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Original Article

Sevoflurane induces long-term memory impairment and increases MeCP2 phosphorylation in developing mice

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Abstract: Sevoflurane is the most widely used volatile anesthetic in pediatric anesthesia. Some pediatric plastic surgery procedures (such as cleft lip repair, the repair of brachial plexus injury, etc.) may require multiple operations, requiring some children to repeatedly inhale this anesthetic. Evidence has suggested that sevoflurane may cause neuronal deficiency. We investigated the long-term effects of sevoflurane use and possible mechanisms underlying neurodegeneration induced by repeated sevoflurane exposure in the developing brain in the present study. Postnatal day 7 (P7) C57BL/6 mice were randomly exposed to either 1.5% sevoflurane or air control for 2 hours/day on 5 consecutive days. Methyl-CpG island binding protein 2 (MeCP2) phosphorylation and brain-derived neurotrophic factor (*BDNF*) expression in the hippocampus were measured by western blotting. Seven weeks after sevoflurane exposure, a fear conditioning test was used to evaluate associative learning processes. Repeated exposure to 1.5% sevoflurane resulted in increased MeCP2 phosphorylation and decreased *BDNF* expression in the hippocampus. This study demonstrates that P7 mice exposed to 1.5% sevoflurane for 2 hours on 5 consecutive days have significant contextual learning and memory impairment after 7 weeks. These data suggest that repeated sevoflurane exposure may cause neurotoxicity in the developing brain by increasing MeCP2 phosphorylation and decreasing *BDNF* expression in the hippocampus.

Keywords: Sevoflurane, memory impairment, Methyl-CpG island binding protein 2, hippocampus

Introduction

Sevoflurane is widely used in pediatric anesthesia. Some pediatric plastic surgery procedures (such as cleft lip repair, the repair of brachial plexus injury, etc.) may require repeated operations, requiring children to inhale this anesthetic repeatedly. Recent findings have suggested the potential of sevoflurane to exert negative effects on brain development. Satomo et al. [1] demonstrated that a single exposure to sevoflurane results in learning deficits in neonatal mice, causing autism-like abnormal social behavior. Wang et al. [2] suggested that a single sevoflurane exposure induces long-term memory impairment in the developing brain. It is possible that the effects of a single sevoflurane exposure are too subtle to cause a substantial or consistent impairment, particularly as measured by a well-learned task. We thus tested whether repeated anesthesia exposure would impair spatial memory and psychomotor performance to a great extent in neonatal mice.

Methyl CpG island binding protein 2 (MeCP2) is a transcriptional repressor and plays an important role in neuron maturation and normal brain function [3]. As a transcription factor, it can selectively bind to methylated DNA and inhibit the expression of some genes [4]. MeCP2 gene mutations can cause the neurodevelopmental disorder Rett syndrome [5], which can lead to cognitive impairment, motor disability, and repetitive stereotyped hand movements. Studies [6, 7] have found that these symptoms may arise from defects in experience-dependent synapse maturation wherein normal synaptic connections fail to be established during a critical developmental period. Han et al. [8] demonstrated that a single sevoflurane exposure causes neurotoxicity in the developing brain, which may be attributed to increased MeCP2 phosphorylation in the hippocampus. A number of studies have reported that MeCP2 serine phosphorylation sites (S421 and S80) play important roles in its function. S421 phosphorylation was found to be associated with the formation of dendritic spines and

activity-dependent *BDNF* transcript generation [4, 9]. These results demonstrate that MeCP2 is important in the maturation of the nervous system and in the normal function of nerve cells.

BDNF is a member of the neurotrophin family of growth factors, which can regulate neuronal survival, differentiation, and synaptic plasticity. It also plays an important role in learning and memory. Head et al. [10] indicated that 1.4% isoflurane exposure for 4 hours can induce apoptosis in primary cultured neurons and postnatal day 5-7 mouse hippocampal slices. This may be explained by reduced tissue plasminogen activator and mature *BDNF* expression, which affect synapse formation during development. One study demonstrated that MeCP2 can bind to promoter III of the *BDNF* gene and detach from the *BDNF* promoter when MeCP2 phosphorylates, thus facilitating *BDNF* transcription and expression [11]. Another study indicated that *BDNF* is upregulated in MeCP2 transgenic animals and downregulated in MeCP2-null animals. This is consistent with the role of MeCP2 as an activator of the *BDNF* promoter and reconciles the existing data on MeCP2 and *BDNF* [12].

