph.D. in Neuroscience

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Radio frequency identification (RFID) is one of the currently introduced wireless radio frequency (RF) systems and is generally used in industrial and everyday life. However, the possible biologic effects of RFID radiation on human health, particularly brain function, remain unclear. Epidemiological studies suggest that exposure to RF electromagnetic field (EMF) may be a dormant risk factor in human health; therefore I expect it may affect the mammalian brain function. To study the effect of RFID exposure on rat brain function, I focused on neuroendocrine system including thyroid hormone system and melatonin, and cerebral metabolism, especially glucose metabolism. For these animal trials, a reverberation chamber was used as a whole-body exposure system. RFID exposure trial was performed during the day for the studies except for a study regarding the pineal melatonin biosynthesis, as RFID exposure during the night is more valid. The whole-body average specific absorption rate (SAR) was 4 W/kg for field of the RFID for all the experiments.

Although some transient changes in serum thyroid hormones were observed in the separate 2, 4, 8, and 16 week exposure experiments, serum level of TSH and thyroid hormones were not influenced in this study. Nocturnal 8 h RFID exposure, at SAR of 4 W/kg, caused a reduction of a 24 h urinary secretion of 6-OHMS, melatonin metabolite, and its diminution degree shows a pineal Aanat transcriptional level-dependent manner. Decreased levels of
AANAT enzyme activity and protein were observed in RFID exposed group compared to sham exposed group. Moreover, level of CREB phosphorylation in pineal gland was reduced after RFID exposure. Consequently, reduced expression of *Aanat* mRNA was also observed in RFID-exposed group. But, no significant change was found after RFID exposure in protein kinase A (PKA) enzyme activity which is known as a key enzyme in phosphorylation of AANAT and CREB in pineal gland at night. These results indicate that nocturnal RFID exposure reduces *Aanat* transcription which is ultimate causes of reduction of melatonin synthesis including protein level of AANAT and, AANAT activity. I investigated the RFID exposure influence on rat cortical glucose metabolism by using $^{18}$F-deoxyglucose positron emission tomography (FDG-PET). The relative cerebral glucose metabolic rate was unchanged in the frontal, temporal and parietal cortices of the RFID-exposed rats, compared with rats in cage-control and sham-exposed groups.

To evaluate the effect of RFID exposure on rat brain function, I had studied three different systems including cerebral glucose metabolism, thyroid system, and pineal melatonin synthesis. Taken together, my scientific data may, at least in part, provide evidence that rat brain can be influenced by RF exposure.

Key words: 914MHz RFID, thyroid hormone, melatonin, FDG-PET, AANAT
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LIST OF ABBREVIATION

RFID, radiofrequency identification
RF, radio frequency
EMF, electromagnetic field
SAR, specific absorption rate
ELF-EMF, extremely-low-frequency electric and magnetic fields
AANAT, arylalkylamine N-acetyltransferase
cAMP, cyclic adenosine monophosphate
6-OHMS, 6-hydroxy melatonin sulfate
PKA, protein kinase A
NE, norepinephrine
T3, triiodothyronine
T4, thyroxine
TSH, thyroid stimulating hormone
FDG-PET, 18F-deoxyglucose positron emission tomography
ROI, regions of interest
GLUT, glucose transporters
CREB, cAMP response element (CRE)-binding protein
This certifies that the dissertation of Hye Sun Kim is approved.

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I. INTRODUCTION

Rapid expansion of the mobile telecommunication services over the last decade has dramatically increased the amount of electromagnetic field (EMF) irradiation and energy in the environment. EMF energy is categorized as thin non-ionizing band of electromagnetic spectrum. EMF technology was designed and developed to optimize energy efficiency and convenience. Many epidemiological reports have suggested that electromagnetic fields (EMF) may have adverse effects on human health (Marino et al., 2003; Lim et al., 2005; Dubey et al., 2010; Sonmez et al., 2010), which might have considerable public health consequences. However, its biologic effect of EMF is still not clear.

Human beings are one of the bioelectrical organisms and their organs, especially heart and brain, are controlled by internal bioelectrical signals. Although several reports have analyzed the responses of the mammalian nervous systems to EMF exposure, no definite evidences has yet been demonstrated the effects of EMF exposure on the central nervous system (CNS) which is regulated by bioelectric and neuroendocrine signals (Marino et al., 2003; Lim et al., 2005; Dubey et al., 2010; Sonmez et al., 2010). Thus, more scientific studies are needed to evaluate effect of EMF on cerebral functions.

A. What is RFID?

1. RFID concepts

Radiofrequency identification (RFID) has recently been introduced, which is an alternative technology with a potential to replace the traditional object identification system. The RFID system comprises of a tag and a reader, which enables identification of an object
from a distance without requiring line of sight. RFID readers are transceivers which transmit
and receive at the same time with the same frequency RFID enable identification of object
from a distance (Kumar et al., 2009). 860-960 MHz band is the most widely used for object
processing management worldwide (Habash et al., 2009).

2. **International guideline of RF energy absorption**

   According to the international guidelines by ICNIRP (International Commission on Non-
   Ionizing Radiation Protection, 1998), the RF energy absorbed workers and for general public
   through whole body was limited by 0.4 W/kg and 0.08 W/kg, respectively. International
guidelines recommended that excessive exposure to RF should not exceed 4 W/kg SAR
   which is threshold for behavioral disruption (Brainard et al., 1999; Hata et al., 2005; Ho and
Chik, 2010). Electric field-induced current and magnetic field- induced current density
within subject is proportional to frequency and field magnitude. Linear dimension of the
subject is approximately proportional to magnetic field-induced current density. Therefore
higher electric field level and higher magnetic field in rat experiment were required to rat
experiment to achieve induced current densities comparable with those in humans at lower
field. For rodent, scaling factor of electric field is 12 and magnetic field are is between 5 and
15, respectively (Brainard et al., 1999). Therefore, 4 W/kg of SAR value was applied in
present animal experiment.

3. **RFID transmitting system**

   Unlike the situation in cellular phones, an RFID transmitting system does not usually
come into contact with body parts, such as the head or hands. Additionally, large RFID
systems irradiate RF to the entire body due to the physical size of the field that is used to
scan for objects. The output power of a cellular phone varies according to the quality of the connection between the base station and the phone, whereas the output power of an RFID system is constant. Therefore, those experiments should be performed using the whole body exposure method instead of focal exposure method.

B. Effects of EMF Exposure on Thyroid System

1. Thyroid system

Thyroid gland is one of the largest endocrine organ and produce thyroid hormones, the principal ones being triiodothyronine (T3) and thyroxine (T4). These hormones are critically important for metabolic homeostasis and are regulated by thyroid stimulating hormone (TSH) secreted from the pituitary gland, which itself is regulated by thyrotrophin-releasing hormone (TRH) produced by the hypothalamus (Koyu et al., 2005a) (Fig. A). Serum concentrations of thyroid hormones are altered by changes in the environment. Thyroid activity has a positive correlation with serum TSH, a major factor influencing the thyroid systems (Cheng et al., 2010).

2. Biological roles of thyroid system

Thyroid hormones and TSH have prominent effects on differentiation, growth, development and metabolism of nearly all organs in the body (Yen, 2001; Pinazo-Duran et al., 2011). Thyroid hormones are required to carry out normal tissue functions and have major effects on oxygen consumption and carbohydrate, lipid, and protein metabolism (Yen, 2001; Weitzel and Iwen, 2011). For this reason, even a small change in circulating thyroid hormone levels is sufficient to alter the body’s functions (Bernal, 2007; Bauer et al., 2008).
3. Effects of EMF Exposure on thyroid system

Animal studies have shown that exposure to RF and EMF may alter the endocrine or nervous systems, particularly, thyroid hormones and TSH secretion (Koyu et al., 2005b; Aydin et al., 2009; Dell'Omo et al., 2009; Dundar et al., 2009; Fonken et al., 2009; Shen and Zhao, 2010). Many epidemiological reports have suggested that electromagnetic fields (EMF) may have adverse effects on human health (Marino et al., 2003; Lim et al., 2005; Dubey et al., 2010; Sonmez et al., 2010). However, the biological health effects of an RFID system have not been studied.

Fig. A. Hypothalamic-Pituitary-Thyroid Axis This is a part of the neuroendocrine system which is responsible for the regulation of body metabolism. Pathway for hormonal regulation of the thyroid hormones was shown. (T3, triiodothyronine; T4, thyroxine; TRH, thyrotrophin-releasing hormone; TSH, thyroid-stimulating hormone) (Pluta et al., 2010).
C. Effect of EMF Exposure on Melatonin Synthesis.

1. Biological roles of melatonin

Melatonin is produced at night by the pineal gland which is a photoneuroendocrine organ of brain. Therefore, pineal gland is central organ in regulation of diurnal rhythm via melatonin secretion. Melatonin plays important roles in reproduction (Dardente, 2012), ageing (Yoo et al., 2012), sleep (Mueller et al., 2011; Harrington, 2012), brain protection (Crupi et al., 2011; Morley-Fletcher et al., 2011; Mueller et al., 2011; Yoo et al., 2012), and oxidative stress (Calamini et al., 2008; Dayoub et al., 2011; Wang et al., 2011; Peng et al., 2012); even a small change in melatonin homeostasis is sufficient to alter the body’s functions.

2. Regulation of melatonin biosynthesis

In vertebrates, the rate of melatonin production is controlled by arylalkylamine N-acetyltransferase (AANAT) enzyme activity, rate-limiting enzyme in melatonin biosynthetic pathway (Gastel et al., 1998; Borjigin et al., 2012). The biosynthesis of the melatonin involves the conversion of serotonin (5-hydrosytryptamine) to N-acetyl serotonin (5-hydroxy-N-acetyltryptamine) catalyzed by AANAT. Melatonin is produced as final metabolic product by methylation of the 5-hydroxy moiety by 5-hydroxyindoles-O-methyltransferase (HIOMT) (Ho and Chik, 2010). In rat pineal gland, nocturnal increase of Aanat gene transcriptional leads to AANAT activity, which mainly leads to parallel increase in melatonin synthesis (Gastel et al., 1998) (Fig. B). It is well known that biochemical pathways of melatonin synthesis by multitude of neurotransmitter and neuromodulator (Karolczak et al., 2005).
3. Transcriptional regulation of Arylalkylamin-N- transferase

In rat pineal gland, *Aanat* gene transcription at night is crucially regulated by increased norepinephrine (NE). Norepinephrine activates the adenylate cyclase / protein kinase-A (AC/PKA) pathway, which lead to the phospholylation of cyclic AMP response element-binding protein (CREB). Phosphorylated CREB is bound to the *Aanat* gene promoter via cyclic AMP-response element (CRE) followed by increase of *Aanat* gene transcription (Ho and Chik, 2010) (Fig. C).

