



Differential Effects of Sevoflurane Exposure on Long-Term Fear Memory in Neonatal and Adult Rats

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Abstract

It remains unclear whether exposure to sevoflurane produces different effects on long-term cognitive function in developing and mature brains. In the present study, Sprague–Dawley neonatal rats at postnatal day (PND) 7 and adult rats (PND 56) were used in all experiments. We performed fear conditioning testing to examine long-term fear memory following 4-h sevoflurane exposure. We assessed hippocampal synapse ultrastructure with a transmission electron microscope. Moreover, we investigated the effect of sevoflurane exposure on the expression of postsynaptic protein 95 (PSD-95) and its binding protein kalirin-7 in the hippocampus. We observed that early exposure to sevoflurane in neonatal rats impairs hippocampus-dependent fear memory, reduces hippocampal synapse density, and dramatically decreases the expressions of PSD-95 and kalirin-7 in the hippocampus of the developing brain. However, sevoflurane exposure in adult rats has no effects on hippocampus-dependent fear memory and hippocampal synapse density, and the expressions of PSD-95 and kalirin-7 in the adult hippocampus are not significantly altered following sevoflurane treatment. Our results indicate that sevoflurane exposure produces differential effects on long-term fear memory in neonatal and adult rats and that PSD-95 signaling may be involved in the molecular mechanism for early sevoflurane exposure-caused long-term fear memory impairment.

Keywords Inhalational anesthetics · Fear memory · Hippocampus · PSD-95

Introduction

Early exposure to anesthetics is a risk factor for the development of learning and memory ability [1]. According to statistics, about 1.5 million infants or young children receive anesthesia every year, and sevoflurane is commonly used in anesthesia for young children and infants [2]. Previous studies have shown that early exposure to sevoflurane can cause learning and memory impairment [3–5]. However, it is unclear whether sevoflurane exposure produces different effects in neonatal and adult animals and the underlying mechanisms are not fully understood.

Our previous study found that postsynaptic density protein 95 (PSD-95) PDZ domain (PSD-95/discs large/zona occludens) is one of the targets of inhalation anesthetics [6], and knockdown of PSD-95 or disruption of PDZ domain-mediated protein–protein interactions facilitates inhalational anesthesia [7, 8]. Moreover, PDZ domain deletion induces significant inhibition on the formation of excitatory synapses and neuronal processes [9]. In addition, kalirin-7 is a subtype of kalirin protein in the *Dbl* (diffuse B lymphoma) gene family, which is co-localized with PSD-95 in the postsynaptic

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density zone, and it modulates development and maturation of excitatory synapses [10–12]. Kalirin-7 can activate small GTPase Rac1 and regulate actin cytoskeleton dynamics. It affects synaptic structure and function by interacting with PSD-95 via its C-tail [13–15]. The Kalrn gene contains 65 exons and splices to produce different forms of proteins, including full-length proteins kalirin-7, karinin-9, and kalirin-12 [16]. Previous studies have shown that kalirin-7 interacts with PSD-95 in the postsynaptic density [16–19], which is involved in synapse formation, hippocampal learning, and memory function [20, 21], indicating that kalirin-7 may participate in synapse changes induced by anesthetics. These results suggest that anesthetics can modulate synapse formation and cognitive function. However, the effects of sevoflurane exposure on hippocampal memory and synapse structure, as well as its underlying mechanisms, remain unclear.

In the present study, we investigate the effects of exposure to sevoflurane on long-term fear memory in postnatal day (PND) 7 neonatal and PND 56 adult rats. Moreover, we explore the involvement of PSD-95 and its bind protein kalirin-7 in the underlying molecular mechanism.

Methods and Materials

Animals

All animal operations conducted in this study were approved by the Animal Care and Use Ethics Review Committee of Zhengzhou University in China. All experiments followed the National Institutes of Health guidelines for the care and use of laboratory animals. Four rats were fed in a cage with a light/dark cycle of 12/12 h and free access to food and water. All efforts are made to minimize animal suffering and reduce the number of animals used.

