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Full Length Article

Anesthesia affects excitatory/inhibitory synapses during the critical synaptogenic period in the hippocampus of young mice: Importance of sex as a biological variable

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A R T I C L E I N F O

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A B S T R A C T

Background: Sex plays an important yet often underexplored role in neurodevelopment and neurotoxicity. While several studies report the importance of sex regarding anesthesia-induced neurotoxicity in neonatal mice, only few have focused on the late postnatal period. Here, to further understand the importance of sex regarding the neurobiological changes after early anesthesia during the critical synaptogenic period, we exposed postnatal day 16, 17 (PND 16, 17) mice to sevoflurane in pediatric patients and performed detailed evaluations in the hippocampus.
Methods: PND 16, 17 mice received a single exposure of oxygen with or without sevoflurane (2.5%) for 2 h. Changes of the hippocampus were analyzed in male and female mice 6 h after exposure: excitatory/inhibitory synaptic transmission, protein/mRNA expression levels of excitatory/inhibitory synaptic molecules (GluR1, GluR2, PSD95, gephyrin, GAD65), and number of excitatory synapses.
Results: Sevoflurane exposure increased the frequency of miniature excitatory postsynaptic currents specifically in male mice (control: 0.07 ± 0.04 [Hz]; sevoflurane: 14.72 ± 0.08 [Hz]), while miniature inhibitory postsynaptic currents were affected specifically in female mice. The protein/mRNA expression levels of excitatory synaptic molecules were also increased specifically in male mice. Unexpectedly, protein/mRNA expression levels of inhibitory synaptic molecules were increased in both sexes, and there was no male-specific increase of excitatory synapse number.
Conclusions: Exposure of mice to sevoflurane during the critical, late postnatal period induces sex-dependent changes in the hippocampus. Although often disregarded, our results confirm the importance of sex as a biological variable when studying the changes triggered by early anesthesia.

1. Introduction

Neurodevelopment is an immensely complicated process that occurs in a strictly regulated sequence of diverse molecular and cellular processes (Silbereis et al., 2016). Disturbance of this highly organized process is considered a key mechanism for neurodevelopmental disorders (Meredith, 2015; Silbereis et al., 2016). Thus, concerns have been raised by preclinical studies, including non-human primates, showing that the use of anesthesia in young animals are associated with unanticipated neurobiological changes at various developmental stages (Briner et al., 2010; Coleman et al., 2017; Creeley et al., 2013; Istaphanous et al., 2013; Jevtovic-Todorovic, 2018; Palanisamy, 2012). However, both clinical and pre-clinical studies have yielded conflicting results regarding the long-term consequences of early anesthesia

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(Chung et al., 2015b; Davidson and Sun, 2018; Lee et al., 2017; O'Leary and Warner, 2017). Although the issue is under heavy debate (Hansen, 2017), many still express concerns regarding early anesthesia.

Sex plays a crucial but often underexplored role in neuroscience (Beery and Zucker, 2011; Cahill, 2006; Shansky and Woolley, 2016). The differences in the neurodevelopment of males versus females (sexually dimorphic neurodevelopment) have been shown to be involved with neurodevelopmental disorders and various neurotoxic insults (Mergler, 2012; Torres-Rojas and Jones, 2018; Werling et al., 2016). Numerous studies report that sex also influences the neurobiological changes after anesthesia in neonatal rodents (postnatal day 7 [PND 7]) (Boscolo et al., 2013; Gonzales et al., 2015; Lee et al., 2014; Murphy and Baxter, 2013; Rothstein et al., 2008; Tan et al., 2014). However, the neurodevelopmental stage of neonatal rodents represents the brain of the human fetus (third trimester) (Workman et al., 2013). Importantly, this does not correlate with the majority of clinical studies that focus on the effects of anesthesia in neonates and infants.

While it is difficult to make a side-by-side comparison between rodents and humans, previous studies imply that older mice represent the neurodevelopmental stage of human infants (Workman et al., 2013). In our previous study, we observed neurological changes after sevoflurane exposure (a widely used inhalation anesthetic agent in pediatric patients) in the medial prefrontal cortex (mPFC) of PND 16, 17 mice (Chung et al., 2017). During this developmental stage, male mice were found to be more sensitive than females to the changes of excitatory synapses in the mPFC (Chung et al., 2017). Considering that males are more susceptible than females to neurodevelopmental disorders in general, such sex-dependent neurobiological changes may contribute to the possible long-term consequences of anesthesia-induced neurotoxicity. However, due to the lack of research, the effect of sex during early anesthesia in this specific neurodevelopmental stage is not well understood.

To further examine the importance of sex with respect to the neurotoxic effects of anesthesia in late postnatal mice, we focused on the hippocampus, a sexually dimorphic brain region (Cahill, 2006) that interacts with the mPFC for memory formation and consolidation (Eichenbaum, 2017). We herein performed a more intensive evaluation through multiple approaches, including measurement of synaptic transmission, expressional analysis of excitatory/inhibitory synaptic proteins and mRNAs, and assessment of synaptic density via electron microscopy. Our findings confirm sex as an important biological variable when studying the neurobiological changes after anesthesia during the critical synaptogenic period.