4. Effect of EMF exposure on melatonin synthesis

It was reported that Electromagnetic field (EMF) exposure is the dormant risk factor controlling melatonin biosynthesis. Extreme low frequency magnetic field (ELF-MF) exposure can reduce the AANAT activity (Selmaoui and Touitou, 1995; Wilson et al., 1999) and consequently, melatonin concentration (Selmaoui and Touitou, 1995; Yellon, 1996; Wilson et al., 1999). In addition to ELF-MF, radiofrequency electromagnetic field (RF-EMF) is also capable of decreasing the melatonin concentration *in vivo* (Kesari et al., 2011, 2012) and *in vitro* (Sukhotina et al., 2006). However, regulation mechanisms underlying these effects of ELF-MF or RF-EMF are largely unknown. Furthermore the biological effect of RFID exposure on melatonin synthesis has not been examined. In order to determine regulation mechanism of exposure to RFID action of pineal gland, this study was undertaken to evaluate whether the melatonin synthesis is directly sensitive to RFID exposure and to examine a possible involvement of phospho-CREB and *Aanat* transcription in the suppressive action of RFID exposure on melatonin synthesis.
Biosynthesis of melatonin involves the conversion of serotonin (5-hydroxytryptamine) to N-acetyl serotonin (5-hydroxy-N-acetyltryptamine) catalyzed by arylalkylamine N-acetyltransferase (AANAT). Melatonin is produced as a final metabolic product by methylation of the 5-hydroxy moiety by 5-hydroxyindoles-O-methyltransferase (HIOMT) (Ho and Chik, 2010).
Fig. C. Transcription of Aanat in the presence of norepinephrine (NE) During night, activation of β1- adrenergic receptor (β1-Adr) on pineal gland does initiate an activation of Aanat mRNA expression through phosphorylation (P) of the transcription factor cyclic adenosine monophosphate (cAMP) response element-binding protein (Creb) by protein kinase A (PKA), cAMP-signal transduction pathway (Ho and Chik, 2010).
D. Effects of EMF Exposure on Cerebral Metabolism

1. Cerebral glucose metabolism

Cerebral metabolism, based on neuronal activity, is closely related to glucose consumption (Zimmermann et al., 1993; Barros et al., 2005; Harris et al., 2005), because glucose is the major energy source of the brain in normal activity. Cerebral glucose metabolism is a more sensitive indicator of neural activity and is more closely related with cerebral metabolic activity (Kwon et al., 2011). There are several reports demonstrating that the correlation between regional glucose metabolism and neural activity is reliable (Barros et al., 2005; Harris et al., 2005). It is still controversial as to whether cell-phone RF-EMF increases the risk of brain cancer (Ragbetli et al., 2009) (Lee et al., 2011).

2. Effects of EMF on cerebral metabolism

Epidemiological studies suggest that exposure to RF-EMF may be a dormant risk factor in human health; it can affect cerebral blood flow (Haarala et al., 2003; Aalto et al., 2006), and glucose metabolism in the brain (Kwon et al., 2011; Volkow et al., 2011). Some studies using immunohistochemical evaluations have demonstrated that RF-EMF radiation induced morphological changes in neural cells, and it has been suggested that RF-EMF radiation prompted changes in neural activity (Maskey et al., 2012). Although the functional activity of neural cells can be suggested from immunohistochemical studies, such anatomical findings are, at best, indirect evidence of neural cell activity. Glucose metabolism, together with oxygen consumption, is an important indicator of neural activity (Rapoport et al., 2012). Recently, the World Health Organization (WHO) changed its position from there being no link between cell-phone RF-EMF and brain cancer to listing the RF-EMF of cell phones in the 'carcinogenic hazard' category (Moussa, 2011). As glucose consumption increases
markedly in cancer (Koolen et al., 2012), it is important to monitor changes in glucose metabolism, especially under conditions of RF-EMF exposure.

3. Small animal PET scanning using $^{18}$F-FDG

Positron emission tomography (PET) scanning is a useful imaging tool for estimating cerebral activities such as cerebral blood flow and cerebral glucose consumption (Lancelot and Zimmer, 2010). Small animal PET (Fig. D) scanning now permits *in vivo* evaluations of physiological processes and biochemical pathways using numerous radiotracers (Xi et al., 2011) (Lancelot and Zimmer, 2010). $^{18}$F 2-fluoro-2-deoxy-d-glucose ($^{18}$F-FDG) is the $^{18}$F-isotope-labeled glucose analog, with a 109-min half-life (Kwon et al., 2011). Intravenously infused FDG is transported into brain cells by reversible glucose transporters (GLUT) in 30-40 min and levels of phosphorylated FDG trapped within the cytoplasm remain relatively constant for about 2 h (Barros et al., 2005; Lancelot and Zimmer, 2010; Kwon et al., 2011). Previous PET studies suggested possible harmful effects of RF-EMF on cerebral metabolism, based on experimental results indicating changes in induced cerebral blood flow (Haarala et al., 2003; Aalto et al., 2006). However, only a few PET studies have demonstrated RF-EMF-related changes in cerebral glucose metabolism in humans (Kwon et al., 2011; Volkow et al., 2011). To date, no animal PET study regarding the influence of RF-EMF exposure on cerebral glucose metabolism has been reported.

In order to determine effects of RFID exposure of brain function, this study was undertaken to examine whether cerebral glucose metabolism is sensitive to RFID exposure by using $^{18}$F-FDG PET scanning in rats.
Fig. D. Schematic representation of the principle underpinning PET (a) The cyclotron creates the positron-emitting radionuclides. (b) These radionuclides are incorporated into molecules during the radiosynthesis step giving the radiotracer ($^{18}$F-FDG) after radiopharmaceutical controls. (c) FDG PET scans are acquired following intravenous injection of the radiotracer. Two photons are detected by the PET camera and simultaneously localized within a fixed period of time by a series of opposing detectors, which correspond to multiple rings of scintillation crystals. (d) By collecting a statistically significant number of radioactive events, mathematical algorithms reconstruct a three-dimensional image that shows the distribution of the positron-emitting molecules in the brain (Lancelot and Zimmer, 2010).
E. Aims of this Study

As a part of investigation of the potential risks of RFID to human health, I hypothesized that 914MHZ RFID exposure may have an influence, at least in part on neuroendocrine systems and cerebral metabolism.

I studied whether thyroid system can be influenced by RFID exposure. For this purpose, I determined

1. whether the 914 MHz RFID exposure to rats can cause any change in serum level of thyroid hormones such as T3 and T4, and thyroid stimulating hormone (TSH).
2. whether morphological changes in thyroid gland follicles can be induced by RFID exposure.

I studied whether melatonin biosynthesis can be influenced by nocturnal RFID exposure. For this purpose, I determined

1. whether urinary level of melatonin metabolite can be changes by diurnal and nocturnal RFID exposure.
2. whether nocturnal RFID exposure-induced reduction of melatonin biosynthesis is related with alteration of AANAT enzyme activity and expression of AANAT protein.
3. whether RFID exposure down-regulates Aanat mRNA expression.
4. which mechanisms of Aanat gene transcription is related to Aanat mRNA expression in nocturnal RFID exposure-induced reduction of melatonin biosynthesis.

I also observed whether cerebral glucose metabolism was affected by exposure to 914 MHz RFID by using FDG-PET scanning in rats.
II. MATERIAL AND METHODS

A. Whole-Body RFID Exposure System

1. Composition of Reverberation chamber

A whole-body exposure system for animal experiments (IRETEC, Anyang, Korea) with 914 MHz RFID was developed using a reverberation chamber (Jung et al., 2008; Wu et al., 2010). This chamber has been validated under the supervision of the Korea Electromagnetic Institute Engineering Society (KIEES, Seoul, Korea; Fig. E). The internal dimensions of the reverberation chamber were 2,400 × 2,325 × 1,616 mm³ and the wall thickness was 125 mm. A wooden table (1400 mm in length, 1000 in width and 700 mm in height) was placed in the center of the chamber and eight cages could be placed on the table without any change in the field uniformity. The size of the cages was 390 mm × 235 mm × 180 mm. The exposure level and time were controlled by a computer.

2. Measurement of field uniformity

The field distribution and field uniformities in the reverberation chamber were tested by direct measurements, using a 3-axis isotropic probe (HI-6105; ETS-Lindgren, Cedar Park, TX, USA).

3. Calculation of SAR value

The specific absorption rate (SAR) distribution for a caged rat was calculated from the measured electric field strength using a commercial finite-difference time-domain tool (XFDTD version 6.5; Remcom, State College, PA, USA) for the incident plane waves in six orthogonal directions with two polarizations, as described previously (Jung et al., 2008).
The calculated SAR values were then averaged and scaled to the measured root mean squared (RMS) electric field strength to estimate the actual SAR for a given input power. It should be noted that electromagnetic waves come from all directions with equal amplitudes in the experimental region of the reverberation chamber. The rat model of the American Airforce Research Lab (AFRL, Dayton, OH, USA) was used for the simulation. To calculate the variation in SAR, I used a scaled rat model from a grown-up AFRL rat model. This model has 36 tissues, a mass of 339 g and a voxel size of $0.39 \times 0.39 \times 0.42 \text{ mm}^3$. For each trial, three or more cages (two rats per cage) were placed on the table in the chamber. Usually, the whole body SAR is calculated for a single rat. SAR values are changed and two rats are exposed. SAR values are dependent on the relative position of two rats. Our analysis shows that the whole body SAR of each rat is 96 % of that for single rat exposure due to the blocking effect, when two rats are very close (1 mm apart). In most cases, however, the distance between two rats will be quite larger than 1 mm. So the whole body SAR in real situation will be 3.84 -4.0 W/kg. Based on the SAR calculations the input power was set to 56 W which produced a whole-body averaged SAR of 4 W/kg in rats. Room temperature of $22 \pm 2 ^\circ C$ was controlled with a ventilation system and its maintenance in both sham chamber

![Fig. E. Schematic of the 914 MHz radiofrequency identification (RFID) exposure device (reverberation chamber)](image-url)
and a chamber for RFID exposure trial was monitored. Data provided by Prof. Jeong-Ki Pack, Department of Radio Sciences and Engineering, College of Engineering, Chungnam National University.

