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Neonatal sevoflurane anesthesia induces long-term memory impairment and decreases hippocampal PSD-95 expression without neuronal loss

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Abstract. – AIM: Volatile anesthetics are widely used in the clinic, and sevoflurane is the most prevalent volatile anesthetic in pediatric anesthesia. Recent findings question the potential risks of volatile anesthetics on brain development. Evidence suggests that sevoflurane may cause neuronal deficiency. This study investigates the long-term effect of sevoflurane in the developing brain.

MATERIALS AND METHODS: We anesthetized 7 day-old rats for 4 h with 2.5% sevoflurane. A Morris water maze was used to evaluate hippocampal function 7 weeks after sevoflurane exposure. Nissl staining was performed to analyze neuronal loss. PSD-95 (postsynaptic density protein-95) expression in the hippocampus was measured using a western blot.

RESULTS: The exposure to 2.5% sevoflurane caused long-term deficits in hippocampal function and decreased hippocampal PSD-95 expression without neuronal loss. This study demonstrates that P7 rats exposed for 4 h to 2.5% sevoflurane have significant spatial learning and memory impairment 7 weeks after anesthesia. In addition, PSD-95 expression in the hippocampus decreased at P56 without neuronal loss.

CONCLUSIONS: These data suggest that sevoflurane causes neurotoxicity in the developing brain, which may be attributed to decreased PSD-95 in the hippocampus.

Key Words:
Sevoflurane, Neurotoxicity, Rat, Developing brain, PSD-95, Hippocampus.

Introduction

Volatile anesthetics are widely used in the clinic, and sevoflurane is the most prevalent volatile anesthetic in pediatric anesthesia. It is suitable for infants and children due to its low blood/gas partition coefficient, rapid onset and offset, aromatic odor, and low airway irritation. However, recent findings question the potential risks of volatile anesthetics on brain development. Exposure of newborn animals to a variety of anesthetics increases neural stem cells and neuronal apoptosis, causing prolonged neurodegeneration in the developing brain[1-11]. Jevtovic-Todorovic et al[7] and others[9,12,13] observed that isoflurane causes widespread apoptotic neurodegeneration in the developing brain, hippocampal synaptic function deficits, and persistent memory impairments. Similar findings were detected in sevoflurane-exposed rat pups[14-18]. Satomoto et al[19] found that exposing neonatal mice to sevoflurane caused learning deficits as well as abnormal social behaviors that resemble autism spectrum disorder. Moreover, a study in human volunteers suggested that 0.25% sevoflurane blocks emotional memory[20].

Postsynaptic density protein-95 (PSD-95) is a membrane-associated guanylate kinase (MAGUK) that is concentrated at glutamatergic synapses[21-24]. It regulates adhesion and receptor function as well as controls *N*-methyl-D-aspartate glutamate (NMDA) and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor clustering[25-32]. PSD-95 enhances maturation of the presynaptic terminal, increases the number and size of dendritic spines[33], and contributes to synaptic organization[34] after neuronal damage from cerebral ischemia[35,36]. These results demonstrate that PSD-95 can orchestrate synaptic development and plays an important role in synapse stabilization and plasticity[33].

To explore the molecular mechanisms of prolonged neurotoxicity induced by sevoflurane, we examined neuronal number and PSD-95 expression in the hippocampus of 7 day-old rats after

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sevoflurane exposure. We also examined their neurobehavioral performance to determine whether a single exposure of sevoflurane can cause long-term cognitive disorders.

Materials and Methods

Animals and General Anesthesia

Male Sprague-Dawley rats (n = 120) at postnatal day 7 (P7) were randomly assigned to sevoflurane anesthesia or mock anesthesia groups. Anesthesia was performed by placing the animal in a plastic chamber flushed continuously for 4 h with 2.5% sevoflurane (Maruishi Pharmaceutical Co. Ltd., Osaka, Japan) in air or air alone at identical flow rates. The temperature of the chamber floor was maintained at 37°C using a heating pad under the chamber. After exposure to sevoflurane or air, rats were housed in standard cages with 4 animals/cage for the duration of the experiment. Ethical approval was obtained from the Zhong Shan Hospital Research Ethics Committee (Shanghai, China).