To investigate the molecular mechanisms of neurodegeneration induced by sevoflurane exposure, we exposed neonatal mice to either sevoflurane or air five consecutive times. Protein expression levels of MeCP2 and *BDNF* in the hippocampus were assessed. We also examined the behavioral performance of the mice to determine whether sevoflurane exposure can cause long-term cognitive disorder.

Materials and methods

Animals and sevoflurane exposure

All animal experiments were performed using protocols approved by the institutional animal use and care committee of the ZhongShan Hospital, Fudan University (Shanghai, China). At postnatal day 7 (P7), male C57BL/6 mice (weight, 3-5 g, Shanghai Laboratory Animal Center, Shanghai, China) were randomly divided into a once-sevoflurane-treated group (OS group), a repeated-sevoflurane-treated group (RS group), and an air-treated control group (C group) for analysis of the effects of sevoflurane on *BDNF* expression, MeCP2 phosphorylation levels, and performance in the fear conditioning test. Mice were placed in a plastic container and continuously exposed to 1.5% sevoflurane (Maruishi Pharmaceutical Co., Osaka, Japan) in air (RS group) or to air alone (C group) for 2 hours/day on 5 consecutive days with a gas flow rate of 2 l/minute. Mice in the OS group were continuously exposed to air alone for 2 hours on the first four days and to 1.5% sevoflurane for 2 hours on the fifth day. While the mice were exposed to sevoflurane or air, the container was heated to 37°C with a heating pad. The concentrations of sevoflurane, oxygen, and carbon dioxide in the container were monitored using a gas monitor (Datex Cardiocap II, Datex-Ohmeda, Madison, WI, USA). The mice were housed six per cage and maintained on a 12-hour light/dark cycle with access to food and water ad libitum. Two hours post-exposure, the mice were sacrificed by decapitation and their hippocampi were removed. In the second part of this study, the mice were housed in standard cages and kept for the fear conditioning test, which was performed 7 weeks later.

Arterial blood gas analysis

Arterial blood samples were obtained from the left cardiac ventricle of the mice immediately after exposure to sevoflurane and were transferred to heparinized glass capillary tubes. Blood pH, partial pressure of carbon dioxide in mmHg ($PaCO_2$), partial pressure of oxygen in mmHg (PaO_2), and lactate (Lac) and bicarbonate (HCO_3) levels were analyzed immediately after blood collection using a GEM Premier 3000 analyzer (Instrumentation Laboratory, Lexington, MA, USA).

Fear conditioning test

Fear conditioning was carried out in a standard fear conditioning apparatus (Panlab, Barcelona, Spain). The floor consisted of 20 steel rods through which a scrambled shock could be delivered. The sidewalls of the observation cage were of black stainless steel and the door was made of Plexiglas. On the first day, the mice freely explored the testing chamber for 5 minutes. The next day, auditory fear conditioning (AFC) training began with a 120 second habituation period, followed by three 0.6 mA shocks (inter-shock interval: 30 seconds). Each shock was paired with a 30 second tone (800 Hz, 80 dB), which was presented before the shock. Ethanol (4%) was used for

Table 1. Arterial blood gas analysis

Arterial blood gas	Control (n = 6)	Sevoflurane (n = 6)
PH	7.38±0.05	7.36±0.04
$PaCO_2$ (mmHg)	28.5±2.8	29.2±3.0
PaO_2 (mmHg)	90.8±6.7	88.9±5.7
SaO_2 (%)	93.3±2.5	92.8±2.6
Lac (mmol/l)	1.16±0.5	1.19±0.4
HCO_3 (mmol/l)	23.9±1.5	24.0±1.4

Exposure to sevoflurane did not induce significant metabolic or respiratory impairment. Analysis of arterial blood gas revealed no significant differences in any of the measured parameters between the sevoflurane group and the control one (t-test, P > 0.05). Pa, Partial pressure; Sa, Saturation.

cleaning in between training sessions. Fear memories to the context were assessed by putting the mice into the same context as the one present during the training for 5 minutes without administering the shocks. To measure fear memories to the tone, visual (gray plastic walls) and odor (4% chlorine) cues in the conditioning chamber were exchanged and mice remained in the chamber for 5 minutes, during which the tone test was continuously on for the last 3 minutes (each pair with a 30 second tone and a 30 second interval). A door-mounted camera recorded the length of time the mice spent freezing. The percentages of time spent freezing during the 5-minute context and 3-minute tone memory test were taken as indicators of fear. Control of the stimuli, data acquisition, and analysis were performed automatically using Image FZ software, which is a modified version of the NIH ImageJ program. Images were captured (one frame per second) and for each pair of successive frames, the area (in pixels) that the mouse moved was measured. If this amount was equal to or above the threshold (ten pixels), then the mouse was considered to be 'moving'; otherwise the mouse was considered to be 'freezing' [13]. Freezing that lasted less than 1 second was not included in our analysis.