2. Methods

The manuscript adheres to the applicable ARRIVE (Animal Research: Reporting In Vivo Experiments) guidelines.

2.1. Animals

All animal studies were approved by the Committees on Animal Research at Chungnam National University Hospital (Daejeon, South Korea, CNUH-014-A0009). C57BL/6 J mice were housed in a room maintained at 24 °C, with a 12-hour light/12-hour dark cycle and were fed *ad libitum*.

2.2. Anesthesia

PND 16, 17 mice were exposed to sevoflurane as previously described (Chung et al., 2017). In brief, the mice were randomly divided into two groups: the control group and the sevoflurane group. Control mice were exposed to a constant flow of fresh gas (FiO_2 1.0, 4 L/min) for 130 min. Mice in the sevoflurane group were exposed to 2.5% sevoflurane (Ilsung, Seoul, Korea) under the same constant flow of fresh gas for 120 min. Mice were recovered under 100% oxygen for another 10 min and were returned to their cages after confirming full recovery. The anesthesia chamber was placed in a water bath set at 36℃ to ensure a constant temperature. The concentration of sevoflurane was monitored using an S/5 compact anesthetic monitor and an m-CAiO gas analyzer module (Datex-Ohmeda, Helsinki, Finland). The effect of our anesthesia protocol on respiratory parameters has been previously reported (Chung et al., 2017).

2.3. Electrophysiology

Whole-cell patch-clamp recordings of hippocampal CA1 pyramidal neurons were performed 6 h after sevoflurane (oxygen) exposure, as previously described (Chung et al., 2015a, 2017). Sagittal slices of the hippocampus (300 μm) were prepared using a VT1200S vibratome (Leica, Wetzlar, Germany) in ice-cold dissection buffer (212 mM sucrose, 25 mM NaHCO3, 5 mM KCl, 1.25 mM NaH2PO4, 10 mM D-glucose, 2 mM Na-pyruvate, 1.2 mM Na-ascorbate, 3.5 mM MgCl2, 0.5 mM CaCl2) continuously aerated with 95% O2/5% CO2. Brain slices were recovered at 32 °C for 30 min in a chamber filled with artificial cerebrospinal fluid (aCSF: 125 mM NaCl, 25 mM NaHCO3, 2.5 mM KCl, 1.25 mM NaH2PO4, 10 mM D-glucose, 1.3 mM MgCl2, 2.5 mM CaCl2) that was aerated with 95% O2/5% CO2. Glass capillaries were filled with the appropriate internal solution for each experiment (for excitatory postsynaptic current recordings: 117 mM CsMeSO4, 10 mM TEA-Cl, 8 mM NaCl, 10 mM HEPES, 5 mM QX-314-Cl, 4 mM Mg-ATP, 0.3 mM Na-GTP, 10 mM EGTA; for inhibitory postsynaptic current recordings: 115 mM CsCl, 10 mM TEA-Cl, 8 mM NaCl, 10 mM HEPES, 5 mM QX-314-Cl, 4 mM Mg-ATP, 0.3 mM Na-GTP, 10 mM EGTA). Recordings were made using a MultiClamp 700 A amplifier (Molecular Devices, CA, USA) under visual control (BX50WI; Olympus, Japan). Data were acquired with Clampex 9.2 (Molecular Devices) and analysed using Clampfit 9 (Molecular Devices).

2.4. Western blotting

Brain sampling and Western blotting were performed as previously described (Chung et al., 2017). In brief, brain samples were obtained 6 h after mice were exposed to sevoflurane or oxygen alone. For ethical reasons, all mice were anesthetized with 3% sevoflurane before brain sampling. The mice were sacrificed by decapitation, and the hippocampus of each mouse was separated from the brain, treated with phosphatase and protease inhibitors and homogenized with a tissue grinder. Homogenized hippocampal tissues were centrifuged at 15,000 × g for 20 min at 4 °C, and supernatants were collected for Western blotting. The following commercial antibodies were used: PSD95 (Thermo Fisher Scientific, MA, USA), Gephyrin (Synaptic Systems, Goettingen, Germany), GAD65 (Abcam, Cambridge, UK), and α-tubulin (Sigma, MO, USA). The antibodies against GluR1 (1193) and GluR2 (1195) were described previously (Kim et al., 2009).

2.5. Real-time PCR

Samples of the hippocampus were obtained 2 or 6 h after mice were exposed to sevoflurane (oxygen). Total RNA was extracted and cDNA was prepared using the M-MLV reverse transcriptase system (Invitrogen, CA, USA). Hippocampal mRNA levels were quantified via real-time PCR, which was performed using cDNA (100 ng), 2X SYBR mix, forward/reverse primers (10 pmol), and a Rotor Gene 6000 (Corbett Life Science, Venlo, Netherlands). The following primers were used: PSD95, 5'-TCTGTGCGAGAGGTAGCAGA-3' (forward) and 5'-ACGGATGAAGATGGCGATAG-3' (reverse); GluR1, 5'-GGAAGTGTGGG GAGATCAGA-3' (forward) and 5'-TCTCACTGCCCAGAGTGATG-3' (reverse); and GluR2, 5'-GACTTCAGGAGCAGGGACAG-3' (forward) and 5'-AGGAGCTGCTAAAACCACGA-3' (reverse); GAD65, 5'- AGATCGCCC CTGTATTTGTG-3' (forward) and 5'- GCATGGCATACATGTTGGAG-3' (reverse); Gephyrin, 5'- TTCTCCTGGCTCCTGTCAGT-3' (forward) and

5’- ACTGCGGTCTTCTGCAAGAT-3’ (reverse), 18 s rRNA, 5’- CTGGTTG ATCCTGCCAGTAG-3’ (forward) and 5’- CGACCAAAGGAACCATAACT-3’ (reverse). mRNA expression levels were normalized using 18 s rRNA as a housekeeping gene.