B. RFID Exposure Conditions

A continuous wave 914 MHz signal was generated using a standard protocol (Class-1, Generation-2) (Fig. F) using an RFID module (Model RFID ANT-08; Kortcom, Anyang, Korea) and amplified by a pre-amplifier (Model RFID PAM-08; Kortcom) and a power amplifier module (Model RFID PA-08; Kortcom). The RFID signals modulated by amplitude shifted keying (ASK) were delivered to the rats continuously without any back-scattered reflection because the rats are not attached to any tag. The Fig. F shows a typical pulse shape and modulated signal (EPC global, 2005). Additionally, the link timing diagram and the real RFID signal captured in the oscilloscope are described in the Fig. F. The maximum output power of the system was 60 W that the exposure level and exposure time are set up by a computer. An 11-bit digital personal identification number diode attenuator (Model 349; General Microwave, Farmingdale, NY, USA) was used to control output power level. The amplified RFID signal was then supplied to the chamber.
Fig. F. Modulation protocol of radiofrequency identification (RFID) Signal (A) baseband and modulated waveforms as generated by an Interrogator for Amplitude Shifted Keying (ASK) modulation (B) Link timing diagram and real signals (i) Interrogator diagram  (ii) Waveforms captured on the oscilloscope
C. Animal Experiments

Sprague Dawley (Deahan Biolink, Seoul, Korea) rat were used. Male rats (6 weeks old, weight 200-250 g at the start of the experiments) were used to avoid the additional complexity of changing hormones in female rats. Animals were kept under laboratory conditions at a temperature of 22±2 °C and maintained under a 12/12-h light/dark cycle. Water and food (Dea-Han Biolink) were supplied ad libitum. Access to tap water and pelleted food was omitted during the exposure times. The RFID experiment was performed using whole body exposure methods since an RFID transmitting system usually comes into contact with whole body.

For all animal experiments for my thesis, rats were randomly assigned to the following three groups: the cage-control group; the sham-exposed group; and the RFID-exposed. Rats in the sham-exposed group were placed in the RFID system without exposure to RFID. Those in the cage-control group were not placed in the RFID exposure system at all. Body temperature was measured using a rectal temperature probe and monitored daily before and after RFID exposure during the initial 2 weeks. Rats (n=10) were weighed biweekly and body changes were monitored for 2–16 weeks, depending on the duration of each trial. The investigation was carried out with the permission of the ethical committee (110405-25) on animal experiments of Ajou University School of Medicine (Suwon, Korea).

1. RFID exposure experiment for thyroid system hormones

Animals were randomly assigned to the following three groups: the cage-control group (n = 6–12); the sham-exposed group (n = 6–12); and the RFID-exposed group (n = 6–12). Three or more cages (two rats/cage) were placed on the table for each trial. All the RFID
exposure experiments were performed during the daytime. The cage containing the rats in the RFID-exposed group were exposed to RFID for 8 h daily, 5 days per week, for 2, 4, 8, and 16 weeks (n = 12 for 2, 4, and 8, weeks and n = 6 for 16 weeks)(Fig. G).

Fig. G. Experimental schematics of effects of RFID on thyroid
2. RFID exposure experiment for melatonin synthesis

Animals were randomly divided into three groups (n=210, n=60-70 in each group), as follows: the cage-control group, sham-exposed group and RFID-exposed group. Animal studies were repeated several times. The RFID exposure experiments were performed during the day time (10:00-18:00) or night time (22:00-06:00). Rats (n=40, n=10 in each group) in Sham-exposed and RFID-exposed group were further divided into two groups according to daily exposure dose; the 1 h RFID-exposed group which is exposed for 1 h (short-duration exposure) and the 8 h RFID-exposed group which is exposed for 8 h (long-duration exposure) daily (Fig. H). The experimental rats in RFID exposure group were also further divided into two according to when the exposure time is in a day, diurnal exposure group which is exposed during the day-time and nocturnal exposure group which is exposed during the night-time, because melatonin is a hormone of darkness and its secretion is markedly increased during the nighttime. After several RFID exposure experiments to measure the urinary melatonin metabolite, I decided to perform only the 8 h (so called, long duration) exposure trial as 8 h RFID exposure was required to induce a significant changes in urinary secretion of a melatonin metabolite and 1 hr (short duration) is not enough.

To exclude thermal effect from experimental results of relationship between melatonin...
and nocturnal RFID exposure, changes in temperature inside the reverberation chamber and rectal temperature in each rat (n=10) before and after RFID exposure were monitored for three times repeatedly and compared during the experiment. The rats were sacrificed 2 day after RFID exposure. Time delay for two days is obliged as 24 hr urine collection after RFID exposure is needed. Especially to study influence of nocturnal RFID exposure, the rats were sacrificed at eleven o’clock during the night to obtain the nocturnal pineal gland. To avoid any unintended light exposure during the specimen collection, only a dim red light was used.

3. RFID exposure experiment for cerebral glucose metabolism

Animals were randomly divided into three groups (n = 6 in each group) as follows: the cage-control group, sham-exposed group and RFID-exposed group. In this study, the RFID-exposure experiment started with a 2-week trial. A 16-week trial was performed sequentially as a separate experiment. These experiments were performed during the day-time hours. The experiment was performed using the whole-body exposure method. The cages in the RFID group were exposed to a RFID field for 8 h daily, 5 days per week, for 2 and 16 weeks. Those in the sham group were placed in the RFID system but with no RF exposure. Those in the cage-control group were not placed in the RFID exposure system at all. All rats were weighed biweekly, and the body changes were monitored for 2 to 16 weeks, depending on the duration of each trial.

D. Enzyme Linked Immunosorbent Assay (ELISA)

Serum level of norepinephrine (NE), T3, T4 and TSH and pineal PKA activity were measured using enzyme-linked immunosorbent assay (ELISA). Commercially available kits were used for T3 and T4 (Calbiotech, Sacramento, CA, USA), TSH ELISA kit (Cusabio
Biotech, Whuan, China) and Norepinephrine ELISA kit (Alfco, Salem, NH, USA), according to the manufacturer’s procedures. Briefly, rats were sacrificed with 10% chloral hydrate and blood from individual rats was collected into serum separator tubes without an anticoagulant (Becton, Dickinson, Franklin Lakes, NJ, USA). Blood was centrifuged (2000 × g, 10 min) to obtain the serum. Standards were assayed in duplicate, and samples were assayed in triplicate. Absolute values were expressed as the mean ± standard deviation (dl/μl for T3, T4, μIU/ml for TSH and nmol/L for NE).

The PKA activity was determined using commercially available PKA activity kit (Enzolife Science, Famingdale, NY, USA) according to the manufacturer’s protocols. Briefly, a pineal gland was individually disrupted by ultrasound in 80 mM buffer (T & I, Seoul, Korea, 20mM MOPS, 50mM ,beta -glycerolphosphate, 50mM sodium fluoride, 1mM sodium vanadate, 5mM EGTA, 2mM EDTA, 1% NP40, 1mM dithiothreitol (DTT), 1mM benzamidine). 20μl lysate was used for PKA activity test. Standards were assayed in duplicate, while samples were experimented in triplicate. PKA activity was adjusted by concentration of total protein by BCA methods. PKA activity of sham-and RFID exposure groups were normalized to that of cage-control group.

E. Haematoxylin and Eosin (H&E) Stain

Immediately after sacrificing the rats, thyroid glands were removed and prepared for standard histological sections or for semi-fine sections. Thyroid glands, with adjacent parts of the trachea and surrounding tissues, were fixed in 4% paraformaldehyde (Sigma, St. Louis, MO, USA) and then processed using standard paraffin embedding procedures. Then, the
glands were cut on a rotation microtome into 3 μm thick sections. Thyroids designated for semi-fine sections were removed from the trachea and were fixed in a 4% paraformaldehyde (Sigma), embedded in epon resin (Merck, Darmstadt, Germany) and cut on a LKB ultramicrotome (8800; LKB, Bromma, Sweden). Histological analysis of the gland was performed on paraffin slices stained with haematoxylin-eosin (H&E) (Sigma). Sections were observed under a bright field microscope (Olympus Optical, BX54, Tokyo, Japan). Bright field images were obtained using Picture Frame Application 2.3 software (Olympus Optical, Tokyo, Japan).

F. Gas Chromatography – Mass Spectrometry (GC-MS)

Urinary melatonin and serotonin secretion was determined using a GC-MS as described previously (Paik et al., 2010). Melatonin is metabolized to 6-hydroxymelatonin sulfate (6-OHMS), a stable metabolite which is excreted into urine (Zimmermann et al., 1993). Simultaneous analysis of melatonin, and serotonin in rat urine were measured using their (N,O)-ethoxycarbonyl/N-pentafluoropropionyl (EOC/PFP) derivatives by gas chromatograph – mass spectrometry (GC-MS) in selected ion monitoring (SIM) mode (GC-SIM-MS). Briefly, Total rat urine samples were collected for 24 h from rats 12h after RFID exposure for 2 weeks. 500μl of rat urine sample after exposure to RFID were prepared by adding Internal standard 1 (IS1; 200ng) and Internal standard 2 (IS2; 500ng were subjected to the aforementioned (N,O)-ethoxycarbonyl/N-pentafluoropropionyl (EOC/PFP) reaction prior to GC-SIM-MS. Data provided by Prof. Man-Jeong Paik, Department of Molecular Science Technology, Ajou University.
G. Liquid Biphasic Diffusion Assay (AANAT activity assay)

AANAT activity was determined as described previously (Chae et al., 1999). Briefly, pineal glands were individually disrupted by ultrasound in 80 µl ice-cold phosphate buffer (50 mM, pH 6.8). Debris was removed by centrifugation (15,000 g, 5 min at 4°C) and the supernatant was transferred to a new tube and stored at -70°C until use. An amount of 4 µl of the supernatant was then incubated in the presence of 5 µl tryptamine-HCl (10 mM, Sigma), 1 ml acetyl CoA (0.5 m, Sigma), and 1 ml [3H]-acetyl CoA (3.6 Ci/mmol, 250 Ci/ml, American radiolabeled chemicals, Inc, USA). Phosphate buffer (50 mM, pH 6.8) was added to yield a final volume of 20 µl. Incubation was done at 37°C for an indicated time and was stopped by diluting the reaction mixture with 180 µl ice-cold phosphate buffer (50 mM, pH 6.8, Sigma). The diluted reaction mixture was rapidly transferred to a vial containing 3 ml of Econofuor (1,2,4-trimethylbenzene >99%, Santa clara, CA, USA), 2,5-diphenyloxazole (0.7%, w/v), 1,4-bis(2-methylstyryl) benzene (0.05%, w/v). The amount of radiolabeled acetyltryptamine was determined in a liquid scintillation counter.

H. Western blot analysis

Rat pineal glands was washed with cold PBS and lysed on cold RIPA buffer (50 mM Tris-HCl (pH 7.6), 150 mM Sodium Chloride, 1% Triton X-100, 1% Na-deoxycholate, 0.1% SDS, 2 mM EDTA) containing protease inhibitor cocktail (T&I, Seoul, Korea), according to the manufacturer’s instruction. Each lysate was centrifuged at 10,000 g for 10 min at 4°C, and the supernatants were collected. Proteins were separated by SDS-PAGE, and transferred to a PVDF membrane. The membrane was incubated with antibodies against rabbit anti – AANAT antibody (1: 2000, Abcam, Cambridge, UK), rabbit anti-phospho-AANAT antibody
(1: 2000, Abcam), goat anti-Actin antibody (1:1000, SCBT, California, USA) ) Rabbit anti-CREB antibody(1:1000 cell signaling, Boston USA), rabbit anti-phospho-CREB antibody (1:1000 cell signaling).