Protein extraction and western blot analysis

Resected hippocampi were placed into 1.5 ml centrifuge tubes and preserved in liquid nitrogen. All procedures were conducted on ice. An NE-PER Nuclear and Cytoplasmic Extraction kit (cat no. 78835; Thermo Fisher Scientific, Waltham, MA, USA) was used to extract protein from the samples. All steps were conducted according to the manufacturer's instructions. Sodium dodecyl sulfate (SDS) was added to the samples prior to boiling for 10 minutes at 100°C. Equal quantities of protein (15 μg) were used to detect the expression of the proteins of interest. Samples were electrophoresed on 10% or 15% SDS polyacrylamide gels, blotted onto polyvinylidine fluoride membranes (Bio-Rad Laboratories, Hercules, CA, USA), and then incubated with the following antibodies overnight at 4°C: Anti-*BDNF* (cat no. AB1779SP, Millipore) at a 1:1,000 dilution in 5% non-fat milk; anti-MeCP2 (cat no. 3456P, Cell Signaling Technology, Danvers, MA, USA) at a 1:4,000 dilution in 5% non-fat milk; anti-phospho-MeCP2-S421 (cat no. AP3693a, Abgent Biotech, Suzhou, China) at a 1:2,000 dilution in 5% non-fat milk; and anti-actin (cat no. A5441, Sigma-Aldrich, St. Louis, MO, USA) at a 1:10,000 dilution in 5% non-fat milk. The following day, the blots were incubated for 1 hour at room temperature with horseradish peroxidase-conjugated secondary goat anti-rabbit or goat anti-mouse immunoglobulin G (Kangchen, Shanghai, China) at a 1:5,000 dilution in 5% non-fat milk. Immunoreactive bands were visualized using the Amersham ECL Prime Western Blotting Detection kit (cat no. RPN2232; GE Healthcare, Chalfont St Giles, UK). The protein signals were quantified using Quantity One software and a GS-800 Calibrated Imaging Densitometer (Bio-Rad Laboratories), and normalized to a corresponding internal reference: MeCP2 for P-MeCP2-S421 and actin for *BDNF*.

Statistical analysis

All data are presented as the mean ± standard error. Data were analyzed with one-way ANOVA followed by Bonferroni's *post hoc* test or two-tailed Student's *t* test in Origin software, version 7.5 (OriginLab, Northampton, MA, USA). $P < 0.05$ was considered to represent a statistically significant difference.

Results

Sevoflurane does not induce metabolic or respiratory deterioration

Blood gas analyses indicated that there was no deterioration in respiration or metabolism in the animals following a 2-hour sevoflurane exposure. All parameters were tested, including

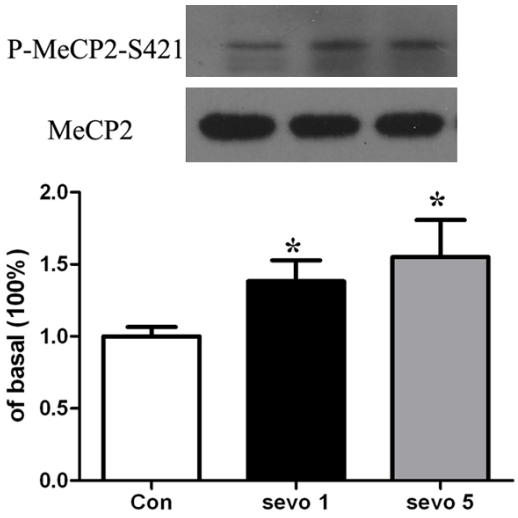

Figure 1. Repeated sevoflurane treatment leads to a significant increase in hippocampal P-MeCP2-S421 expression levels at postnatal day 7. *P < 0.05, sevo5 group (n = 6) vs. control group (n = 6). Single sevoflurane treatment leads to a significant increase in hippocampal P-MeCP2-S421 expression levels at postnatal day 7. *P < 0.05, sevo1 group (n = 6) vs. control group (n = 6). MeCP2, methyl-CpG island binding protein 2.