2.6. ***Transmission*** *electron* ***microscopy (EM)***

Coronal slices of the hippocampus (200 μm) were sectioned and immediately pre-fixed in 2.5% glutaraldehyde-2% paraformaldehyde in 0.15 M sodium cacodylate buffer (pH 7.4) for at least 2 hours2 h at 4 °C. The samples were washed with sodium cacodylate buffer, post-fixed in 2% osmium tetroxide-1.5% ferrocyanide in 0.15 M cacodylate buffer (pH 7.4) for 1 h, incubated with 1% TCH for 30 min and treated with 2% OsO_4 for 30 min. The samples were *en bloc* stained with 1% uranyl acetate overnight at 4 °C and then with lead citrate for 30 min at 60 °C. The tissues were then dehydrated in an ethanol and propylene oxide series, embedded in an Epon 812 mixture, and subjected to polymerization with pure resin at 70 °C for 24 h. Ultrathin sections were obtained with an ultramicrotome (Ultra Cut-UCT; Leica, Vienna, Austria) and then collected on 150 mesh copper grids. The sections were visualized using a Bio-HVEM system (JEM-1400Plus at 120 kV and JEM-1000BEF at 1000 kV; JEOL, JAPAN), and images of the CA1 stratum radiatum (208 μm^2) were obtained from each mouse. The excitatory synapses, which were identified as having a pronounced postsynaptic density (PSD) facing a presynaptic bouton containing at least three vesicles (Briner et al., 2010), were counted by a blinded observer.

2.7. *Statistical* ***analysis***

Sample size was estimated from previous studies for synaptic transmission (Li et al., 2015), real-time PCR (Spalloni et al., 2006), and EM (Briner et al., 2011). The experimentally important difference was set to a 40% change, with $\alpha = 0.05$ (two-tailed) and power of 80%. Sample size for western blotting was based on a previous study (Chung et al., 2017). Data was analyzed using the R statistical software package (3.1.2; R Core Team, Austria). Two-way ANOVA was used to evaluate the interactions between the effects of anesthesia and sex. Nested model was performed to separately compare male and female results. Only the western blot results of male and female mice were compared separately with independent *t*-test or Welch’s *t*-test. Multiple testing was not used due to the fact that male and female mice could not be compared simultaneously (limited number of samples that can be compared on a single SDS-PAGE gel). The results of our statistical analyses are included as supplementary data.

3. Results

3.1. *Sevoflurane exposure induces sex-dependent changes of excitatory/inhibitory synaptic transmission in PND 16, 17 mice*

To further evaluate the significance of sex regarding synaptic transmission after early anesthesia, we measured synaptic transmission in CA1 hippocampal pyramidal neurons. Miniature excitatory postsynaptic currents (mEPSCs) and miniature inhibitory postsynaptic currents (mIPSCs) were measured 6 h after exposure to sevoflurane (Fig. 1). Two-way ANOVA revealed interaction between sex and anesthesia only for mEPSC frequency. The same analyses show significant effect of sevoflurane exposure for mEPSC frequency, and significant effect of sex for mIPSC amplitude and frequency. When separately evaluated (nested modeling), mEPSC frequency was increased only in male mice (Fig. 1 A–D), while female mice exhibited decreased amplitude and increased frequency of mIPSCs (Fig. 1 E–H).

3.2. *Excitatory/inhibitory synaptic proteins are sex-dependently up-regulated following sevoflurane exposure in PND 16, 17 mice*

We next evaluated whether sevoflurane exposure could sex-dependently affect the expression levels of synaptic proteins in mice. In protein samples obtained from the hippocampus at 6 h after sevoflurane exposure, two subunits of the AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) receptor, GluR1, and GluR2, were significantly increased only in male mice (Fig. 2). The male-specific increase of AMPA receptor subunits are consistent with the male-dependent increase of excitatory synaptic transmission (Fig. 1). Unexpectedly, while inhibitory synaptic transmission was only increased in female mice (Fig. 1.), the expression of inhibitory synaptic proteins was increased in both male (gephyrin and GAD [Glutamic acid decarboxylase] 65) and female mice (gephyrin) (Fig. 2).