Arterial Blood Gas Analyses

The pups underwent arterial blood sampling from the left cardiac ventricle, and the samples were transferred to heparinized glass capillary tubes. Blood pH, $PaCO_2$, PaO_2, lactate (Lac), and bicarbonate (HCO_3) were analyzed immediately after blood collection using a GEM Premier 3000 (Instrumentation Laboratory; Lexington, MA, USA). Samples were obtained immediately upon completion of anesthesia.

Morris Water Maze

We followed the procedure established by Morris et al[37]. Briefly, the Morris water maze (MWM), a circular pool (2 m diameter; 0.6 m depth), was filled with water (19-21°C). A transparent platform (15 cm diameter) was placed in a constant position (in the middle of one of the pool's quadrants, 1-2 cm below the water surface to render it invisible) for each set of trials. Rats were subjected to 3 consecutive training days (3 series of 8 trials) to familiarize them with finding and perching on the hidden platform that was maintained in the fixed location. At the start of each trial, rats were placed in the pool facing the wall and were allowed to swim for 60 s or until the platform was found. If the rat did not find the platform during the trial, it was guided to the platform and remained on it for 15 s. On the test day (day 4), rats were given one 60-s probe trial in which the platform was removed from the pool to evaluate the number of times the animal crossed the previous location of the platform, time spent in the target quadrant, swimming speed in the target quadrant, and overall swimming speed.

Nissl Staining

After 8 weeks, rats from each experimental group were perfused transcardially after anesthetizing with 40 ml of normal saline, followed by 30 ml of 4% paraformaldehyde (PFA). The brains were fixed in 4% PFA overnight at 4°C. Coronal sections (20 m in thickness) were cut using a vibratome (VT1200; Leica, Wetzlar, Germany). Sections were mounted on microscope slides (Fisher, Fair Lawn, NJ, USA) and air-dried. The slides were soaked for 3 min in cresyl violet working solution (0.02% buffer solution, 0.2% sodium acetate, and 0.3% acetic acid), dehydrated with alcohol and xylene, and mounted[38]. All images were acquired using an Olympus IX71 microscope (Tokyo, Japan) and displayed as maximum-intensity projections of Z-stack images created using Image-Pro software (Media Cybernetics, Silver Spring, MD, USA). Cell counts were performed without knowledge of the experimental conditions. Nissl-positive neuronal cells were manually and blind-test counted within the hippocampal dorsal ganglia, CA1 (cornu ammonis 1), and CA3 regions. Total cell counts were averaged from at least 3 sections of each hippocampal region per animal.

Western Blot Analyses

On P7 and P56, rats were decapitated and the hippocampi were homogenized and sonicated in 2% SDS (sodium dodecyl sulfate) buffer containing 50 mM Tris (pH 6.8), 1 mM EDTA, 1 mM sodium fluoride, 1 mM sodium orthovanadate, and complete protease inhibitor mixture (Sigma P8340; Sigma Aldrich Co., St. Louis, MO, USA; 1:100 dilution). The homogenates were centrifuged at 15,000 × *g* for 20 min at 4°C. Protein concentrations were estimated using a Bradford assay (Bio-Rad, Hercules, CA, USA). Samples were treated for 5 min with Sodium Dodecyl Sulphate (SDS) buffer at 95°C, electrophoresed on a 10% SDS polyacrylamide gel, and blotted to a PVDF membrane (Millipore, Bedford, MA, USA). Blots were incubated overnight at 4°C with the rabbit PSD-95 antibody (Cell Signaling Technology, Danvers, MA, USA; 1:1000 dilution)

or a monoclonal -actin antibody (Cell Signaling Technology, Danvers, MA, USA; 1:4000 dilution). Blots were incubated for 1 h at room temperature with HRP-conjugated secondary goat anti-rabbit immunoglobulin G (IgG; Cell Signaling Technology, Danvers, MA, USA; 1:4000 dilution). Immunoreactive bands were visualized using Bio-Rad Universal Hood 76S/08298 (Bio-Rad, Hercules, CA, USA). PSD95 immunoreactivity was normalized to that of -actin. Each experiment was repeated at least 5 times.

Statistical Analysis

All data are expressed as the mean ± SEM. Data were analyzed using unpaired Student's *t*-tests using Origin 7.5. $p < 0.05$ was considered statistically significant.