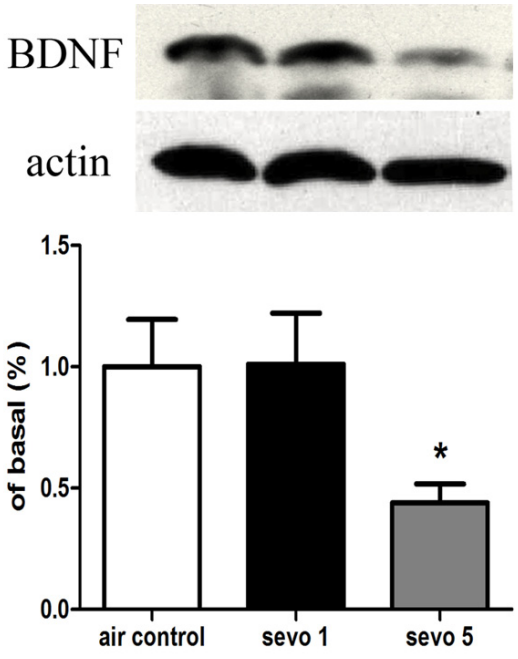

Figure 2. Repeated sevoflurane treatment leads to a significant decrease in hippocampal *BDNF* expression levels at postnatal day 7. *P < 0.05, sevo5 group (n = 6) vs. control group (n = 6). Single sevoflurane treatment did not cause significant changes in hippocampal *BDNF* expression levels at postnatal day 7. P > 0.05, sevo1 group (n = 6) vs. control group (n = 6). *BDNF*, brain-derived neurotrophic factor.

pH, $PaCO_2$, PaO_2, oxygen saturation, and Lac and HCO_3 levels. No significant differences in any of the parameters were detected between the sevoflurane and control groups (P > 0.05, **Table 1**).

Repeated sevoflurane treatment increases MeCP2 phosphorylation at the serine 421 locus in the hippocampus

Western blot analysis of hippocampal MeCP2 phosphorylation in the sevoflurane-treated and control groups was performed 2 hours following exposure to sevoflurane or air. The results indicate that mice repeatedly treated with sevoflurane mice (n = 6) exhibited an increase in hippocampal MeCP2 phosphorylation at the serine 421 locus (P-MeCP2-S421) when compared with control mice (n = 6) (P < 0.05, **Figure 1**). The results also indicate that there were significant differences in the levels of P-MeCP2-S421 between the single sevoflurane-treated (n = 6) and the air-treated (n = 6) groups (P < 0.05, **Figure 1**).

Repeated sevoflurane treatment decreases BDNF expression in the hippocampus

Western blot analysis of hippocampal *BDNF* expression levels was performed 2 hours following exposure to sevoflurane or air. The results indicated that there were significant differences in the levels of *BDNF* expression between the repeated sevoflurane-treated (n = 6) and the air-treated (n = 6) groups (P < 0.05, **Figure 2**). The results also indicate that there were no significant differences in the levels of *BDNF* expression between the single sevoflurane-treated (n = 6) and the air-treated (n = 6) groups (P > 0.05, **Figure 2**).

Repeated sevoflurane treatment decreases the average percentage of freezing time in the context test of the fear conditioning paradigm

The average percentage of time spent freezing over the entire 5 minutes of the context test was calculated. The results indicate that compared to control mice, mice treated repeatedly with sevoflurane exhibited a decrease in context freezing. The average percentage of time spent freezing was decreased in the repeated sevoflurane-treated group (n = 9) compared with the control group (n = 8) (P < 0.01, **Figure 3**). The results also indicate that there

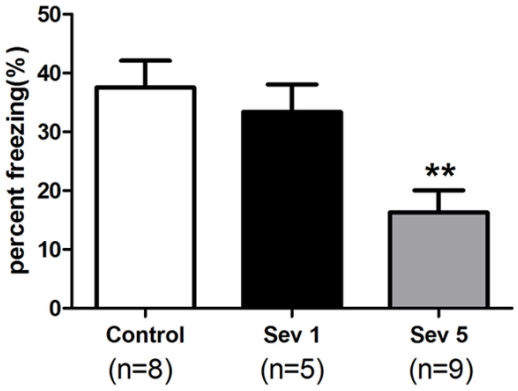

Figure 3. In the context test of the fear conditioning paradigm, repeated sevoflurane treatment leads to a significant decrease in the average percentage of time spent freezing at postnatal day 7. *P < 0.01, sevo5 group (n = 9) vs. control group (n = 8). Single sevoflurane treatment did not cause significant changes in the average percentage of time spent freezing at postnatal day 7. P > 0.05, sevo1 group (n = 5) vs. control group (n = 8).