I. Reverse Transcription and Polymerase Chain Reaction (RT-PCR)

Rat pineal glands were collected at night, and total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). 500 nanogram of total RNA per pineal gland was reverse-transcribed by using SuperScript III RT (Invitrogen), according to the manufacturer’s instruction. Quantitative RT-PCR was performed on first cDNA using primers (Bioneer, Deajoen, Korea) designed to detect pineal Aanat mRNA. Aanat primer sequences used were: sense primer 5’- TGA GCC CGA AGC CTT TAT CTC AGT-3’, and antisense primer 3’- TGT GGC ACC GTA AGG AAC ATT GCA-5’. Primers against Gapdh were used in separate reaction to normalized for total cDNA contents. Gapdh primer sequences used were: sense primer 5’- TCC CTC AAG ATT GTC AGC AA- 3’, and antisense primer 5’- AGA TCC ACA ACG GATA CAT T- 3’. The amplified products were separated by electrophoresis on a 1% agarose gel, and detect under UV light. Band intensities were analyzed using Quantity One 1-D analysis software, v 4.6.5 (Bio-Rad., Hercules, CA, USA).

J. Micro FDG PET

PET scanning was performed on the day after the last exposure to RFID. Each rat (which had been fasted for at least 12 h, n=6) was anesthetized with isoflurane (15 mg/kg, Sigma) and 63 MBq of FDG (Carecamp, Suwon, Korea) was injected through the tail vein. After 30 min to allow distribution of the intravenously infused FDG uptake, brain images of the rat
were acquired in a high-resolution eXplore Vista PET scanner (GE Healthcare, Piscataway, NJ, USA). Each rat was scanned for 30 min in a prone position with its brain centered in the axial and transaxial fields of view (Fig. I).

PET images were reconstructed with a three dimensional ordered subsets expectation maximization algorithm, with corrections for decay, detector dead time, scatter and random coincidences. The final image resolution in the central field of view (FOV) was less than 1 mm at full-width-half-maximum (Liu et al., 2009; Sung et al., 2009). For semiquantitative evaluation of the data obtained, a Xeleris functional imaging work station (GE Healthcare) was used. A ring-shaped region of interest (ROI) was drawn on each side of the frontal, temporal and parietal cortices and cerebellum, as described previously (Cheney et al., 2001). The data obtained by placing ROI included the mean counts per pixel. To express the PET images numerically, the PET image obtained was analyzed manually by drawing ROI. Relative FDG uptake was calculated for each ROI using the average tissue activity in the region, standardized by mean cerebellar hemispheric activity. Data evaluation was performed in a blinded manner by one scientist in a day.
PET scanning was performed on the day after the last exposure to RFID. Each rat (which had been fasted for at least 12 h, n=6) was anesthetized with isoflurane (15 mg/kg, Sigma) and 63 MBq of FDG (Carecamp) was injected through the tail vein. After 30 min to allow distribution of the intravenously infused FDG uptake, brain images of the rat were acquired in a high-resolution eXplore Vista PET scanner (GE Healthcare). Each rat was scanned for 30 min in a prone position with its brain centered in the axial and transaxial fields of view.
K. Statistical Analysis

The SPSS software (ver. 12.00 for Windows; SPSS Inc., Chicago, IL, USA) was used. Body mass, temperature and serum levels of T3, T4, TSH, melatonin hormone level, enzyme activity, protein and mRNA levels, ROI-based data, are shown as mean ± standard deviation. Group means were compared with the Mann-Whitney U-test for pairs. The general linear model (GLM), followed by a univariate-test were used for statistical comparisons over time. The significance of the differences between groups of $^{18}$F-FDG uptake levels and melatonin related molecular levels were determined by one way analysis of variation (ANOVA) test followed by post hoc testing using Tukey method for statistical comparison. P value < 0.05 was seemed to indicate statistical significance.
III. RESULT

Part A. Effects of RFID Exposure on Thyroid System; T3, T4 and TSH in Rat

1. Effect of RFID exposure on body temperature

To monitor thermal effect from experimental results by RFID exposure, room and body temperature were measured before and after RFID exposure. There were no significant differences in room temperatures between the sham-chamber and the chamber for RFID exposure (p>0.05). Before exposure to RFID, measured body temperature of the rats in the sham-exposed (n=10) and in the RFID-exposed groups (n=10) were 36.9 ± 0.2 °C and 36.9 ± 0.1 °C, respectively. After exposure to RFID, measured body temperatures in the sham-exposed and in the RFID-exposed groups were 37.1 ± 0.3 °C and 37.0 ± 0.3 °C, respectively. No significant differences in body temperature were observed between the sham-exposed and RFID-exposed groups (p>0.05).

2. Effect of RFID exposure on body mass

To determine effect of RFID on gain of body mass, I measured rat body mass biweekly. The average body mass of the experimental animals (n=6) was 204 ± 0.8 g at the start of the experiment. Body masses before exposure to RFID were 216 ± 3.7 g in the sham-exposed group (n=6) and 216 ± 2.0 g in the RFID-exposed group (n=6). At 2 weeks after exposure to RFID, the average body masses in the sham-exposed and in RFID-exposed groups were 299 ± 2.4 g and 293 ± 1.1 g, respectively. At 16 weeks after exposure to RFID, the average body masses in the sham-exposed (n=6) and in RFID-exposed groups (n=6) were 523 ± 9.6 g and 516 ± 3.5 g, respectively. The body mass of rats in each of the group was increased
constantly and no significant differences were observed between the sham-exposed and RFID-exposed groups. No significant differences in body mass were observed for any of the exposure conditions over a span of 16 weeks (p>0.05).

3. Changes in the SAR value.

Changes in SAR values according to the gain of body mass was determined and calculated SAR values at the input power of 56 W (data provided by Prof. Jeong-Ki Pack, Department of Radio Sciences and Engineering, College of Engineering, Chungnam National University). Whole-body averaged SAR was 4 W/kg (Table 1) and the calculated average SAR in the neck was 5.1 W/kg for a rat with a body mass of 339 g. The calculated whole-body averaged SAR was 4.6 W/kg for a body mass of 225 g and 3.2 W/kg for a body mass of 525 g. The corresponding average SARs in the neck was 3.6 W/kg for a body mass of 225 g and 5.7 W/kg for a body mass of 525 g.

4. Effect of RFID exposure on thyroid system hormones

To test the effect of RFID exposure on thyroid system, I measured RFID exposure on thyroid hormones and thyroid stimulating hormone level. Changes in the thyroid hormones and the TSH absolute profile after RFID exposure are summarized in Table 2. However, no changes in T3, T4, or TSH were observed over time between the sham-exposed and RFID-exposed groups (P = 0.45, 0.50 and 0.53, respectively, univariate test). The normalized serum T3 concentrations in the cage control, sham-exposed and the RFID-exposed groups, at 2, 4, 8, and 16 week period are shown in Fig.1A. No differences in serum T3 concentrations were observed between the groups (Mann-Whitney U- test). The normalized serum T4 concentrations in the sham-exposed and the RFID-exposed groups, at 2, 4, 8, and 16 week
period are presented in Fig. 1B. Figure shows that the T4 concentrations in the rats exposed to the RFID for 8 h daily, for 4 weeks, were significantly lower than those in the sham-exposed group (p < 0.05 Mann-Whitney U-test). The absolute values were 3.78 ± 0.25 dl/μl in the sham-exposed group and 2.85 ± 0.27 dl/μl in the RFID-exposed group. However, no significant differences in serum T4 concentrations between the sham-exposed and the RFID-exposed groups at the other times conditions (p>0.05, Mann-Whitney U-test). No significant differences in serum TSH concentrations were observed between the sham-exposed and RFID-exposed groups, at 2, 4, 8, or 16 week period (p >0.05, Mann-Whitney U-test; Fig. 1C).

5. Effect of RFID exposure on thyroid gland follicles

To observe the effect of RFID exposure on thyroid gland follicles, I observed thyroid after RFID exposure. No histological changes in morphological observation of the thyroid gland were observed among the groups (Fig. 2).
Table 1. Measurement of SAR value at 914MHz RFID according to the input RFID field strength

<table>
<thead>
<tr>
<th>Output Power [W]</th>
<th>Field Strength [V/m]</th>
<th>SAR [W/kg]</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>97.97</td>
<td>1.24</td>
</tr>
<tr>
<td>20</td>
<td>128.02</td>
<td>2.11</td>
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<tr>
<td>30</td>
<td>151.41</td>
<td>2.96</td>
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<tr>
<td>40</td>
<td>166.79</td>
<td>3.59</td>
</tr>
<tr>
<td>50</td>
<td>174.10</td>
<td>3.91</td>
</tr>
<tr>
<td>51</td>
<td>175.00</td>
<td>3.95</td>
</tr>
<tr>
<td>52</td>
<td>175.89</td>
<td>3.99</td>
</tr>
<tr>
<td>53</td>
<td>176.78</td>
<td>4.03</td>
</tr>
<tr>
<td>54</td>
<td>177.66</td>
<td>4.07</td>
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<td>4.11</td>
</tr>
<tr>
<td>56</td>
<td><strong>179.41</strong></td>
<td><strong>4.15</strong></td>
</tr>
<tr>
<td>57</td>
<td>180.28</td>
<td>4.19</td>
</tr>
<tr>
<td>58</td>
<td>181.15</td>
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</tr>
<tr>
<td>59</td>
<td>182.01</td>
<td>4.27</td>
</tr>
<tr>
<td>60</td>
<td>182.87</td>
<td>4.32</td>
</tr>
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</table>
Table 2. Changes in the thyroid hormones and the TSH absolute profile after the 914 MHz RFID exposure.