Anesthesia

Rats were placed in a plexiglas box of 20 cm × 40 cm × 15 cm, and copper plates with variable temperature were set in the box, so that the ambient temperature of all rats was automatically maintained at 36.5°C. Sevoflurane was pumped into the chamber using 100% oxygen (2 L/min) and a sevoflurane vaporizer (Datex-Ohmeda, General Medical Devices, USA) from an intake hole connected to an anesthetic gas detector (Datex-Ohmeda, General Medical Devices, USA). All rats with sevoflurane exposure were anesthetized for 4 h at 1.0 MAC (minimum alveolar anesthetic concentration) as previously described [22–24].

In the present study, we used male Sprague–Dawley PND 7 pups and adult PND 56 rats to carry out all experiments. Anesthesia for PND 56 rats was set at 2.4% to achieve 1.0

MAC [24], and anesthesia for PND 7 rats was set at the following concentrations to achieve 1.0 MAC as described previously [22, 23]: 4.2–5.5% for the first hour, 3.3–4.2% for the second hour, and 2.7% for the third and fourth hours. After anesthesia, the rats continued to breathe oxygen until wake up. At the same time, control rats were placed in the same anesthesia box and exposed only to air rather than sevoflurane. After exposure to sevoflurane or air, the PND 7 and PND 56 rats were placed in a cage until PND 63 for further experiments, including fear condition testing, transmission electron microscopy analysis, and western blot determination.

Fear Conditioning

The contextual condition includes a rectangular glass room (25 cm × 31 cm × 25 cm), while the tone experiment consists of a different box with a diameter of 45 cm and a white plastic floor. Fear conditioning training and testing were performed at different days. In the training phase, the two groups of rats were given 3 times of sound stimulations (2000 Hz, 90 db, 30 s) after a 3-min adaptation exploration period, accompanied by electrical stimulation (1 mA, 2 s). The electrical stimulation starts at the last 2 s of the sound and ends at the same time. The interval between sound stimulation is 1 min. On the testing day, contextual and tone test sessions were performed, respectively. In the contextual test session, the rats were placed in the same environment with the training day but without sound and electric stimulation. While in the tone test session, the rats were given 3 times of sound stimulation (2000 Hz, 90 db, 30 s) after the 3-min adaptation period but without electrical stimulation. The fear conditioning test scores were calculated by a video system and automated motion-sensitive analysis software (Clever system, Reston, VA, USA) [25, 26]. The behavior changes of each animal in different chambers were recorded as video images through a camera, and then automatically analyzed by the software. We recorded a reference video before putting rats into the chambers. Any change in the video signal greater than that in the reference video was recorded as a change of behavior. Fear behavior was measured as the percentage of time frozen during the conditioned stimulus using automatic motion-sensitive software.

Synapse Determination by Transmission Electron Microscopy

To further observe the effects of sevoflurane on hippocampal synapses, we performed transmission electron microscopy to determine the synapse ultrastructure changes in PND 7 and PND 56 rats at PND 63. The brains were placed in 2.5% glutaraldehyde and fixed with 1% tetroxide for further fixation. After dehydration with gradient ethanol, tissue sections were

embedded in Epon618 and then were stained with heavy metals, uranyl acetate, and lead citrate for further analysis. The synapse morphology in CA1 region of the hippocampus was analyzed as described previously [27, 28]. We analyzed synapse ultrastructure in hippocampal CA1 stratum pyramidale, and all the synapses in 100 μm^2 of CA1 pyramidal neurons were examined. In brief, each grid was observed on an 80 kV Phillips CM 120 TEM, and the digital image captured with XR80-8 megapixel CCD. We set the size of X and Y dimensions of the analyzed bricks at 10 μm . For further PSD length and thickness measurement, the dimensional scale of X and Y was set at 250 nm. We calculated the number of PSD in the slices of 20 nm thickness. An average of ten images per animal was taken and each animal contributed one data point to obtain the median PSD. Any PSD falling within the framework of the CA1 region was counted. The number of PSD profiles divided by 100 μm^2 was calculated. The rules of PSD analysis were used as described previously [29]. The PSD length and thickness were measured by NIH ImageJ software version 1.51. Generally, the photomicrograph was enlarged to $\times 25,000$, and the cytoplasmic outline was tracked and the area was divided by the length of the postsynaptic membrane to get the average thickness and length of each PSD.