3.3. *The mRNAs encoding synaptic molecules are sex-dependently up-regulated after sevoflurane exposure in PND 16, 17 mice*

We further evaluated sex-dependent sevoflurane-induced changes of the synaptic system by examining the mRNA expression levels of synaptic molecules. Total RNA was extracted at 2, 6 h after sevoflurane exposure, and mRNA levels were quantified by RT-PCR using 18 s rRNA as a housekeeping gene (Fig. 3). Significant interactions between sex and anesthesia were seen for most synaptic molecules at 2 h (Fig. 3 A), but no significant interaction was seen 6 h (Fig. 3 B) (two-way ANOVA). The same analyses at 2 h show significant effect of both anesthesia and sex for gephyrin and GluR2 (Fig. 3. A). There was also a significant effect of sex for gephyrin, and a significant effect of anesthesia for GluR2 at 6 h (Fig. 3. B). When separately evaluated (nested model), significant changes were observed in the levels of GluR2, gephyrin and GAD65 at 2 h after sevoflurane exposure only in male mice (Fig. 3 A). The increased mRNA expression of excitatory/inhibitory synaptic molecules well correlate with changes of protein expression in male mice (Fig. 2 A, B). However, such correlation between mRNA and protein expression levels were not shown in female mice, as mRNA expression of inhibitory synaptic molecules were not increased (Fig. 2 C, D). The increases shown in male mice were transient, as the mRNA levels were normalized at 6 h post-exposure (Fig. 3 B).

3.4. *Sex-independent increase of excitatory synapse number after sevoflurane exposure in PND 16, 17 mice*

To explore the mechanism underlying the male-dependent increase of excitatory synaptic transmission in sevoflurane-exposed mice, we performed electron microscopy (EM) and compared the number of excitatory synapses in the CA1 stratum radiatum of the hippocampus (Fig. 4A, B). While there was no significant interaction between sex and anesthesia regarding synapse number, there was both a significant effect of sex and anesthesia (two-way ANOVA). Unexpectedly, there was no increase of synapse number in both male and female mice when separately evaluated (nested modeling) (Fig. 4C).

4. Discussion

Sex is now regarded as an important biological variable by funding organizations such as the European Commission (EC), Canadian Institutes of Health Research (CHIR), and the US National Institutes of Health (NIH). The significance of sex has also been recognized in the field of neuroscience (Beery and Zucker, 2011; Cahill, 2006; Mergler, 2012; Shansky and Woolley, 2016). While it is difficult to determine how sex may affect anesthesia-induced neurotoxicity in young children due to the predominance of males in clinical studies (Lin et al., 2017), the significance of sex has been repeatedly reported in neonatal rodents (Boscolo et al., 2013; Gonzales et al., 2015; Lee et al., 2014; Murphy and Baxter, 2013; Rothstein et al., 2008). However, there are very few