<table>
<thead>
<tr>
<th>Duration of exposure (weeks)</th>
<th>Group</th>
<th>T3 (ng/dL)</th>
<th>T4 (ng/dL)</th>
<th>TSH (μIU/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>Control</td>
<td>31±3.47</td>
<td>2.7±0.26</td>
<td>1.04±0.41</td>
</tr>
<tr>
<td></td>
<td>Sham</td>
<td>46.50±1.63</td>
<td>4.08±0.27</td>
<td>1.12±0.27</td>
</tr>
<tr>
<td></td>
<td>RFID</td>
<td>44.83±1.66</td>
<td>3.60±0.24</td>
<td>0.72±0.18</td>
</tr>
<tr>
<td>P value (Sham vs. RFID)</td>
<td></td>
<td>0.26</td>
<td>0.17</td>
<td>0.52</td>
</tr>
<tr>
<td>4</td>
<td>Control</td>
<td>36.33±1.44</td>
<td>3.42±0.16</td>
<td>1.37±0.25</td>
</tr>
<tr>
<td></td>
<td>Sham</td>
<td>39.18±1.68</td>
<td>3.78±0.25</td>
<td>1.04±0.18</td>
</tr>
<tr>
<td></td>
<td>RFID</td>
<td>42.27±1.88</td>
<td>2.85±0.27</td>
<td>1.14±0.14</td>
</tr>
<tr>
<td>P value (Sham vs. RFID)</td>
<td></td>
<td>0.23</td>
<td>0.01 *</td>
<td>0.71</td>
</tr>
<tr>
<td>8</td>
<td>Control</td>
<td>16.54±1.06</td>
<td>2.65±0.11</td>
<td>0.78±0.13</td>
</tr>
<tr>
<td></td>
<td>Sham</td>
<td>20.64±1.97</td>
<td>2.61±0.15</td>
<td>1.11±0.25</td>
</tr>
<tr>
<td></td>
<td>RFID</td>
<td>19.90±1.79</td>
<td>2.42±0.10</td>
<td>1.75±0.24</td>
</tr>
<tr>
<td>P value (Sham vs. RFID)</td>
<td></td>
<td>0.75</td>
<td>0.35</td>
<td>0.12</td>
</tr>
<tr>
<td>16</td>
<td>Control</td>
<td>32.69±0.17</td>
<td>6.58±0.3</td>
<td>0.88±0.04</td>
</tr>
<tr>
<td></td>
<td>Sham</td>
<td>29.09±0.61</td>
<td>6.01±0.16</td>
<td>0.89±0.16</td>
</tr>
<tr>
<td></td>
<td>RFID</td>
<td>29.40±0.43</td>
<td>6.07±0.33</td>
<td>0.89±0.16</td>
</tr>
<tr>
<td>P value (Sham vs. RFID)</td>
<td></td>
<td>0.72</td>
<td>0.74</td>
<td>0.87</td>
</tr>
</tbody>
</table>
Fig. 1. Comparison of average serum concentrations of thyroid hormones and thyroid stimulating hormone (TSH) To determine the effect of RFID exposure on thyroid hormone system, serum level of thyroid hormones and thyroid stimulating hormone was measured by ELISA. Blood was collected after RFID exposure. The amount of T3, T4 and TSH were measured using T3 and T4 (Calbiotech, Sacramento, CA, USA) and TSH ELISA kits (Cusabio Biotech, Whuan, China). CTL: cage control group; Sham: sham-exposed group;
RFID: RFID-exposed group. (A) No significant differences in serum triiodothyronine (T3) concentration were observed between the sham- and radiofrequency identification (RFID) - exposed groups. (B) Serum concentrations of thyroxine (T4) in the RFID-exposed rats for 8 h per day for 4 weeks, were significantly lower than those in the sham-exposed rats (p < 0.05). (C) No significant differences in serum concentrations of TSH were observed between the sham- and RFID-exposed groups (p > 0.05). Data represents hormone concentrations ± S.D of 12 rats for 2, 4 and 8 weeks and six rats for 16 weeks. *Mean difference was significant at P < 0.05 level.
Fig. 2. Photomicrographs of thyroid gland paraffin sections of the thyroid gland To observe the effect of RFID exposure on thyroid gland follicles, I observed thyroid gland follicles by microscope. Photomicrographs of thyroid gland paraffin sections of the thyroid gland stained with hematoxylin and eosin (H&E) as described in materials and methods. No histological changes in morphological observation of the thyroid gland were observed among the groups. All photomicrographs are of the same magnification. Magnification is 40×, Scale bar, 25 μm.
Part B. Effect of RFID Exposure on Melatonin Synthesis

1. Effect of RFID exposure on melatonin biosynthesis in rat pineal gland

To determine the effect of RFID exposure on melatonin synthesis, urinary 6-OHMS and serotonin were analyzed. No significant change in concentration of urinary 6-OHMS and serotonin was observed in both of 1 h and 8 h day-time RFID-exposed group (n=10) compared to sham-exposed group (n=10) (p>0.05, Fig. 3). Concentration of 6-OHMS in 24-h urine was decreased only in the 8 h nocturnal RFID-exposed group (n=10) (F1, 18= 8.68, p=0.00; Fig. 4A), but not in the 1 h RFID-exposed group (n=10). Concentration of urinary serotonin in both 1 h and 8 h nocturnal RFID-exposed groups was slightly increased, however significant differences were not observed (P>0.05)(Fig. 4B). These findings suggested that 8 h (so called, long duration) exposure is required to study influence of RFID exposure on melatonin biosynthesis, as 1 hr (short duration) is not enough to induce a significant changes in urinary secretion of a melatonin metabolite. Therefore I decided to examine the experiments only with long-duration (8 h) exposed groups to study the mechanism of RFID exposure-induced suppression of melatonin biosynthesis.

2. Effect of nocturnal RFID exposure on body temperature

I measured body temperature (n=10) before and after RFID exposure daily to exclude thermal effect from results by nocturnal RFID exposure. The measured average body temperatures before nocturnal RFID exposure, the average rectal temperatures in the sham-exposed and in the RFID-exposed groups were 36.9 ± 0.2 °C and 36.5 ± 0.2 °C, respectively. The measured average rectal temperatures in the sham-exposed and in the RFID-exposed groups were 37.2 ± 0.2 °C and 37.2 ± 0.2 °C, respectively. No significant differences were
observed between the groups. Based on the data, I confirmed that RFID exposure in this experimental condition did not induce any thermal effect.

3. Effect of nocturnal RFID exposure on concentration of serum norepinephrine (NE)

I wondered whether RFID exposure-induced reduction of melatonin metabolite concentration was caused by changes in serum norepinephrine level. Therefore I measured concentration of serum norepinephrine. The measured serum norepinephrine concentration after nocturnal exposure to RFID was 204.9±2.1 nmol/L in the cage-control group (n=6), 203.8±3.7nmol/L in the sham-exposed group (n=6), and 215.0 ±1.6 in the RFID-exposed group (n=6). No significant changes in the serum level of norepinephrine were observed in RFID-exposed group compared to sham-exposed and cage-control groups (P>0.05; Fig. 5). These findings suggested that serum NE level did not changed by RFID exposure and serum NE is not a initial RFID exposure related inducer to start changes in pineal gland.

4. Effect of RFID exposure on AANAT activity

As I observed a RFID exposure-induced reduction of melatonin metabolite in 24 h urine, I thought a possibility that RFID exposure can cause any disturbance in melatonin synthesis pathway including conversion from serotonin to melatonin. Before confirming my curiosity, I started to measure a AANAT activity in normal rat pineal gland which was already well know and I observed a tendency of daily pattern of AANAT activity which was increased fivefold during the night compared to during the daytime in rat pineal gland. I studied to measure AANAT activity in rat pineal gland after nocturnal RFID exposure. A significant
reduction of AANAT activity, as I expected, was observed in RFID-exposed rats (n=4) compared to sham-exposed group (n=4) (F1, 6= 52.55, P=0.00, Fig. 6).

5. Effect of RFID exposure on expression level of AANAT protein

As suggested previously, AANAT activity is an essential prerequisite for melatonin production (Huang et al., 2010). Hence, I measured level of pineal AANAT protein after exposure to the RFID. Comparatively low level of AANAT protein was observed in RFID-exposed group (n=5) compared to sham-exposed group (n=5) (F1,8=6.67, p=0.03; Fig. 7A). In addition, I measured level of phospho-AANAT, which was significantly decreased in RFID exposed group compared to sham-exposed and cage-control groups (n=5) (F1.6=8.13, p=0.02, Fig. 7B).

6. Effects of RFID exposure on regulation of Aanat gene transcription

As I observed a significant decrease level of pineal AANAT protein, I deservingly measured level of Aanat gene transcription and found a significantly reduced level of Aanat gene transcription, as expected in RFID-exposed group (n=5) compared to sham-exposed (n=5) and cage-control groups (n=5) (F1.8=11.87, p=0.00, Fig. 8). Although expression level of Aanat mRNA was decreased also in sham-exposed group compared to in cage-control group, but its difference were not significant. As it is known that phospho-CREB is one of the transcriptional regulators of Aanat mRNA expression (Simonneaux et al., 2006; Ho and Chik, 2010), I examined the protein expression level of pineal phospho-CREB after RFID exposure. Relative level of phospho-CREB protein was decreased in RFID-exposed group (n=3) compared to in sham-exposed (n=3) and cage-control (n=3) groups. However, No
change in total amount of CREB protein in all three groups were observed (Fig. 9). According to the previous mechanism studies of Aanat gene transcription, it is known that phosphorylation of CERB protein was regulated by PKA in the rat pineal gland (Maronde et al., 1999). Therefore, I measured a pineal PKA activity after RFID exposure. Measured pineal PKA activity after RFID exposure was 203.8±3.7 nmol/L in the sham-exposed group (n=6) and 215.0 ±1.6 in the RFID-exposed group (n=6). No significant change in pineal PKA activity in RFID-exposed group compared to sham exposed group was also observed (P>0.05; Fig. 10).

These findings suggest that nocturnal 8 h exposure to RFID may suppress melatonin synthesis, which might be related with inhibition of phospho-CREB-dependent Aanat gene transcription.
Fig. 3. Changes in urinary level of 6-OHMS and serotonin after RFID exposure during the day. To determine the level of melatonin and serotonin, 24 h-urine after diurnal RFID exposure was collected with metabolic cage at 8 a.m. and urinary level of 6-OHMS and serotonin were analyzed by GC-MS as described in materials and methods. Urinary excretion of (A) 6-OHMS and (B) serotonin was unchanged both in long duration (8 h) and short duration (1 h) diurnal RFID exposure rats. Data are represented as means ± standard deviation for at least 10 samples. *p<0.05 compared with the value obtained from sham-exposed group.
Fig. 4. Changes in urinary level of 6-OHMS and serotonin after RFID exposure during the night