Western Blot

After exposure to sevoflurane or air, PND 7 and PND 56 rats were sacrificed by cervical dislocation to harvest hippocampus at PND 63. According to our previous study [30], the tissue was homogenized in lysis buffer on ice for 30 min, and then centrifuged at 700 g for 15 min at 4 °C. The supernatant was diluted and the protein concentration was measured by the Bicinchoninic Acid (BCA) method before gel electrophoresis. The proteins were transferred to nitrocellulose membranes and then blocked with Tris–HCl-buffered saline (TBS) containing 0.1% Tween-20 and 5% nonfat milk at room temperature for 1 h. The primary antibodies against kalirin-7 (Cat. #18–202–335,512, Genway, USA) [31] and PSD-95 (Cat #sc-32290, Santa Cruz, USA) were used overnight at 4 °C to determine the expression levels of kalirin-7 and PSD-95 in the hippocampus of PND 7 and PND 56 rats. The horseradish peroxidase-conjugated anti-mouse or anti-goat immunoglobulin were used as secondary antibodies and incubated at room temperature for 1 h. Proteins were detected by enhanced chemiluminescence and intensities were quantified with the ImageJ software [32, 33]. β -actin (Cat #A5316, Sigma-Aldrich, USA) served as a loading control.

Statistical Analysis

Data are expressed as the mean \pm S.E.M. Statistical analyses were performed with the GraphPad Prism 8 software (GraphPad Software, LLC, San Diego, CA). Student's *t* test was used to analyze data and the level of statistical significance was set at $P < 0.05$.

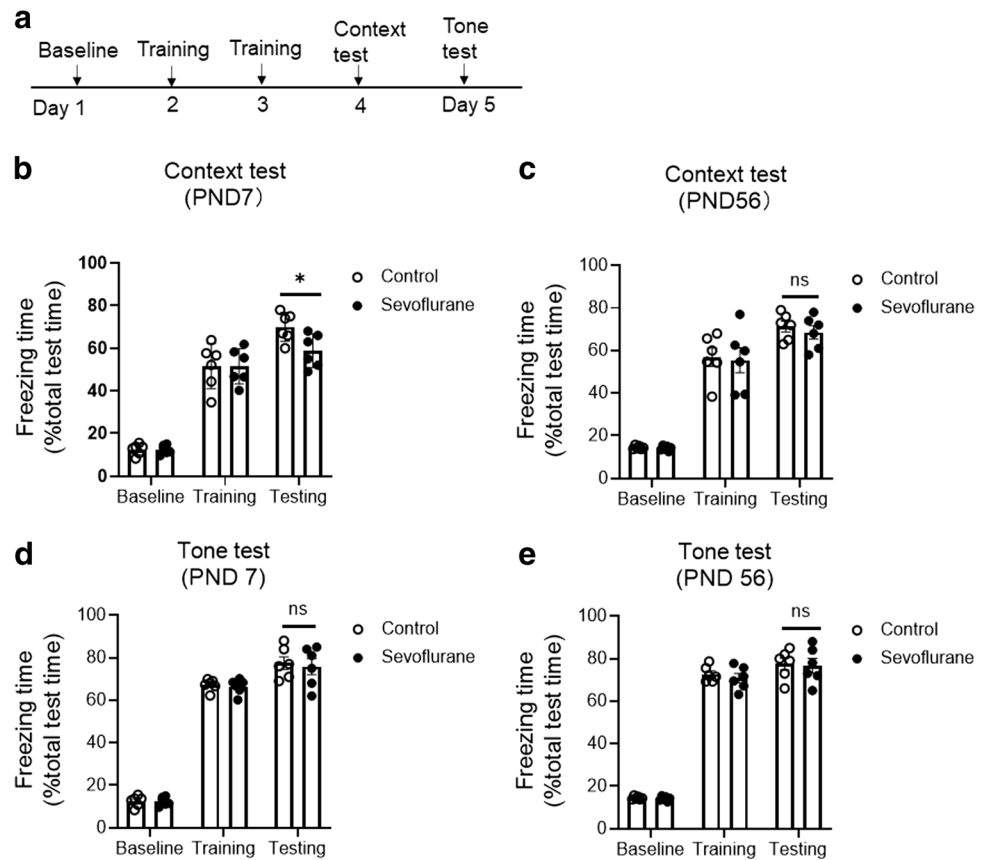
Results Sevoflurane Anesthesia Impairs the Hippocampus-Dependent Fear Memory in PND 7, but not PND 56 Rats