Results

Sevoflurane Does Not Induce Metabolic or Respiratory Impairment

The pups appeared pink throughout the 4 h sevoflurane exposure, suggesting no impairment in respiration or metabolism. Animals recovered rapidly from anesthesia and displayed no neurologic symptoms or signs of discomfort. There was no mortality during or after anesthesia. Furthermore, to assess the ventilation and oxygenation efficiency, we performed blood gas analyses at the end of anesthesia (n = 9). Control samples were obtained from rats exposed to air-oxygen mixture alone during the same period. Blood gas analyses indicated no signs of metabolic or respiratory impairment. All parameters tested, including pH, arterial oxygen tension, and arterial carbon dioxide tension, did not differ significantly from the control (*t*-test, all *p* values > 0.05; Table I).

Table I. Arterial blood gas analyses.

Arterial blood gas	Control	Sevoflurane
pH	7.4 ± 0.05	7.38 ± 0.04
$PaCO_2$ (mmHg)	28.4 ± 3.0	30.1 ± 4.0
PaO_2 (mmHg)	91.0 ± 6.8	88.8 ± 5.6
SaO_2 (%)	93.6 ± 2.6	92.7 ± 2.5

Exposure to sevoflurane does not induce significant metabolic or respiratory dysfunction. Arterial blood gas analyses revealed no significant differences in any of the measured parameters between the sevoflurane-exposure group and controls (*t*-test, *p* values > 0.05).

Neonatal Sevoflurane Exposure Results in Late-Onset Learning and Memory Deficits

After exposure to 2.5% sevoflurane or air at P7, rats were tested in the Morris water maze at P56 to evaluate spatial learning and reference memory.

Rat spatial memory was impaired 7 weeks (P56) after sevoflurane exposure (Figure 1). Both anesthesia groups successfully learned the task, but the sevoflurane-exposure group had a higher escape latency (n = 15, $p < 0.05$) (Figure 1 A). During the probe trial, sevoflurane-exposed rats spent less time in the target quadrant searching for the missing platform than did the control rats (n = 15, $p < 0.05$) (Figure 1 B). The decreased number of times the sevoflurane-exposed rats crossed the former location of the platform reflected impairment in special memory (n = 15, $p < 0.05$) (Figure 1 C). However, sevoflurane-exposed rats had the same swimming speed compared with to control rats (n = 15, $p > 0.05$) (Figure 1 D), indicating that motor deficits did not contribute to differences in escape latencies, number of times of crossing the platform location, or time spent in the target quadrant.

Sevoflurane Does Not Reduce the Number of Healthy Pyramidal Neurons in the Hippocampus

Using Nissl staining, we investigated whether sevoflurane increased hippocampal neuronal loss 49 days after exposure. As shown in Figure 2, compared with pyramidal neurons in the hippocampus of the control group, there were no remarkable neuropathological changes, including neuronal loss and nucleus shrinkage, in each region of the hippocampus (n = 12, $p > 0.05$).

Sevoflurane Time-Dependently Decreases PSD-95 Expression in the Hippocampus

Western blot analyses of hippocampal PSD-95 in the sevoflurane-exposure group and control group were performed immediately after the rats recovered from anesthesia, and 49 days after anesthesia (P56). The results showed that, compared with control rats, sevoflurane-treated rats exhibited a time-dependent decrease in hippocampal PSD-95. PSD-95 expression was significantly different between the sevoflurane and control groups (n = 12, $p < 0.05$) in the hippocampus of rats at P56, but not at P7 (n = 12, $p > 0.05$) (Figure 3).