To determine the level of melatonin and serotonin after nocturnal, 24 h-urine was collected with metabolic cage at 8 a.m. and urinary level of 6-OHMS and serotonin was analyzed by GC-MS as described in materials and methods. (A) Urinary excretion of 6-OHMS was significantly reduced in long duration (8 h) nocturnal RFID exposed group \( (P=0.003) \), but not in short duration (1 h) group. (B) Urinary level of serotonin was unchanged. Data are represented as means ± standard deviation for at least 10 samples. *\( p<0.05 \) compared with the value obtained from sham-exposed group.
Fig. 5. Changes in serum norepinephrine (NE) level after nocturnal RFID exposure To determine the effect of RFID exposure on NE concentration, I measured serum NE level by ELISA as described in materials and methods. Blood was collected at night under a red dim light after nocturnal RFID exposure. The amount of NE was measured using ELISA kit (Alfco, Salem, NH, USA). No significant change in the serum level of norepinephrine was observed in RFID-exposed group compared to sham-exposed and cage-control groups. Experimental data of sham-and RFID exposed groups were normalized to that of cage-control group. CTL: cage control group; Sham: sham-exposed group; RFID: RFID-exposed group. Data represent hormone concentration ± S.D of 6 rats.
Fig. 6. Effects of nocturnal RFID exposure on arylalkylamine N-acetyltransferase (AANAT) enzyme activity. To determine AANAT enzyme activity changes after RFID exposure, AANAT activity was measured by liquid biphasic diffusion assay (Chae et al., 1999). Briefly, nocturnal harvested pineal gland was individually disrupted by ultrasound in 80 µl ice-cold phosphate buffer (50 mM, pH 6.8). Debris was removed by centrifugation (15,000 g, 5 min at 4°C) and the supernatant was transferred to a new tube. An amount of 4 µl of the supernatant was then incubated in the presence of 5 µl tryptamine-HCl (10 mM), 1 ml acetyl CoA (0.5 m), and 1 ml [3H]-acetyl CoA (3.6 Ci/mmol, 250 Ci/ml). Phosphate buffer (50 mM, pH 6.8) was added to yield a final volume of 20 µl. Incubation was done at 37°C for an indicated time and was stopped by diluting the reaction mixture with 180 µl ice-cold phosphate buffer (50 mM, pH 6.8). The diluted reaction mixture was rapidly transferred to a vial containing 3 ml of Econofuor (1,2,4-trimethylbenzene >99%), 2,5-diphenyloxazole (0.7%, w/v), 1,4-bis(2-methylstyril) benzene (0.05%, w/v)). The amount of radiolabeled acetyltryptamine was determined in a liquid scintillation counter. AANAT activity was
reduced only in nocturnal RFID exposed group as compared to sham-exposed and control group. CTL: cage control group; Sham: sham-exposed group; RFID: RFID-exposed group. Data are represented as means ± standard deviation for at least 4 samples per group. *p<0.05 compared with the value obtained from sham-exposed and control groups.
Fig. 7. Effects of RFID exposure on arylalkylamin N-acetyltransferase (AANAT) protein level

To determine the effect of RFID on protein expression of AANAT in rat pineal gland, I examined protein level of AANAT and phospho-AANAT after RFID exposure by western analysis. The level of AANAT protein and phospho-AANAT significantly reduced in long duration RFID-exposed group. The density of each band was quantified by imageJ software and relative ratio of AANAT (1:2000, Abcam) to actin (1:1000, Abcam) and phospho-AANAT (1:2000, Abcam) to actin was quantitatively analyzed. CTL: cage control group; Sham: sham-exposed group; RFID 1, 2: long duration RFID-exposed group. Data are represented as means ± standard deviation for at least 5 samples per group. *p<0.05 compared with the value obtained from sham-exposed and control groups.
Fig. 8. Effects of RFID exposure on arylalkylamin N-acetyltransferase (Aanat) mRNA level

To determine the effect of RFID exposure on expression of Aanat mRNA, I examined transcriptional level of Aanat mRNA by RT-PCR. Pineal gland was nocturnally harvested and total RNAs were isolated at night. (A) Level of Aanat mRNA was analyzed. (B) The DNA bands were quantified by Quantity One 1-D analysis software, v 4.6.5 and changes were represented in graph. Gapdh shows comparative intensities of Aanat PCR product. CTL: cage control group; Sham: sham-exposed group; RFID: RFID-exposed group. Data are represented as means ± standard deviation for at least 5 samples. *p<0.05 compared with the value obtained from sham-exposed and control groups.
Fig. 9. Effects of RFID exposure on phosphorylated CREB (phospho-CREB) protein level

To determine the effect of RFID exposure on protein expression of phospho-CREB, I examined protein level of phospho-CREB and after RFID exposure by western analysis. The density of each band was quantified by imageJ software and the relative ratio of phospho-CREB (upper, 1:1000, Cell signaling) to CREB (lower, 1:1000, Cell signaling) in rat pineal glands after nocturnal RFID exposure was determined. CTL: cage control group; Sham: sham-exposed group; RFID: RFID-exposed group. Level of phosph-CREB was decreased in RFID exposed group compared to sham-exposed group. Data are represented as means ± standard deviation for at least 3 samples per group.
Fig. 10. Effects of RFID exposure on protein kinase A (PKA) activity To evaluate whether pineal PKA is related to the phosphorylation of CREB, PKA activity was analyzed by ELISA with pineal tissue which was harvested at night under red dim light after RFID exposure. Relative percentage of PKA activity to total pineal protein was quantitatively analyzed. The PKA activity was determined by slide phase enzyme-linked immune absorbent assay (ELISA) using a commercially available PKA activity kit (Enzolife Science, Famingdale, NY, USA) according to the manufacturer’s protocols. Pineal PKA activity in RFID-exposed group was not different from those in sham-exposed and cage-control groups (P>0.05). CTL: cage control group; Sham: sham-exposed group; RFID: RFID-exposed group. Data represent hormone concentration ± S.D of 6 rats. PKA activity of sham- and RFID exposure groups were normalized to that of cage-control group.
Part C. Effects of RFID exposure on cerebral glucose metabolism in rat:

A (F-18) FDG micro PET Study

1. Effect of RFID exposure on cerebral glucose metabolism

To determine effect of RFID exposure on cerebral glucose metabolism, the FDG-PET images were analyzed (n=6, Fig. 11). I performed RFID exposure for 2 weeks and gained PET image after RFID exposure. Analyzed average ROI values in the frontal cortex in the sham-exposed and RFID-exposed groups were 1.03±0.03 and 0.93±0.01, respectively (F 2,15 = 1.24, P = 0.33). Analyzed average ROI values for the temporal cortex in the sham-exposed and RFID-exposed groups were 0.95±0.01 and 0.98±0.00, respectively (F2,15 =1.16, P = 0.21). Analyzed average ROI values for the parietal cortex in the sham-exposed and RFID-exposed groups were 0.88±0.01 and 0.91±0.01, respectively (F2,15 =1.36, P = 0.64; Fig. 11E). There were no significant differences in the measured average relative ROI values between the sham-exposed and RFID-exposed groups. Therefore, I examined whether cerebral glucose metabolism was influenced by RFID exposure for 16 weeks.

The average ROI values were obtained after the 16-week exposure experiment. Analyzed values for the frontal cortex in the sham-exposed and RFID-exposed groups were 1.09±0.02 and 1.09±0.00, respectively (F 2,15 = 2.64, P = 0.95). Analyzed values for the temporal cortex in the sham-exposed and RFID-exposed groups were 0.89±0.01 and 0.94±0.01, respectively (F 2,15 = 2.89, P = 0.11). Analyzed values for the parietal cortex in the sham-exposed and RFID-exposed groups were 0.92±0.01 and 0.97±0.01, respectively (F 2.15 = 0.84, P = 0.10; Fig. 11F). There were no significant differences in the measured average relative ROI values at 16 weeks between the sham-exposed and RFID-exposed groups.
In addition, I compared the ROI values obtained from each cortex of each hemisphere of the rats in the 2-week and 16-week exposure trials. There was no significant difference between the right and left hemispheres in RFID-exposed group. Changes in the relative ROI and P values are summarized in Table 3. There were no significant differences in average relative ROI values for each side of the cortical region of the RFID-exposed rats in the 2- or 16-week periods.
Fig. 11. A-D. The analysis of PET image and relative ROI values after RFID exposure at 2 and 16 weeks. To evaluate the effect of RFID exposure on cerebral glucose metabolism, micro PET image after RFID exposure was obtained using 18F-FDG, as a radiotracer for PET scanning. PET scanning was performed on the day after the last exposure to RFID. Each rat (which had been fasted for at least 12 h, n=6) was anesthetized with isoflurane (15 mg/kg, Sigma) and 63 MBq of FDG (Carecamp, Suwon, Korea) was injected through the tail vein. After 30 min to allow distribution of the intravenously infused FDG uptake, brain images of the rat were acquired in a high-resolution eXplore Vista PET scanner (GE.
Healthcare, Piscataway, NJ, USA). Each rat was scanned for 30 min in a prone position with its brain centered in the axial and transaxial fields of view (Fig. 7). For semiquantitative evaluation of the data obtained, a Xeleris functional imaging work station (GE Healthcare) was used. A ring-shaped region of interest (ROI) was drawn on each side of the frontal, temporal and parietal cortices and cerebellum, as described previously (Cheney et al., 2001). To express the PET images numerically, the PET image obtained was analyzed manually by drawing ROI. Relative FDG uptake was calculated for each ROI using the average tissue activity in the region, standardized by mean cerebellar hemispheric activity. The PET images show the distributions of cerebral FDG uptake at 2 weeks (A. coronal; B. axial) and 16 weeks (C. coronal; D. axial) after RFID exposure in the cage-control, sham-exposed, and RFID-exposed rats. E-F. Comparison of FDG uptake values between the sham-exposed and RFID-exposed groups in the frontal, temporal, and parietal cortices after RFID exposure for 2 weeks (E) and 16 weeks (F) demonstrated that there was no significant difference in any measurement between the groups (p > 0.05). ROI values of the cortex (mean ± standard deviation) were normalized to that of the cerebellum. Data represent average ROI values ± standard deviation of six rats. * mean difference was significant at P < 0.05 level.
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*P indicate P-value
IV. DISCUSSION

Part A. Effects of RFID Exposure on Thyroid System; T3, T4 and TSH in rat

In this study, I examined the effects of a sub-chronic exposure to RFID, on serum levels of thyroid hormones and TSH. The estimated whole-body average SAR varied from 3.2–4.6 W/kg depending on the age/ mass of the animals for the field of the RFID reader. The major finding in this experiment is that the exposure to RFID, at least under our experimental conditions, did not induce any significant changes in secretion of T3, T4, or TSH in the rats (Fig. 1).

In this study, the exposure duration started at 2 weeks. However, no significant changes in thyroid hormones or TSH at both 1h and 8 h exposures per day were observed. Because serum T3, T4, and TSH levels remained unchanged in both the 1 h exposed- and 8 h exposed-groups after the 2 week trial, I performed an 8 h daily exposure trial only in further experiments. RFID exposure duration was extended gradually from 2 to 16 weeks to verify the changes in T3, T4 and TSH after exposure to RFID. I observed that serum T4 concentrations in RFID-exposed groups for 4 weeks were significantly lower than those in the sham-exposed groups (Fig. 1B). However, an 8 week-long exposure did not cause any significant changes in the thyroid hormones. Although TSH levels in 8 weeks exposed groups tended to increase, the difference was not significant (Fig. 1C). These findings forced me to conduct further experiments with a 16 weeks exposure, keeping the same conditions. But, no significant differences in serum T3, T4, or TSH levels were observed between the groups after the 16 week exposure.

Although some transient changes in serum thyroid hormones were observed in the
separate 2, 4, 8, and 16 week exposure experiments, serum TSH level remained unchanged (Fig. 1C). Secretion of thyroid hormones was maintained with a certain range in this study. These findings suggest that homeostatic secretion of thyroid hormones was still observed for 8 h daily for 16 weeks at 4 W/kg whole-body averaged SAR. These findings were also supported by the morphological findings. Previous reports also noted that RF does not cause any significant morphological changes (Rajkovic et al., 2003).