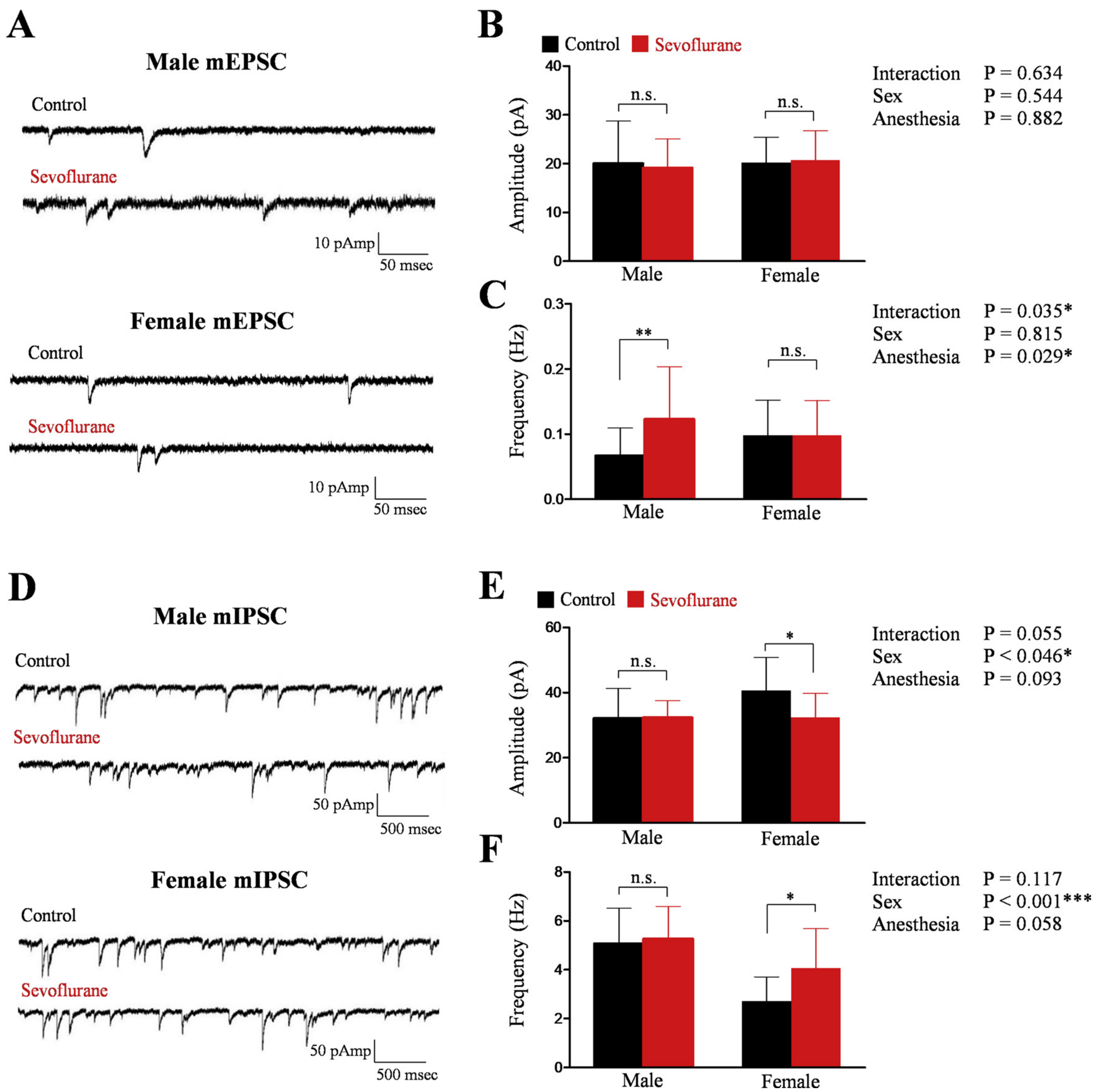

Fig. 1. Sex-dependent changes of excitatory/inhibitory synaptic transmission in CA1 hippocampal pyramidal neurons are observed 6 h after sevoflurane exposure of postnatal day 16 and 17 (PND 16, 17) mice. (A) Representative image of miniature excitatory postsynaptic currents (mEPSCs) in male and female mice (n = 21 cells from five control male mice and n = 22 cells from four sevoflurane-exposed male mice mice; n = 14 cells from three control female mice and n = 15 cells from three sevoflurane-exposed female mice). (B, C) Sevoflurane exposure does not affect mEPSC amplitude, but increases the frequency of miniature excitatory postsynaptic currents (mEPSCs) only in male mice (two-way ANOVA and nested model, *$P < 0.05$ and n.s. = not significant). (D) Representative image of miniature inhibitory postsynaptic currents (mIPSCs) in male and female mice (n = 16 cells from 4 control male mice and n = 14 cells from three sevoflurane-exposed male mice; n = 14 cells from three control female mice and n = 14 cells from three sevoflurane-exposed female mice). (E, F) Sevoflurane exposure decreased mIPSC amplitude and increased mIPSC frequency only in female mice (two-way ANOVA and nested model, *$P < 0.05$, ***$P < 0.001$). Values are presented as mean ± SD.

reports focusing on late postnatal mice, a period that may be more comparable to the neurodevelopment of postnatal human infants. Here, we provide multiple lines of evidence showing that sex is indeed an important biological variable when studying the neurobiological changes from sevoflurane exposure by examining the hippocampus.

Since we previously reported that sevoflurane exposure in the late postnatal period could male-dependently increase excitatory synaptic transmission in the mPFC (Chung et al., 2017), we speculated that sex-dependent changes of the excitatory synapses might also occur in different brain regions. Thus, we evaluated the hippocampus at the same time point used in our previous study (6 h after anesthesia). However, since transcription precedes translation, we presumed that changes of mRNA levels could appear earlier than the changes of protein expression. Accordingly, we additionally evaluated mRNA expression levels 2 h after sevoflurane exposure.

While our results agree that sex is an important variable when studying the effects of anesthesia in young mice, the hippocampal data obtained in the present study differ somewhat from the mPFC data obtained in our previous study (Chung et al., 2017). First, in contrast to our present hippocampal data, early sevoflurane exposure altered inhibitory synaptic transmission in the mPFC of male mice. Second, while the expression levels of excitatory synaptic proteins in the mPFC were increased by sevoflurane in both male and female mice, only male mice showed increases among excitatory synaptic proteins of the hippocampus. Although we do not yet understand the basis of these differences, we speculate that they may reflect fundamental differences in the ultrastructure and composition of post-synapses of different brain regions and cell types (Harris and Weinberg, 2012). Differences in the gene expression patterns of brain regions may also influence the consequences of early anesthesia (Lein et al., 2007). Thus, our previous and present results together suggest that the sex-dependent neurobiological changes triggered by sevoflurane exposure may differ between brain