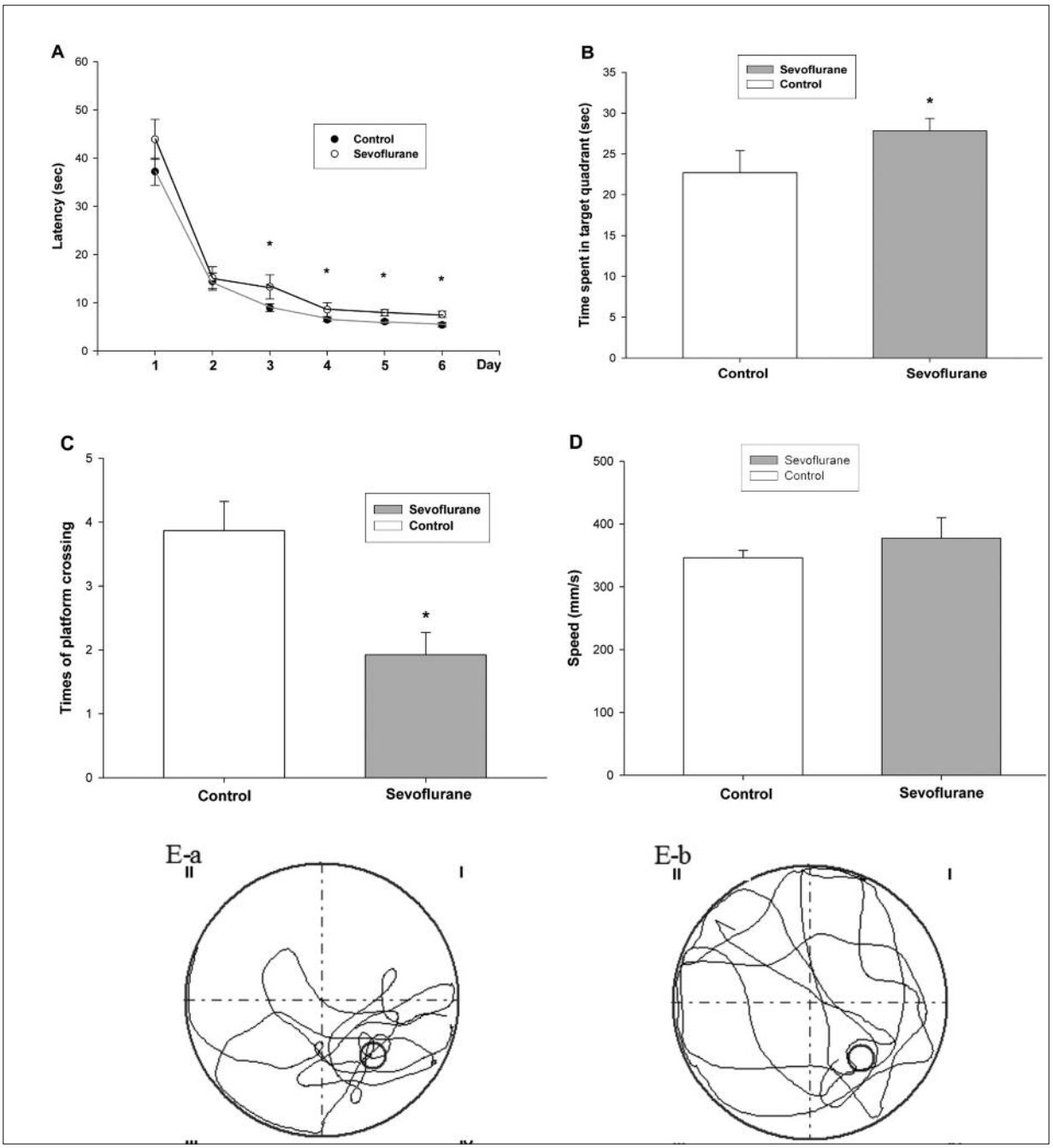

Figure 1. Effect of neonatal sevoflurane exposure on spatial learning. ***A,*** Rats were evaluated at P56 for their ability to determine the location of a hidden platform. A *t*-test of escape latency (the time to find the hidden platform) indicated that the performance of sevoflurane-exposed rats was significantly inferior to that of control rats during place training. ***B,*** Probe trial performance of sevoflurane-exposed and control rats during testing. During the probe trial, sevoflurane-exposed rats spent less time in the target quadrant searching for the missing platform than did the controls ($p < 0.05$). ***C,*** During the probe trial, sevoflurane-exposed rats spent less time crossing the former location of the platform than did the controls ($p < 0.05$). ***D,*** Swimming speed during the probe trial. Sevoflurane-exposed rats had a similar swimming speed to the control rats ($p > 0.05$). ***E-a,*** Typical swimming pattern in the probe trial for control rats. ***E-b,*** Typical swimming pattern in the probe trial for sevoflurane-exposed rats.