Several studies have shown that exposure to RF-EMF may not alter the endocrine system, particularly T3, T4, and TSH. According to a human study, serum concentrations of T3 and T4 are not affected by microwave exposure (2,450 MHz, 8 h/day, at 2, or 10 mW/cm², male) (Abhold et al., 1981). It has also been reported that exposure to 900 MHz global system for mobile (GSM) for 24, 48, and 96 h does not modify iodide accumulation in FRTL-5 cells (cultured thyroid cells), with respect to the basal condition (Dimida et al., 2011). The results from the present study, using RFID, from an RFID reader, also showed no alterations in the thyroid function from EMF.

Some studies have reported that RF-EMF can change secretion of thyroid hormones or TSH. One study observed that a 21% decrease in serum TSH levels in human male volunteers, who were chronically exposed to 900 MHz GSM cell phone fields 2 h/day, 5 days/week for 4 weeks (de Seze et al., 1998). However, TSH levels during the or after RF exposure remained within the physiological range. These findings suggest that cell phones emitting the radiofrequency can induce an imbalance in thyroid hormonal secretion, although this level is not detrimental and can be managed by the homeostatic mechanisms of the human body. An animal study showed that serum T3, T4, and TSH values in a 900 MHz EMF group (30 min/day, 5 days/week, for 4 weeks at 2 W/kg with male rats) were
significantly lower than those in a sham-exposed group. However, they reported that their findings were insufficient to conclude that RF-EMF had biological or detrimental effects. It was also reported that microwave (2450 MHz) irradiation reduces blood T3 levels on days 16 and 21 and T4 levels on day 21 (Sinha, 2008). That study suggested that low energy microwave irradiation can have a harmful effect on thyroid secretory function (Sinha, 2008). However, the influence of RF-EMF on the thyroid system remains inconclusive.

I used male rats, as the hormonal cycle of female rats can result in experimental variations, as well as the increased possibility for thyroid disorders in susceptible female rats (Kilfoy et al., 2009). This could be one of the limitations of this experiment. Previous reports have shown that EMF-exposed female rats may develop harmful thyroid system effects (Krassas et al., 2010; Negro and Mestman, 2011). The present study was composed of four separate experimental trials that were performed sequentially, according to the experimental results. Previous reports suggested that the geomagnetic activity and seasonal changes may also play a role in the entrainment of the serum hormone levels, including melatonin and thyroid hormones (O'Connor and Persinger, 1996; Bermudez et al., 2005; Boggs et al., 2011). As the serum levels of thyroid hormones and TSH were different in each group and in each trial, I normalized the absolute values in this study to compare such differences (Fig 1C). Although the pattern of change in secretion and serum concentrations of thyroid hormones area varied according to the previous studies, a seasonal variation occurs. The present results showed the same pattern. The small sample size was also a limitation of this trial.

Based on our results, I suggest that even long duration exposure to RFID at a SAR of 4 W/kg has no significant effects on the thyroid system in male rats.
Part B. Effect of RFID Exposure on pineal Melatonin Synthesis

I focused on the effects of nocturnal RFID exposure on melatonin synthesis, especially changes of pineal AANAT enzyme in rats. I examined the effect of RFID exposure with a 4W/kg SAR and rats were exposed for 8 hours daily to mimic the occupational environment in humans. A previous study indicated that the photoperiod length influences on the duration of night time melatonin rise in mammals (Hazlerigg and Wagner, 2006) and a seasonal change may entrain the hormone levels, including melatonin (Wehr et al., 1993; Hazlerigg and Wagner, 2006). Thus The current animal experiment to study influence on melatonin biosynthesis was mostly performed during the winter season, as it is impossible to keep eight hour naturally dark during the night time in the summer. In addition, 24-h urinary 6-OHMS, a stable melatonin metabolite was analyzed by GC-MS to detect the RFID exposure induced change in melatonin secretion and also to avoid analysis error with a time lag from blood collection time point (Graham et al., 1998; Hong et al., 2001). I expected that although RF including RFID can influence on pineal melatonin production, it may be subtle in degree and it is known that serum level of secreted melatonin is not only extremely small in amount but also variable during the day.

The major findings of this study can be summarized as follows: (A) nocturnal RFID exposure cause a decreased urinary secretion of melatonin metabolite, (B) Aanat gene transcription was directly related with the RFID exposure-induced suppression of melatonin synthesis and (C) a reduced phospho-CREB, not a PKA was involved in a mechanism causing a decrease in Aanat mRNA after RFID exposure in the rat pineal gland.
Several studies reported the influence of EMF on melatonin synthesis and AANAT activity (Sukhotina et al., 2006; Kesari et al., 2012). So far, a few researches were undertaken to examine the effect of RF-EMF on melatonin synthesis (Koyu et al., 2005b; Sukhotina et al., 2006; Kesari et al., 2011). A recent study indicated that the serum concentration of melatonin was significantly reduced in the rat pineal gland in a 900 MHz microwave-exposed group (Kesari et al., 2011). Another in vitro study demonstrated that melatonin release from the isolated pineal gland of Djungarian hamsters was reduced by 1800 MHz EMF irradiation (Sukhotina et al., 2006). The authors of these two reports suggested that the changes in melatonin synthesis were caused by exposure to EMF (Sukhotina et al., 2006; Kesari et al., 2012). However, they did not show the definite evidence that of the effect of EMF on melatonin synthesis. The authors have not examined the EMF exposure related mechanism of melatonin synthesis (Sukhotina et al., 2006; Kesari et al., 2012).

In some studies, to study EMF exposure-induced changes in melatonin production, serum level of melatonin was measured in blood obtained with a single puncture technique during the nighttime (Koyu et al., 2005b; Kesari et al., 2011, 2012). In their study, EMF exposure experiments were performed during the daytime (Koyu et al., 2005b; Kesari et al., 2011, 2012). Even though blood were obtained during the night, it seems not suitable to study a subtle change in serum melatonin level, besides this hormone is unstable and its serum concentration is extremely low (Cocco et al., 2005).

In this study, I hypothesized that rats had to be exposed to RFID during nighttime instead of during the daytime. I also hypothesized that for a proper studying the mechanism of RFID exposure influence on melatonin synthesis, RFID exposure trial has to be performed during
the night as the rat is nocturnal animal. RFID exposure-induced reduction of the AANAT activity could be subtle and its influence on melatonin is of importance during the night.

According to my data, no change was observed in urinary melatonin metabolite level in rats, which were exposure during the daytime (Fig. 3). Its level reduced significantly only in rats in which were nocturnally exposed for 8 h daily, but no change in 1 hr RFID-exposed rats. These findings indicated that a long duration nocturnal but not diurnal RFID exposure can induce a reduction of melatonin synthesis in the rat pineal gland.

It is known that significant elevation of AANAT activity more than 100-fold at night has to be preceded to induce de novo synthesis of Aanat mRNA in rat, which take gives a lag time to induce AANAT activity in the rat pineal gland (Ganguly et al., 2002). Because of this reason, I started a nocturnal RFID exposure experiment at 22:00, 4 h after onset of darkness in the late evening during the winter time.

In this study, the rat pineal glands were harvested under a dim red light at 11:00 p.m. to avoid any unintended light exposure. I observed a reduced AANAT activity in the pineal gland which was harvested during the night after nocturnal RFID exposure, as reported previously (Selmaoui and Touitou, 1995)(Fig. 6). It is known that changes in AANAT activity reflect changes in level of AANAT enzyme molecules in several vertebrate species including rats (Huang et al., 2010). I also found that decrements of the AANAT and phospho-AANAT protein in the same tissue (Fig. 7).

It was suggested previously that PKA plays key role in inhibition of AANAT proteosomal degradation through phosphorylation of AANAT protein (Huang et al., 2010). Therefore, I expected a possibility of RFID exposure-induced activation of AANAT proteosomal degradation. However I failed to find any significant change in PKA activity after nocturnal
RFID exposure under this particular experimental condition (Fig. 10). Based on these data, I carefully excluded the possibility that RFID exposure-induced reduction of the AANAT activity is related with PKA activity.

To evaluate which molecular mechanism is fundamentally influenced by RFID exposure, I studied Aanat gene transcription pathway in rat pineal gland as suggested previously (Cena et al., 1991; Klein et al., 1997; Gauer et al., 1999; Garidou et al., 2003). I also found the decrease in the Aanat mRNA in RFID exposed group (Fig. 8). In accordance with previous studies showing a reduction of CREB phosphorylation in vivo which precede the decline of Aanat mRNA level at night (Ganguly et al., 2002; Karolczak et al., 2005; Ho and Chik, 2010), I also observed the reduced level of phospho-CREB after nocturnal RFID exposure (Fig. 9). Although it was suggested previously that PKA plays key role in AANAT protein phosphorylation of CREB in pineal gland at night (Huang et al., 2010), no change in PKA activity was observed in RFID-exposed group (Fig. 10). According to my experimental data, I suggest that RFID exposure-induced suppression of melatonin biosynthesis, at least in part, was closely related with a repression of Aanat mRNA expression after RFID exposure in the rat pineal gland.

RF including RFID is quite similar to light because both can be classified as non-ionizing radiation although its specific frequency is different each other (Khaki et al., 2008). It is well known that light radiation has a clear impact on regulation of melatonin production during the night (Leitgeb, 2012). It is also known that melatonin synthesis can be influenced by physical factors such as geomagnetic field and light (Csernus et al., 2005; Jin et al., 2011). Especially, light at night is known to entrain daily circadian rhythm (Csernus et al., 2005) by alteration of Aanat gene transcription in pineal gland of broilers (Jin et al., 2011).
reported that the alteration of Aanat mRNA expression caused by light at night (Jin et al., 2011), and UV-A irradiation (Rosiak et al., 2005; Ho and Chik, 2010). The findings obtained from my study indicated that RFID exposure during the night can not only suppress melatonin biosynthesis, but also have a similar effect of sleep deprivation. It is of clinical importance because sleep under exposure to RF including RFID may have a cause a detrimental effect on health unconsciously.

There are several limitations in this study. Only male rats were employed to avoid other hormonal influences, therefore; its influence on male rat alone was determined in this study.

Although, I used urine which was collected for 24 hours instead of blood obtained with a single puncture at a single time point, it can still be insufficient to detect subtle changes in melatonin biosynthesis than 8 to 12 h urine which was collected during the night, as it is known that its production and secretion are peak during the night. To evaluate an immediate effect of RFID exposure on melatonin synthesis, immediate harvest of pineal gland without time delay is essential. However, for this trial, a time delay for two days between termination of experiment and tissue collection was obliged to collect urine for 24 hrs after termination of exposure experiment. I have not done, however, an in vitro study may be needed with isolated rat pineal gland to study the mechanism of RFID exposure-induced suppression of melatonin. I measured level of serum NE, however, to determine the effect of RFID exposure on start signal of Aanat transcription in pineal gland, pineal NE concentration measurement may be more crucial. I had not observed yet whether or not the RFID exposure induced change in melatonin synthesis is reversible.

Several limitations as mentioned above may guide a research direction to study further and some are going on. I suggest, however, the data from this study provide enough evidences to
claim the adverse effects of high energy long duration RFID exposure on melatonin synthesis.