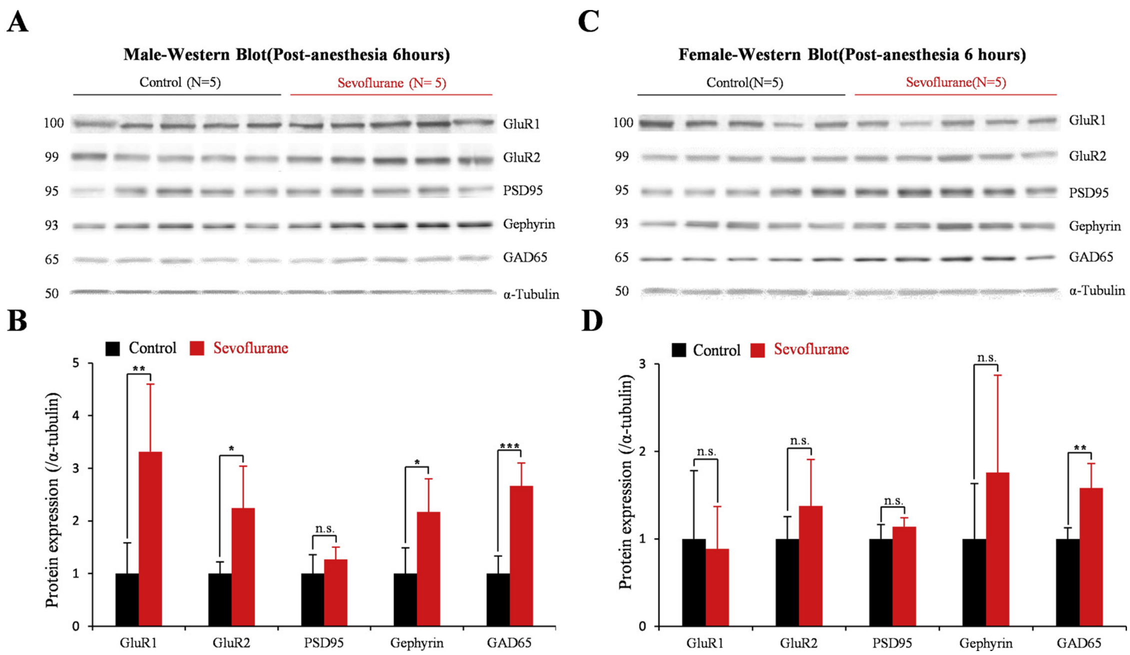

Fig. 2. Excitatory, inhibitory synaptic proteins are sex-dependently up-regulated at 6 h after sevoflurane exposure of postnatal day 16, 17 mice. (A, B) Sevoflurane exposure increases the expression of AMPA receptor subunits (GluR1, GluR2), gephyrin and GAD65 in male mice. (C, D) Only the expression level of GAD65 was increased after sevoflurane exposure in female mice. (*P < 0.05, **P < 0.01, ***P < 0.001 and n.s. = not significant, as assessed by Student's t test or Welch's t test). Values presented as mean ± SD. Shown are the AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate receptor) receptor subunits, GluR1 and GluR2; PSD95, postsynaptic density protein 95; Gephyrin; GAD65, Glutamic acid decarboxylase 65.