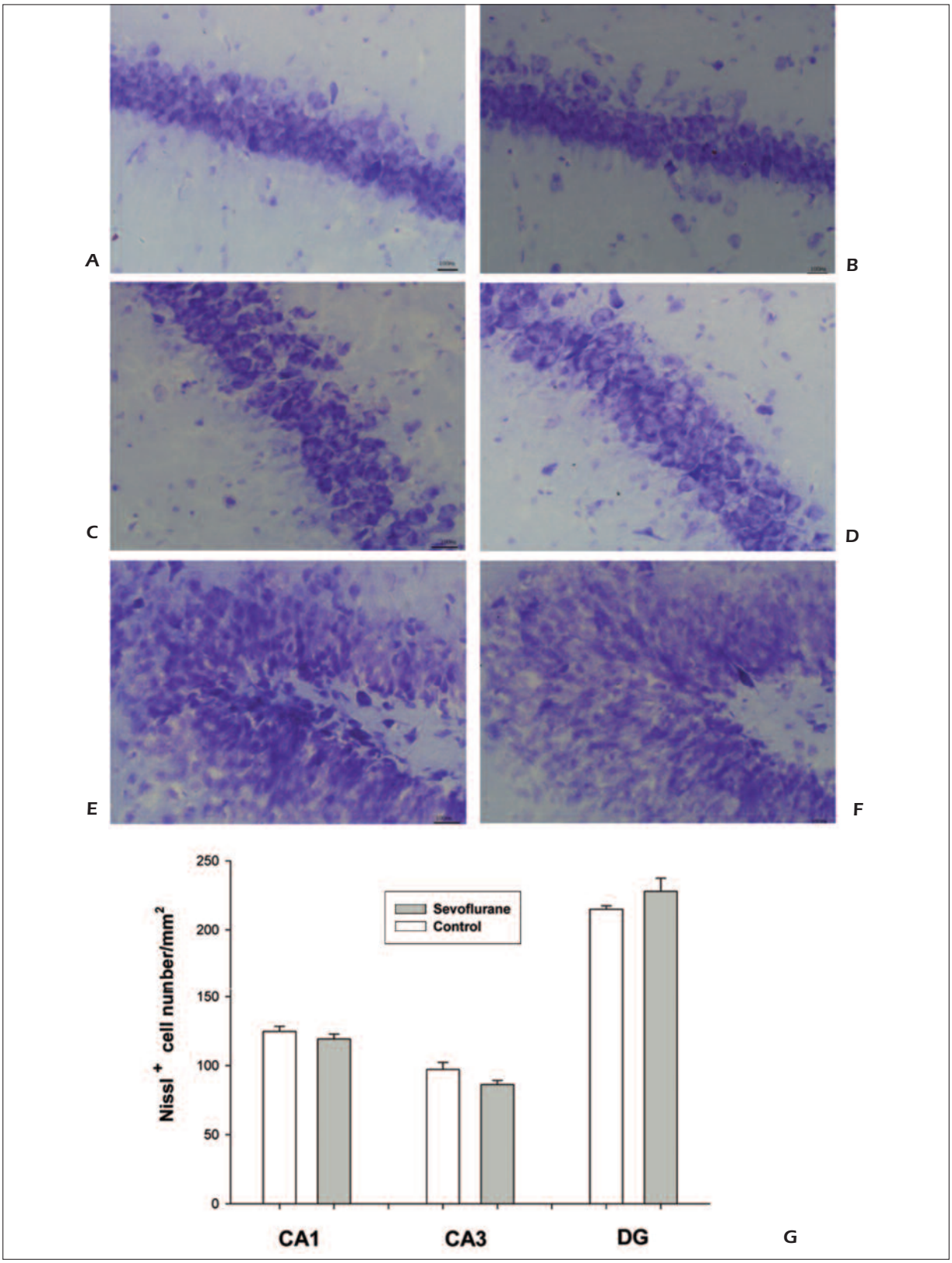

Figure 2. Neonatal sevoflurane exposure did not cause significant neuronal loss. Sections of CA1, CA3, and DG were Nissl stained (×40): X. No remarkable neuropathological changes, including neuronal loss and nucleus shrinkage, in each hippocampal region were observed.