To investigate the effect of sevoflurane anesthesia on the brains of PND 7 and PND 56 rats, we performed fear conditioning test to determine hippocampus-dependent and independent fear memory. The contextual test showed significantly reduced freezing time in the PND 7 rats after early exposure to sevoflurane, compared to the air exposure control group (Fig. 1b, $n = 6$, $P = 0.0256$, $t = 2.63$), while the PND 56 rats with sevoflurane exposure had no significant change in the freezing time compared to the control group (Fig. 1c, $n = 6$, $P = 0.5012$, $t = 0.6991$). Next, we carried out the tone test to determine the effect of sevoflurane on hippocampus-independent memory, and we found that neither PND 7 or PND 56 rats had no change in freezing time compared to their control groups (Fig. 1d, $n = 6/\text{group}$, $P = 0.7174$, $t = 0.3450$; Fig. 1e, $n = 6/\text{group}$, $P = 0.8552$, $t = 0.1875$).

Postsynaptic Density and Synapse Number Are Reduced Significantly in PND 7, but not PND 56 Rats with Early Exposure to Sevoflurane

To further investigate the mechanisms by which sevoflurane exposure impairs hippocampus-dependent fear memory, we performed hippocampal synaptic structure analysis with transmission electron microscope. As shown in Fig. 2a–d, synapse number, postsynaptic density length and thickness in CA1 region of the hippocampus were reduced sharply in PND 7 rats with early exposure of sevoflurane compared to the control group ($n = 3/\text{group}$, synapse number: $P = 0.009$, $t = 8.76$; PSD length: $P = 0.0407$, $t = 3.098$; PSD thickness: $P = 0.0172$, $t = 5.503$). However, sevoflurane anesthesia had no significant effect on the number of synapses, the length and the thickness of synaptic zones in the hippocampus of PND 56 rats compared to the control group (Fig. 2e–h, $n = 3/\text{group}$, synapse number: $P = 0.2686$, $t = 1.342$; PSD length: $P = 0.09287$, $t = 0.0954$; PSD thickness: $P = 0.1.22$, $t = 0.2905$).

Fig. 1 Sevoflurane exposure impairs the hippocampus-dependent fear memory in PND 7, but not PND 56 rats. **a** Experimental timeline. **b** and **d** In the contextual test, sevoflurane anesthesia significantly reduced freezing time in PND 7 rats compared to control group (**b**), while sevoflurane inhalation had no significant change in freezing time in the tone test (**d**). **c** and **e** Sevoflurane exposure had no significant change in freezing time in both contextual and tone tests in PND 56 adult rats compared to air exposure control groups. $n = 6/\text{group}$. $*P < 0.05$ vs. the control group



Sevoflurane Anesthesia Decreases the Expression of Kalirin-7 and PSD-95 in the Developing Brain, but not Adult Mature Brain

To reveal if postsynaptic proteins kalirin-7 and PSD-95 are involved in sevoflurane-induced memory impairment, we examined their expression in the hippocampus after sevoflurane treatment. We observed that the expression of kalirin-7 in the hippocampus decreased sharply in the PND 7 group (Fig. 3a and b, $n = 3/\text{group}$, $P = 0.0047$, $t = 8.477$). However, in the PND 56 rats with or without sevoflurane, kalirin-7 expression in the hippocampus had no significant change. (Fig. 3c and d, $n = 3/\text{group}$, $P = 0.9318$, $t = 0.0937$). Likewise, sevoflurane inhalation significantly decreased PSD-95 expression in the PND 7 rats (Fig. 3e and f, $n = 3/\text{group}$, $P = 0.0003$, $t = 13.36$), but in the PND 56 rats, PSD-95 expression in the hippocampus had no significant change (Fig. 3g and h, $n = 3/\text{group}$, $P = 0.5534$, $t = 0.6709$).