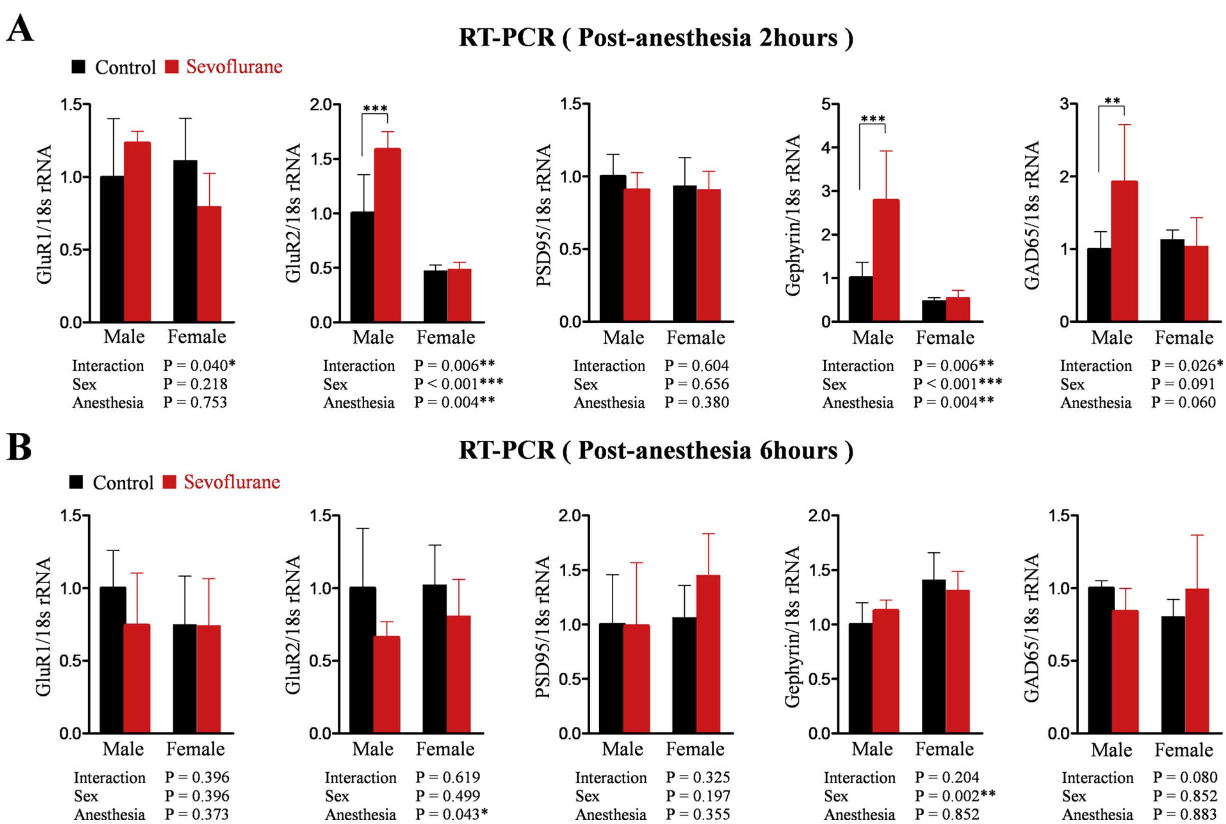

Fig. 3. The mRNAs encoding hippocampal synaptic molecules transiently and sex-dependently increase after sevoflurane exposure in postnatal day 16, 17 mice. (A) The expression levels of mRNAs at 2 h after sevoflurane. AMPA receptor subunit GluR2, gephyrin, and GAD65 were increased specifically in male mice, as determined by real-time PCR analysis (n = 5 per group; two-way ANOVA and nested model, *P < 0.05, **P < 0.01, ***P < 0.001) (B) The expression levels of mRNAs at 6 h after sevoflurane. mRNA expression levels of synaptic molecules were similar to the control levels in both male and female mice, as determined by real-time PCR analysis (n = 5 per group; two-way ANOVA and nested model, **P < 0.01). 18 s rRNA was used as an internal control and mRNA expression levels were normalized to 18 s rRNA. Values are presented as mean ± SD. Shown are the AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate receptor) receptor subunits, GluR1 and GluR2; PSD95, postsynaptic density protein 95; Gephyrin; GAD65, Glutamic acid decarboxylase 65.