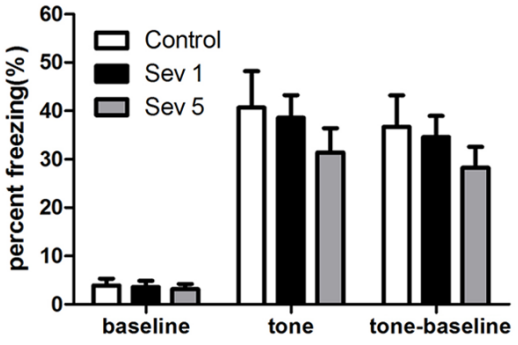

Figure 4. In the tone test of the fear conditioning paradigm, the average percentages of time spent freezing during the 120-second baseline period were not significantly different among the three groups (P > 0.05). The average percentages of time spent freezing during the tone and the tone-baseline periods were not significantly different among the three groups (P > 0.05).

were no significant differences in context freezing between the single sevoflurane-treated (n = 5) and the air-treated (n = 8) groups (P > 0.05, **Figure 3**).

Repeated sevoflurane treatment does not lead to a change in the average percentage of freezing time in the tone test of the fear conditioning paradigm

During the 120-second baseline period of the cue test, no differences were observed among the three groups (P > 0.05, **Figure 4**). The average percentages of time spent freezing during the tone and the tone-baseline periods were not significantly different among the three groups (P > 0.05, **Figure 4**).

Discussion

In the present study, we found that repeated sevoflurane exposure may increase MeCP2 phosphorylation and decrease *BDNF* expression in the hippocampi of P7 mice. In addition, the mice that underwent repeated sevoflurane exposure may have a reduced learning ability in the fear conditioning test as adults compared to control mice. This indicates that repeated sevoflurane exposure may impair the developing brain in mice.

Behavioral impairment was observed in mice after sevoflurane exposure on P56. The poor performance of sevoflurane-exposed mice in the fear conditioning test was manifested as less time spent freezing, which is considered to be a reliable indicator of fear in rodents. It is widely accepted that the contextual fear conditioning test depends on the hippocampus and the tone test is more dependent on the frontal cortex, the hippocampus, and the amygdala [14, 15]. Therefore, we conclude that repeated neonatal sevoflurane exposure causes long-term hippocampal impairment. This is consistent with data from other labs. In the current study, we used 1.5% sevoflurane, which did not inhibit respiration and circulation in mouse pups. Arterial blood analyses confirmed that none of the mice experienced hypoxemia or hypercapnia during the 2-hour sevoflurane exposure. There were no significant differences in any of tested parameters between the sevoflurane group and the control group. The results exclude the possibility of hypoxemia and hypercapnia affecting the outcomes of our experiments.

Recent reports indicate that early exposure to sevoflurane causes widespread neurodegeneration in the developing brain [16]. However, the exact mechanisms of action underlying the effects of sevoflurane remain unknown. The results of our previous study suggested that MeCP2 may be important in neuronal degeneration following neonatal sevoflurane exposure [8].

MeCP2 is mainly considered to be a transcriptional repressor, is critical in the maturation of the nervous system, and plays an important role in learning and memory [17]. MeCP2 phosphorylation at the serine-421 locus is a key signal, which may cause downstream changes in the signaling pathway. Phosphorylation at this site was found to be important in the formation of dendritic spines and activity-dependent *BDNF* transcription [6, 18]. *BDNF*, a member of the neurotrophin family of growth factors, can regulate neuronal survival, differentiation, and synaptic plasticity, and plays an important role in learning and memory as well. Previous studies have found that *BDNF* can be regulated by MeCP2. Studies have demonstrated that MeCP2 can bind to promoter III of the *BDNF* gene and can detach from the *BDNF* promoter when it is phosphorylated, facilitating *BDNF* transcription and expression [11, 19]. However, in our present study, we found that P-MeCP2-S421 expression levels increased and *BDNF* expression decreased in the hippocampus following repeated sevoflurane exposure. This indicates that MeCP2 may regulate *BDNF* expression in an indirect way, leading to various effects on brain function in mice. Further study of these aspects is required in order to determine how MeCP2 regulates the *BDNF* signal pathway and how the two pathways are involved in the regulation of neuronal degeneration following neonatal sevoflurane exposure.

In conclusion, our study demonstrated that repeated sevoflurane exposure may cause neuronal impairment in the developing mouse brain. The MeCP2 and *BDNF* signaling pathways play important roles in this process and may contribute to the impairment of hippocampal function. Further investigation remains to be carried out to determine how such impairment happens in the mouse hippocampus. These studies may be helpful for regulating the use of anesthesia in clinical settings.

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Disclosure of conflict of interest

None.

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