Discussion In the present study, we observed that early exposure to sevoflurane in neonatal rats, but not adult rats, impairs hippocampus-dependent fear memory. Using transmission electron microscope, we further showed that sevoflurane anesthesia reduces post-synaptic density and synapse number in neonatal rats. Moreover, we found that

sevoflurane exposure in neonatal rats not only significantly decreases the expression of postsynaptic proteins PSD-95, but also its binding protein kalirin-7 in the hippocampus.

To date, it is unknown whether sevoflurane anesthesia has long-term effects on hippocampal-dependent contextual fear memory. Our results in this study demonstrate that sevoflurane exposure-caused impairment of such fear memory is age-dependent. We show that early exposure to sevoflurane in the PND 7 rats significantly reduces freezing time in the contextual fear memory test, but the sevoflurane treatment has no effect on the fear memory in adult rats. These results suggest that sevoflurane anesthesia is a risk factor for hippocampal-dependent fear memory impairment in the developing brain. We also observed that exposure to sevoflurane does not alter tone fear memory in both neonatal and adult rats, indicating that sevoflurane anesthesia has no effect on hippocampal-independent fear memory. Because the freezing level in tone-cued fear test is much higher than that in contextual fear test, “ceiling” levels of performance might be a potential reason for the lack of an effect on cued fear in PND 7 rats. Previous studies have shown that sevoflurane anesthesia does not impair acquisition learning and memory in the Morris Water Maze testing [34] and that early exposure to sevoflurane does not cause learning and memory disorders in monkeys during childhood [35]. In the present

Fig. 2 Postsynaptic density and synapse number are reduced in PND 7, but not PND 56 rats after sevoflurane anesthesia. Hippocampal synaptic ultrastructure was analyzed with transmitted electron microscope. **a** Representative images of synapses in hippocampus of PND 7 rats. **b–d** Sevoflurane reduced synapse density (**b**), PSD length (**c**), and PSD thickness (**d**) in PND 7 rats. **e** Representative images of synapses in hippocampus of PND 56 rats. **f–h** Sevoflurane anesthesia had no significant change in hippocampal synapse density (**f**), PSD length (**g**), and PSD thickness (**h**) of PND 56 rats compared to control groups. $n=3/\text{group}$. $*P<0.05$ vs. the control group