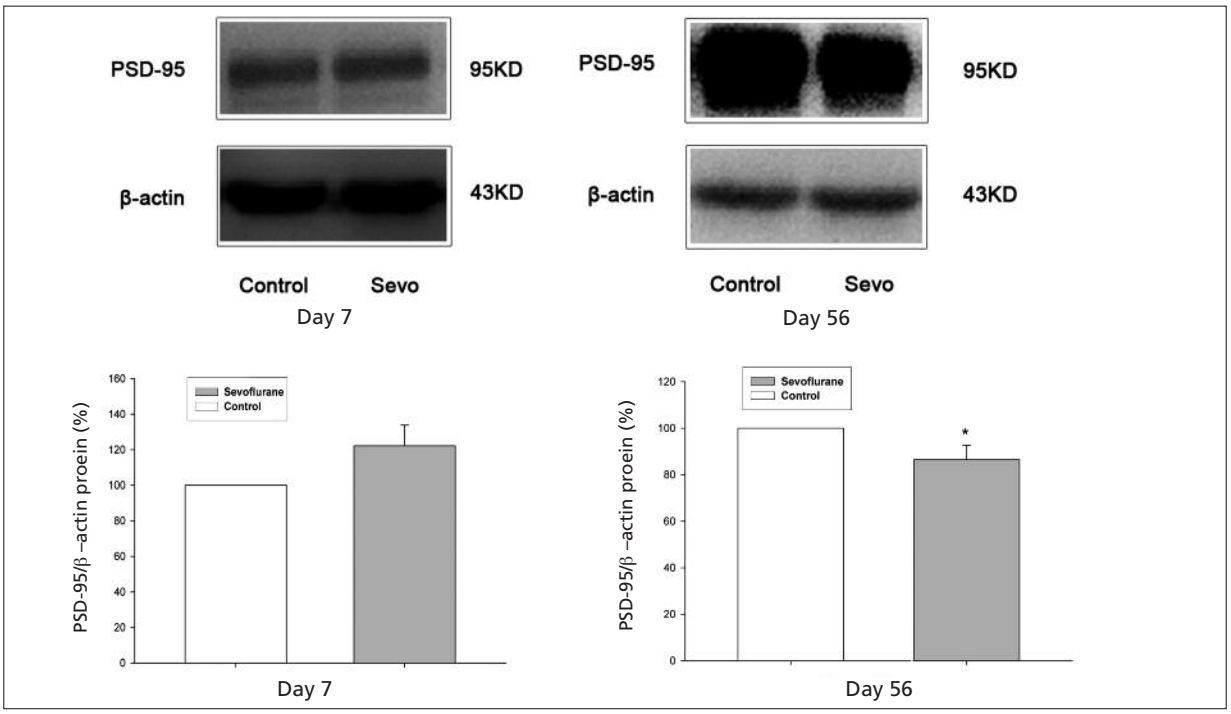

Figure 3. Compared with control rats, sevoflurane-treated rats exhibited a time-dependent decrease in hippocampal PSD-95 expression. A significant difference in PSD-95 expression was detected in the hippocampi of rats in the sevoflurane group and control group ($p < 0.05$) at P56, but not at P7 ($p > 0.05$).

Discussion

This study demonstrates that P7 rats exposed for 4 h to 2.5% sevoflurane have significant spatial learning and memory impairment 7 weeks after anesthesia. In addition, PSD-95 expression in the hippocampus decreased at P56 without neuronal loss.

Behavioral impairment was observed in rats after sevoflurane exposure on P56. The poor performance of sevoflurane-exposed rats in the Morris water maze was manifested as higher escape latency, less time spent in the target quadrant, and fewer number of times the platform was crossed. It is widely accepted that spatial learning and reference memory detected by the Morris water maze represent hippocampal function[37,39,40]. Therefore, we conclude that neonatal sevoflurane exposure causes long-term hippocampal impairment. This is consistent with data from other labs[14,41]. In this study, we used 2.5% sevoflurane that did not inhibit respiration and circulation in rat pups. Arterial blood analyses confirmed that none of the rats experienced hypoxemia or hypercapnia during the 4-h sevoflurane exposure. In addition, no differences in the arterial blood gas analyses between sevoflurane-exposed and control rats were detected. These results exclude the contribution of hypoxemia or hypercapnia to behavioral impairment. The similar swimming speeds of sevoflurane-exposed and control rats suggest that learning and memory deficits in anesthetized rats do not result from poor locomotor performance. From these data we hypothesize that sevoflurane itself triggers neurodegeneration.