In conclusion, I suggest that nocturnal RF including RFID exposure, as if exposure to the light at night may cause a decrease of melatonin synthesis via a suppression of Aanat gene transcription in rat pineal gland. Consequently, nocturnal exposure to RF can entrain the daily circadian rhythm.
Fig. 12. Proposed action mechanism of suppressive effect of RFID exposure on reduced secretion of urinary melatonin metabolite (modified figure, Ho and Chik, 2010) At night, in a normal condition, the noradrenergic (NE) input to the pineal gland increased. Activation of \( \beta_1 \)-adrenergic receptors (\( \beta_1 \)-AR) leads to increase in cellular cAMP levels. Protein kinase A (PKA) becomes activated. This sequential pathway will lead a phosphorylation of cAMP-responsive element binding protein (CREB) and AANAT transcription which is the rate limiting step to produce melatonin. The proposed signaling pathway from CREB to melatonin is as follows. Under the nocturnal RFID exposure condition, phospho-CREB decreased markedly although amount of CREB was unchanged, that leads a suppression of \( Aanat \) transcription. Therefore, as a result of this sequence, biosynthesis of melatonin decreased.
Part C. Effects of RFID Exposure on Cerebral Glucose Metabolism in Rat:

A (F-18) FDG Micro PET Study

In this animal study, I evaluated the effects of exposure to RFID by using FDG PET scanning to monitor any RF-EMF-induced changes in cerebral glucose metabolism in rats. For the study, two separate exposure experiments were conducted. The results showed that under these experimental conditions RFID exposure did not induce any significant change in cerebral glucose metabolism in rats (Fig. 11). To my knowledge, this is the first reported investigation using FDG PET scanning after whole-body exposure to RFID in rats.

Only two PET studies on the influence of RF-EMF on cerebral glucose metabolism have been reported (Kwon et al., 2011; Volkow et al., 2011). The RF-EMF-emitting conditions were similar in frequency, 800-900 MHz, to that in the present study, and those studies were performed in humans. In one, it was reported that exposure to cell-phone radiation, with a frequency of 837.8 MHz for 50 min, induced increased brain glucose metabolism in the region closest to the antenna (Volkow et al., 2011). However, the other human study claimed that exposure to a pulse-modulated 902.4-MHz global system for mobile communication (GSM) signal for 33 min suppressed glucose consumption in the anterior and posterior parts of the right temporal cortex ipsilateral to the radiation source (Kwon et al., 2011). As a source of RF-EMF, Volkow et al. used a cell phone. In contrast, Kwon et al. used a cell phone without a battery to exclude any thermal effect. In both human studies, exposure duration was as short as 30 and 50 min and they observed direct and immediate effects of cell-phone radiation on neural activity with FDG PET scanning at the time of cell-phone use.
In this study, PET scanning was performed on the rats at 1 day after the RFID exposure experiment, because it was impossible practically to do both a whole-body RFID exposure experiment and PET scanning at the same time. This study aimed to assess the effects of relatively long-term exposure rather than immediate or transient effects of exposure to RFID. I assumed that with exposure duration of RFID of 2 or 16 weeks, a 24-h delay before the PET scan would not be an important issue as the exposure durations are significantly longer than one day break between the end of exposure experiment and PET scan. I measured the rectal temperature before and 8 h after exposure trials in both sham-exposed and RFID-exposed groups. The rectal temperature dose not changed overtime in all rats. As the animals can move freely during the RFID exposure, their body temperature can be controlled automatically to keep homeostatic status by sweating, breathing and circulating body fluid including blood. During the PET study, those rats were anesthetized to restrict their movement to prevent glucose consumption in the peripheral part of the body.

Glucose metabolism is unevenly distributed throughout rat brain tissues, with higher rates in the cortex and basal ganglia (Barros et al., 2005). Although RFID-EMF is distributed to the whole body, glucose consumption could differ on one side of the brain, such as the dominant hemisphere, so I compared the relative values of the sides of both hemispheres and observed no significant differences between them (Table 3). It has been reported that there was no significant difference between right and left hemispheres in normal brain (Cottone et al., 2013).

Previously, a number of PET studies with RF-EMF exposure from cell phones have focused on changes in regional cerebral blood flow (CBF) (Huber et al., 2002; Haarala et al., 2003; Huber et al., 2005; Aalto et al., 2006; Mizuno et al., 2009). One study
suggested that exposure to a 902-MHz active cell phone caused a relative decrease in regional cerebral blood flow (rCBF) in the bilateral auditory cortex (Haarala et al., 2003). Another report suggested that exposure to a GSM cell-phone signal for 30 min induced a regional decrease in CBF in the inferior temporal cortex (Aalto et al., 2006). A change in rCBF could be indirect evidence of cerebral metabolism; thus, these experimental results after RF-EMF exposure might suggest possible changes in neural activity (Huber et al., 2002; Haarala et al., 2003; Huber et al., 2005; Aalto et al., 2006; Mizuno et al., 2009).

In the present study, RFID exposure was performed during the day-time hours, although the rat is typically a nocturnal animal. The present study consisted of two separate experimental trials that were performed sequentially. In the animal PET scanning system used, the number of pixels is relatively low, so the quality of the images was not as high as I would have liked.

In conclusion, RFID exposure did not cause a significant long lasting effect on glucose metabolism in the rat brain. Based on these results, I suggest that this study is high scientific value with regard to the first PET study with RFID exposed rat regard
V. SUMMARY AND CONCLUSION

1. RFID exposure, at least under our experimental conditions, does not induce any significant changes in secretion of T3, T4 and TSH levels in the RFID exposed group compared to the sham-exposed group.

2. Nocturnal 8h exposure to whole body SAR 4W/kg strong RFID elicits reduction of a 24h urinary melatonin secretion and its diminution degree shows a pineal Aanat gene transcriptional level-dependent manner. Nocturnal RFID exposure-induced inhibition of pineal Aanat gene transcription may be related with reduced PKA-independent CERB phosphorylation.

3. RFID exposure did not cause a significant long lasting effect on glucose metabolism in the rat brain.

Conclusion: To evaluate the effect of RFID exposure on rat brain functions, I have studied three different systems including cerebral metabolism, thyroid system and pineal melatonin synthesis. I obtained some positive data from these trials, at least under our experimental conditions. These studies may provide evidence that rat brain is sensitive to RF exposure including RFID.
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국문요약

914MHz 대역 Radiofrequency Identification 전자파 노출이 흰쥐 호르몬계 및 뇌대사에 미치는 영향

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무선통신전자파 (Radiofrequency electromagnetic field, RF-EMF) 노출이 인체에 잠재적 위해 요소일 가능성이 있다는 역학보고가 있지만, 생체 영향에 대한 연구 자료는 아직 제한적이다. RFID (Radio frequency identification, RFID) 는 최근 도입되어 산업 및 일상 생활에서 흔히 사용되는 무선 통신 시스템의 일종으로 전자파 (Radiofrequency, RF) 를 방출한다. 전자파 노출 동물 실험을 위하여 Reverberation chamber를 구축하였다. 실험용 흰쥐는 225-250g 중량의 수컷 Sprague-Dawley 종을 활용하였다. 동물 실험은 1)갑상선 호르몬계 영향 연구, 2) 송과체 멜라토닌 생성 영향 및 관련 기전 연구, 3) FDG-PET 검사를 이용한 뇌 피질 당 대사 영향 연구 등으로 구분하여 수행하였다. 갑상선 호르몬계 연구와 뇌 피질 당 대사 연구는 RFID 전자파 노출 실험을 낮 시간대(10:00-18:00)에, 멜라토닌 관련 연구는 해당 호르몬의 특수성을 감안하여 어두운 밤 시간대(22:00-06:00)에 수행하였다. 조직 내 변화 여부도 해당 시간대에 관찰하기 위해, 멜라토닌 관련 연구용 조직은 심야 시간대에 채취하였다. 모든 연구는 specific absorption rate (SAR, 전자파흡수율) 4 W/kg 강도의 RFID 전자파를 전신에 노출하는 방법으로 수행하고 전신 SAR 값은 흰쥐의 몸무게 변화에 따라 3.2-4.6 W/kg 내에 분포하도록 전자파 노출량을 조절하였다. 모든 전자파 노출실험은 일일 8 시간.
주당 5일간 수행하였다. 갑상선 호르몬계 실험을 위한 전자파 노출 기간은 2, 4, 8, 및 16주 동으로 구분하여 순차적으로 수행하고, 멜라토닌 생성 조절 실험은 2주 동안 노출하는 환경에서 반복하여 수행하였다. 당 대사 영향 실험은 2주와 16주 동안 수행하였다. 아주대학교 동물실험연구윤리위원회의 심의를 거쳤으며 (110405-25) 실험은 그 규정을 준수하여 수행하였다.

연구 결과를 실험 별로 정리하면 아래와 같다. 1. RFID 전자파를 2, 4, 8, 및 16주 동안 낮시간 대에 노출한 흰쥐 갑상선계 호르몬에 미치는 영향에 대한 분석 결과를 보면, 갑상선호르몬 (T3, T4)에는 일시적인 영향을 주지만 정상 범위 이내에서 유지되는 소견을 보였고, 갑상선자극호르몬 (TSH)은 각각의 실험에서도 대조군의 수치와 차이를 보이지 않아, RFID 전자파가 갑상선 호르몬계에 유해한 영향을 미치지 않는다는 소견을 확인하였다. 2. RFID 전자파 야간 노출 군의 24시간-뇨 내 멜라토닌 대사 물질이 대조군에 비해 현저히 낮았다. 이러한 변화의 원인이 송과체내 Arylalkylamine N-acetyltrasferase (AANAT)의 activity 변화, AANAT의 단백질 및 mRNA의 발현 감소와 유관함을 확인하였다. 또한 Aanat gene transcription의 감소가 CREB의 phosphorylation의 감소와 관련이 있으나, PKA activity와는 무관하다는 소견을 확인 하였다. 이러한 연구 결과는 전자파에 의한 멜라토닌 생성의 기전을 규명하는 의미있는 결과라고 생각된다. 3. 18F-deoxyglucose positron emission tomography (FDG-PET) 검사를 이용한 RFID 전자파 노출 영향 분석상 전두엽, 측두엽, 후두엽 등의 뇌피질 부위에서의 뇌 피질 당 대사량이 유의미한 차이를 보이지 않아 전자파가 뇌피질에는 별 영향을 주지 않는다는 소견을 확인하였다.

본 연구자가 수행한 연구 결과를 바탕으로 RFID 전자파 노출이 노출 조건과 환경에 따라 중추신경계와 관련한 내분비계에 영향을 줄 가능성이 있음을 확인하여, 관련 분야에 대한 깊이 있고 체계적인 연구가 더 수행될 필요성이 있음을 제안하고자 한다.

핵심어: 914MHz RFID, 갑상선계호르몬, 멜라토닌, FDG-PET, AANAT