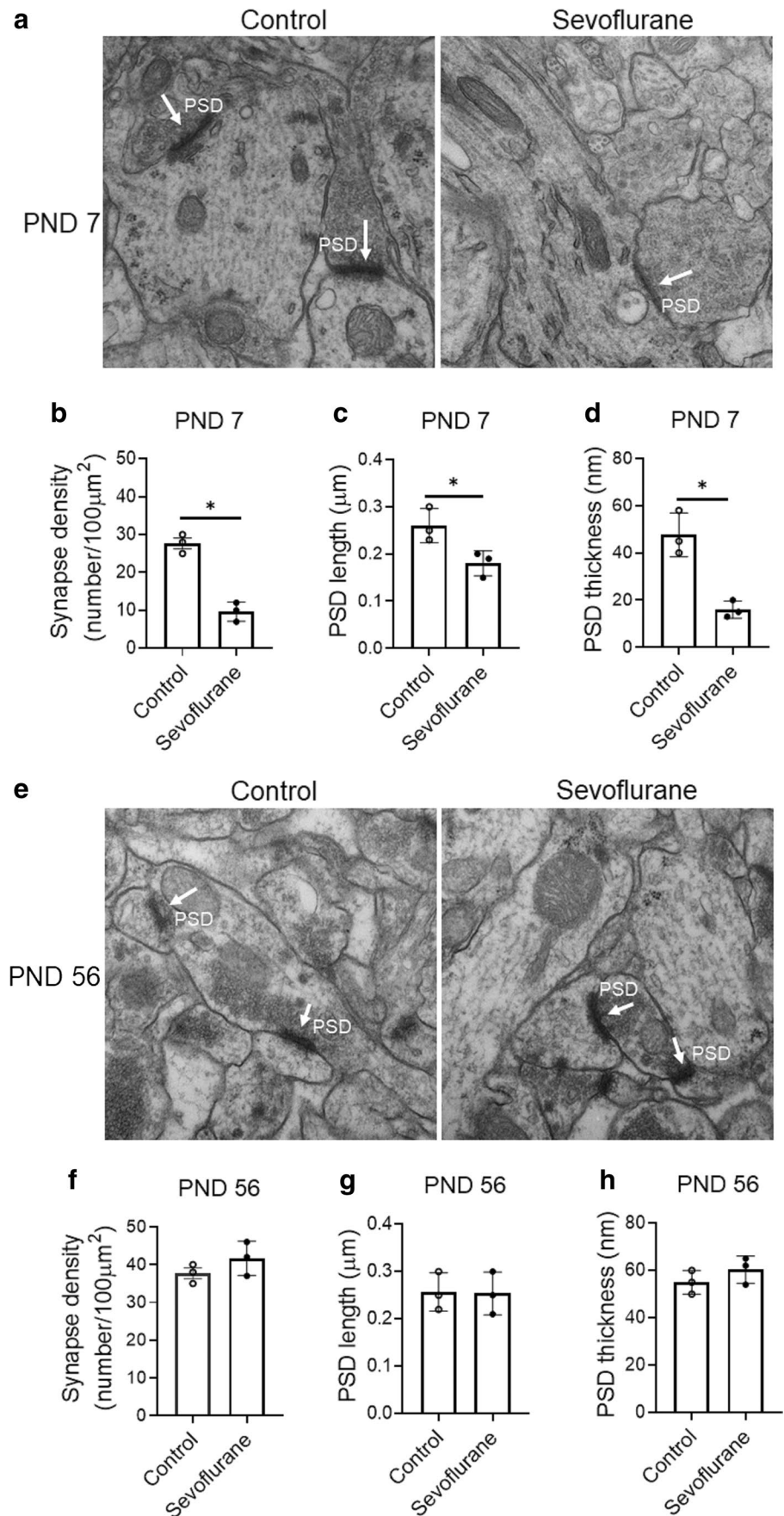
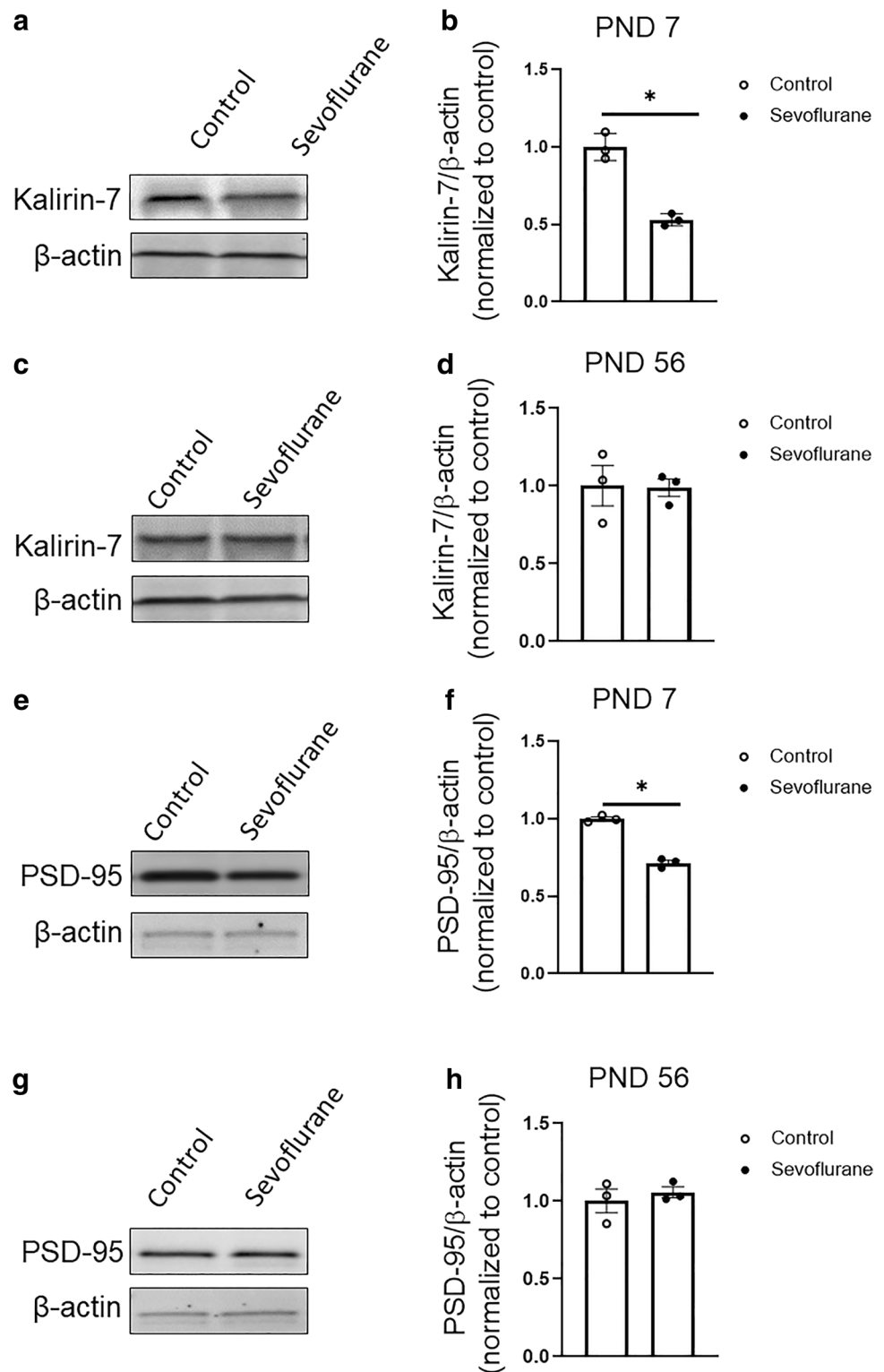