Brain growth spurts in prenatal and postnatal stages[42]. In humans, the largest increase in brain weight in both sexes occurs during the first 3 years of life[43]. In rats, brain growth is most rapid during the first 2 weeks of postnatal life[44]. Any influence imposed during the period of brain growth affects brain development[2,45-48]. In the present study we used rats at P7 as immature animals to study sevoflurane-induced neuronal impairment.

Although the exact mechanism of action of sevoflurane remains unknown, alteration of synaptic transmission involving the γ-aminobutyric acid type A (GABA) and NMDA receptors seems to play an important role[49-53]. The GABA and NMDA receptors are essential for development of an ordered neural map[54-57]. Neurotransmitters or compounds that act on NMDA or GABA receptors may contribute to the impairment of brain development and synaptogenesis[12,13,58-62].

We hypothesized that sevoflurane impairs hippocampal function via the GABA receptor and/or NMDA receptor.

We observed no increased neuron loss 7 weeks after sevoflurane exposure. Similarly, Loepke et al[1] found increased rates of brain cell death in the hours after neonatal isoflurane exposure without a detectable reduction in neuronal density in adulthood. Bercker et al[15] revealed that sevoflurane did not cause increased cell death although the animals were deeply anesthetized. In addition, we detected a time-dependent reduction of PSD-95 expression in the hippocampus after neonatal sevoflurane exposure. Hippocampal PSD-95 expression did not decrease immediately after sevoflurane exposure, but occurred 7 weeks later. The decrease in PSD-95 may be due to dysplasia of the glutamatergic synapse or a lower density of PSD-95 on excitatory postsynaptic membranes.

Recent investigations revealed some mechanisms may be involved in the cognitive impairment. Xie Z[72] found nitrous oxide plus isoflurane can promote neurotoxicity by inducing apoptosis and increasing -amyloid protein (A) levels[73]. Law A's finding suggested a correlation between aged cognitive impairment and change in messenger RNA expression for the neuronal nitric oxide synthase and haem oxygenase-2 systems in hippocampus. Our study may provide another possible explanation for neuron degeneration. PSD-95 may play an important role in neuronal degeneration after neonatal sevoflurane exposure.

PSD-95 is a membrane-associated guanylate kinase (MAGUK) that is concentrated at glutamatergic synapses[21-24]. It regulates NMDA and AMPA receptor clustering[33,63-65] and plays an important role in the transport, localization, and assembly of supramolecular signaling complexes[66,67]. PSD-95 may recruit ion channels and neurotransmitter receptors to intercellular junctions formed between neurons[68]. It also enhances maturation of the presynaptic terminal, increases the number and size of dendritic spines[33], and contributes to synaptic organization[34,69]. PSD-95 interacts with neuronal nitric oxide synthase (nNOS)[63]. Overexpression of PSD-95 in hippocampal neurons can drive maturation of glutamatergic synapses[33]. Mice lacking PSD-95 have severely impaired spatial learning[69]. Feng Tao[74] has demonstrated that PSD-95 PDZ domain-mediated protein-protein interations are disrupted by clinically relevant concentrations of inhaled anesthetics. It is in good agreement with our result.

Xie Z[75] found sevoflurane can elevate levels of beta-site amyloid precursor protein-cleaving enzyme and A beta *in vitro* and *in vivo*. A 42 could induce decreases in PSD-95[76]. So, A may has a role in the decreases in PSD-95. Neonatal exposure to sevoflurane causes neurohistopathological changes and decreases nNOS protein levels in the rat hippocampus[77]. Early exposure to sevoflurane decreases nNOS in the neonatal hippocampus[16]. This change of nNOS may disrupt nNOS-PSD protein interaction, resulting in the decreases of PSD-95.

Conclusions

In the present study, decreased PSD-95 expression may contribute to the impairment of hippocampal function. The lack of the PSD-95 bond to the NMDA receptor allows αCaMKII (α-calcium calmomodulin kinase II) to find free binding sites on NR2A (the NMDA receptor: NR2A) subunits that are no longer occupied by PSD-95. PSD deficiency leads to increased levels of CaMKII bound to the NR2 subunits of NMDA receptor[70,71]. These molecular events are associated with increased neuronal impairment.

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Disclosure of Interest

None.

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