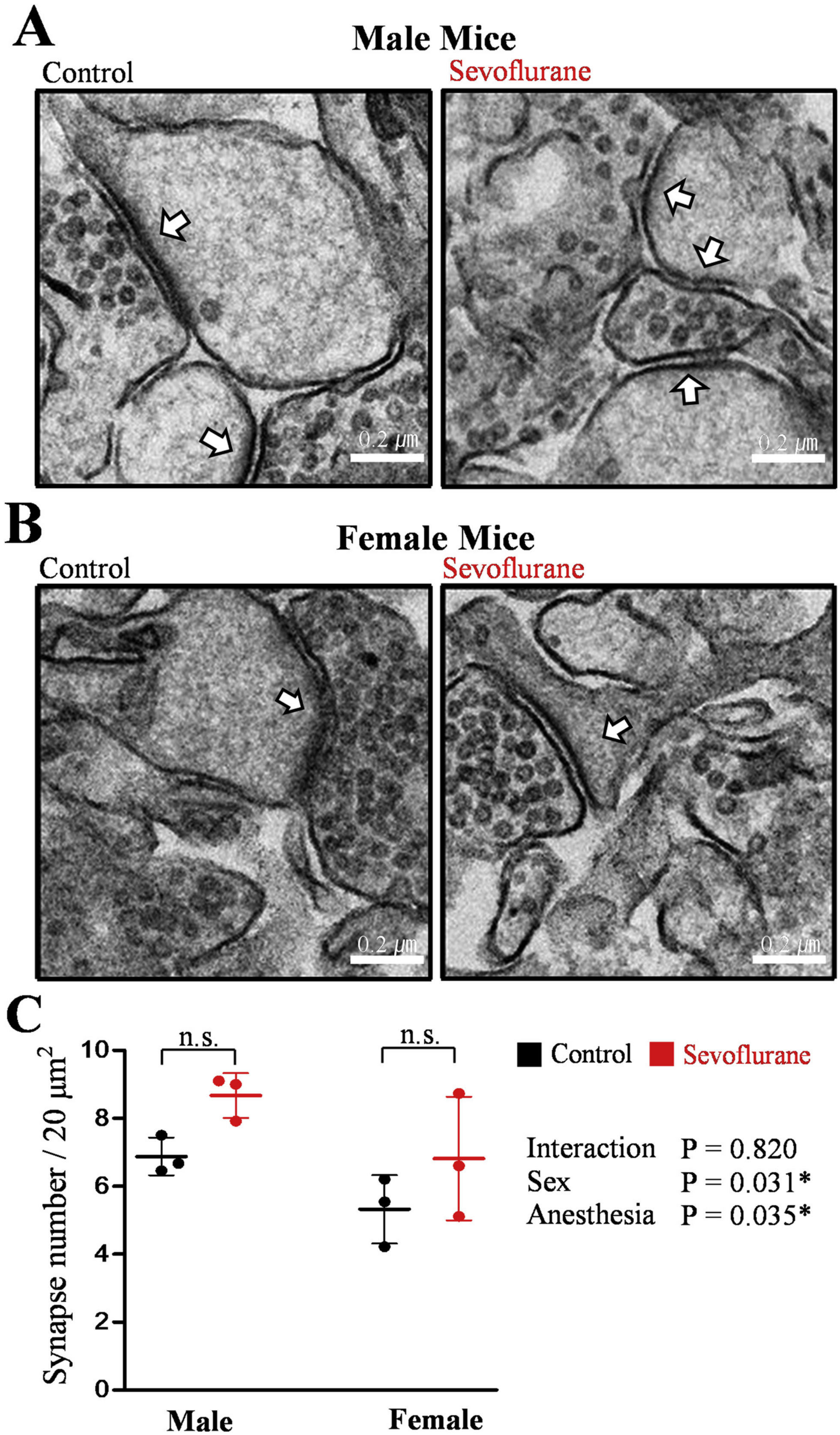

Fig. 4. Electron microscopic images display sex-independent increase of excitatory synapse number after sevoflurane exposure in PND 16, 17 mice. (A, B) Representative images of excitatory synapses in a male and female mouse at 6 h after sevoflurane or oxygen exposure. (C) There was both a significant effect of sex and anesthesia, but no significant interaction. Sevoflurane exposure did not increase synapse number in both male and female mice when separately evaluated (n = 3 per group; two-way ANOVA with nested model, *$P < 0.05$). Arrows indicate excitatory synapses. Values are presented as mean ± SD.

regions.

Another important finding is that early sevoflurane exposure alters inhibitory synapses in multiple brain regions. Previous studies show that anesthesia in late postnatal mice induces dendritic spine formation (Briner et al., 2010, 2011; De Roo et al., 2009). Since excitatory synapses form mostly on dendritic spines, this would logically lead to changes in excitatory synaptic transmission. Unlike excitatory synapses, however, inhibitory synapses are present at the cell body, dendritic shaft, and dendritic spines, making it impossible for researchers to monitor changes morphologically among inhibitory synapses. To our knowledge, our previous mPFC data and the present hippocampal data are the only direct evidence showing that early anesthesia in PND 16, 17 mice can alter both excitatory and inhibitory synaptic transmission. Interestingly, mIPSC frequencies of mPFC and hippocampal pyramidal neurons were affected only in female mice, suggesting that female mice are more sensitive to changes of inhibitory synapses.

There are several important inconsistencies between our results after sevoflurane exposure in PND 16, 17 mice. First, while male mice displayed sex-dependent increases of excitatory synaptic transmission and expression levels of excitatory synaptic molecules, the increase of excitatory synapse number occurred sex-independently. It is possible that sevoflurane exposure affects the expression of AMPA receptors at postsynaptic sites sex-dependently, thus increasing synaptic transmission even without a significant increase of synapse number. Secondly, changes regarding inhibitory synapses are inconsistent in both male and female mice. In male mice, sevoflurane did not affect inhibitory synaptic transmission but increased the expression levels of inhibitory synaptic molecules (gephyrin, GAD65). In female mice, sevoflurane exposure affected inhibitory synaptic transmission, but did not affect the expression of gephyrin, an inhibitory postsynaptic scaffold protein. Although we are presently unable to explain such inconsistences, other studies have reported discrepancies between the expression of inhibitory synaptic molecules and inhibitory synaptic transmission (Levi et al., 2004; Mo et al., 2015). Another confusing fact is that our RT-PCR results are only partially consistent with our western blot results. Previous studies show that translation of plasticity regulators can be regulated independent of transcription (Li et al., 2012). It is possible that sevoflurane enhances protein translation or stability without affecting transcription. Since translation initiation is mediated by multiple factors such as eIF2 or PERK, further evaluations seem necessary.

Our previous study showed that PND 16, 17 mice given a single exposure to sevoflurane did not exhibit long-term behavioral consequences (Chung et al., 2017). Although there have been conflicting results, several previous studies (both preclinical and clinical) have indicated that multiple exposures to anesthesia may carry a higher risk of inducing long-term behavioral consequences (Hu et al., 2017; Zhang et al., 2015). Future studies utilizing various anesthetic protocols (choice of drug, drug dose, number of exposures, etc) may help understand the importance of sex on possible long-term consequences.

The present study has several limitations. First, since we utilized the same anesthetic protocol applied in our previous study (Chung et al., 2017), the present study shares the limitations of the prior work. There were certain unavoidable changes in physiological parameters during anesthesia, such as a slight increase of arterial carbon dioxide and reduction of blood pH, which may have affected our present results. Also, the use of 100% oxygen, which was applied to avoid possible hypoxemia, may have exacerbated the production of oxygen free radicals. As a second limitation, we were unable to analyze the number of inhibitory synapses separately in the images obtained from our electron microscopy. Excitatory synapses (also called asymmetric synapses) are relatively easy to identify based on the presence of a prominent PSD in the post-synaptic region and presynaptic vesicles in the presynaptic region. Unfortunately, inhibitory synapses (also called symmetric synapses) are much more difficult to identify and comprise less than 10% of the total synapses in the hippocampus (Harris and Weinberg, 2012). Measurement of excitatory synapses from single sections obtained from our electron microscopy is also another limitation, since it is less accurate than using a three-dimensional method (Merchan-Perez et al., 2009).

In conclusion, we herein show that exposure of mice to sevoflurane during the critical late postnatal period induces sex-dependent changes in the hippocampus. Our results confirm the importance of sex as a biological variable, although often disregarded when studying the neurobiological changes triggered by early anesthesia. Further studies regarding sex-dependent changes in the brain, especially after multiple anesthetic exposures, may provide valuable insights on anesthesia-induced neurotoxicity.

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Conflicts of interests

None.

Transparency document

The Transparency document associated with this article can be found in the online version.

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