Fig. 3 Sevoflurane inhalation decreases the expressions of hippocampal kalirin-7 and PSD-95 in PND 7, but not PND 56 rats. Western blot was performed to examine the expression levels of kalirin-7 and PSD-95 proteins in the hippocampus of PND 7 and PND 56 rats after sevoflurane exposure. **a–d** Hippocampal kalirin-7 expression was significantly reduced in PND 7 rats with sevoflurane application (**a** and **b**), but there was no significant change in hippocampal kalirin-7 expression in PND 56 rats exposed to sevoflurane (**c** and **d**). **e–h** Sevoflurane inhalation significantly decreased hippocampal PSD-95 expression in the PND 7 rats (**e** and **f**), but hippocampal PSD-95 expression did not change significantly in PND 56 rats compared to control group (**g** and **h**). $n=3$ /group. * $P<0.05$ vs. the control group



study, we specifically investigate the long-term effects of sevoflurane anesthesia on fear memory in rats. However, a previous study using adolescent mice reports different results, in which sevoflurane inhalation for 3 h not only does not reduce memory but also enhances the consolidation of

long-term memory by increasing the composition of the cytoskeleton in the hippocampus [36]. Although different animal species may lead to variable results, the more likely possibility is that sevoflurane has different effects on the long-term hippocampal cognitive ability in animals at

different growth and development stages, including newborn, adolescent, and adulthood. In addition, it is unknown whether sevoflurane anesthesia for 4 h causes hypercapnia and acidosis. A previous report demonstrates that inhalation of sevoflurane for 1 h causes hypercapnia and acidosis in rat pups, but has no effect on long-term cognitive behavioral disorders [37]. However, although 1 h of anesthesia may cause changes in the internal environment, such as hypercapnia, there is no change in cognition; and when the time of anesthesia is increased to 4 h, internal environmental interference may be compensated, thereby leading to long-term cognitive impairment [22]. Interestingly, another study [38] shows that motor coordination and spatial learning and memory are not impaired in adult Sprague–Dawley rats exposed to 2.5% sevoflurane for 1 h, which is consistent with our findings. We found that adult rats exposed to 2.4% sevoflurane have no fear memory impairment, but long-term fear memory is impaired in PND 7 rats after 4 h of sevoflurane anesthesia. This further suggests that different sensitivities to anesthetics lead to different effects on learning and memory in neonatal and adult rats. Therefore, the type of anesthetics and the age of patients should be considered when pediatric anesthesia is carried out. Accumulating data from animal anesthesia research remind us that pediatric patients should not be anesthetized for a long time to avoid long-term cognitive impairment.

A previous study has shown that exposure to general anesthesia in PND 7 rats leads to severe and long-term ultrastructural changes in the developing subiculum [14], suggesting that early exposure to anesthetics may cause synapse abnormalities in young animals. In our study, we found that sevoflurane exposure reduces hippocampal post-synaptic density and synapse number in neonatal rats, but not adult rats. This is consistent with previous reports [39–41], in which sevoflurane is responsible for the damnification of synapse plasticity and synaptogenesis. Therefore, it is reasonable to propose that sevoflurane exposure in early age could cause cognitive dysfunction (such as fear memory impairment) by damaging synaptic structure and function in the developing brain. Interestingly, sevoflurane anesthesia in 4-week-old mice causes long-term cognitive function enhancement due to increased expression of Rac1 protein and F-actin constitution [36], suggesting that changes of hippocampal ultrastructure caused by sevoflurane directly lead to different cognitive behaviors in animals.

Furthermore, previous studies have shown that kalirin-7 plays an important role in synaptic plasticity by interacting with PDZ-containing PSD-95 and may be involved in sevoflurane-induced fear memory deficits [10, 15, 42]. It has been reported that sevoflurane exposure can reduce PSD-95 in young mice [43]. To further investigate the molecular mechanism for the fear memory impairment in neonatal rats after sevoflurane exposure, we examine

the expression kalirin-7 and PSD-95, a well-characterized post-synaptic protein that contributes to inhalational anesthesia, in the rat hippocampus. We found that sevoflurane treatment decreases the expression of both kalirin-7 and PSD-95 in the hippocampus of neonatal, but not adult rats. Previous studies have demonstrated that kalirin-7 is essential for synaptic structure formation and targeting [11, 15], as well as synapse plasticity [13, 44], which suggests that kalirin-7 is a potential target for anesthetics to regulate synaptic function. Therefore, our results suggest that reduced expression of kalirin-7 and PSD-95 in the hippocampus could be an important molecular mechanism by which sevoflurane anesthesia causes hippocampus-dependent fear memory in young animals. In a previous study using PSD-95 mutant mouse lacking guanylate kinase domain [45], PSD-95 deletion does not affect retrieval of recent fear memories, but impairs their extinction and precision. Thus, different domains in PSD-95 may differentially contribute to fear memory.

In the present study, we focus on the analysis of synaptic ultrastructure and PSD-95/kalirin-7 expression in the hippocampus, because we observed that sevoflurane-caused fear memory impairment is hippocampus-dependent. Additional analysis of synaptic ultrastructure and PSD-95/kalirin-7 expression in the brain areas required for cued fear can determine the specificity of sevoflurane's effects on synaptic plasticity.

In conclusion, our results show that early exposure to sevoflurane in the developing brain impairs hippocampus-dependent fear memory, which may be mediated by sevoflurane-produced alterations of hippocampal synaptic structure and decreased expression of kalirin-7 and PSD-95 in the hippocampal synapses.

Author Contribution All authors read and approved the manuscript. C.L., H.L., and S.L.: conceptualization; Y.M., Q.W., and S.L.: methodology; C.L., F.T., X.L., and H.L.: data analysis; C.L.: writing (original draft); F.T. and S.L.: writing (review and editing); and C.L.: funding acquisition.

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Data Availability All data generated or analyzed during this study are included in this published article.

Declarations

Ethics Approval and Consent to Participate The local independent Ethical Committee approved the study protocol.

Consent for Publication Not applicable.

Conflict of Interest The authors declare no competing interests.

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