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ORIGINAL ARTICLE

Sevoflurane attenuates stress-enhanced fear learning by regulating hippocampal BDNF expression and Akt/GSK-3β signaling pathway in a rat model of post-traumatic stress disorder

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Abstract
Purpose Post-traumatic stress disorder (PTSD) is a psychiatric disease that may occur after intense psychological trauma or physiological stress. Accumulating evidence suggests that brain-derived neurotrophic factor (BDNF) and the serine/threonine kinase (Akt)/glycogen synthase kinase-3β (GSK-3β) signaling pathway are critically involved in brain plasticity, including hippocampal-dependent learning and memory, while sevoflurane impairs memory processing. Thus, we hypothesized that sevoflurane can suppress fear learning by regulating the expression of BDNF and the Akt/GSK-3β signaling pathway in a rat model of PTSD.
Method Rats were exposed to sevoflurane during or after a 15 foot-shock stressor. Thereafter, rats were subjected to a single foot-shock in a totally different environment. The fear response was recorded in response to the 15 foot-shock and the single foot-shock environments. In another set of experiments, the brain tissue was harvested and subjected to biochemistry studies.
Results Our data suggested that increasing sevoflurane concentrations decreased stress-enhanced fear learning (SEFL) when given during but not after the stressor. Furthermore, administration of lithium chloride (100 mg/kg, intraperitoneally) 30 min before the contextual fear conditioning reversed the inhibitory effect of 0.8 % sevoflurane on SEFL as well as phosphorylated (p)-Akt, p-GSK-3β and BDNF expressions.
Conclusion Our data suggested that increasing sevoflurane administration during but not after the stressor can impair SEFL in a rat model of PTSD, which may be due, at least in part, to the regulation of hippocampal BDNF expression and the Akt/GSK-3β signaling pathway.

Keywords Fear learning · Sevoflurane · BDNF · Akt · GSK-3β

Introduction

Post-traumatic stress disorder (PTSD) is a psychiatric disease that may occur after intense psychological trauma or physiological stress [1–3]. Sufferers continue to re-experience trauma-related anxiety and distress even after the cessation of the trauma [3]. The hallmark symptoms of PTSD include nightmares, hyperarousal, chronic fear, and emotional numbing, which leads to a profound social burden due to its high rates of comorbidity with major depression, increased risk of suicide, and marked psychosocial and occupational impairments [1–4]. Unfortunately, no specific pharmacological approach is currently to available treat PTSD symptoms.

The phosphorylation of glycogen synthase kinase-3β (p-GSK-3β) in the hippocampus has been proposed to play an important role in the process of memory formation and memory reconsolidation [5]. Serine/threonine kinase (Akt), also known as protein kinase B, is a major upstream modulator of GSK-3β in the neurotrophin-dependent signaling pathway, which directly phosphorylates GSK-3β at amino acid serine 9 and deactivates it [5]. On the other hand, brain-derived neurotrophic factor (BDNF) is an important neurotrophin that is involved in synaptic plasticity including hippocampal-dependent learning and memory [6, 7],

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long-term potentiation [8] and neurogenesis [8, 9] by binding to tyrosine kinase B receptors. These findings suggested that hippocampal BDNF and the Akt/GSK-3β signaling pathway may be involved in fear conditioning learning.

Sevoflurane is a commonly used inhaled anesthetic, which provides amnesia, unconsciousness, and immobility by interfering with γ-aminobutyric acid receptor subtype A, *N*-methyl-D-aspartate acid receptors, and glycine receptors [10]. It has been suggested that subanesthetic doses of sevoflurane administration before or during learning impairs memory formation in humans [11] and rodents [12]. In the present study, we thus hypothesized that sevoflurane can affect the formation of stress-enhanced fear learning (SEFL). Furthermore, we investigated whether pre-administration of lithium chloride (LiCl), a well-known inhibitor of GSK-3β, would influence the effect of sevoflurane administration on SEFL in a rat model of PTSD.

Materials and methods

Animals and housing

The study protocol was approved by the Ethics Committee of Jinling Hospital, Nanjing University and the experiment was performed in accordance with the Guidelines for the Care and Use of Laboratory Animals from the National Institutes of Health, USA. Adult male Sprague–Dawley rats (250–300 g) were purchased from the Experimental Animal Center of Jinling Hospital. All rats were housed under a 12:12-h light–dark cycle, with room temperature of 22 ± 2 °C and free access to food and water.

PTSD model

The PTSD model was established according to a previous study reported by Rau et al. [4] with minor modification. The experimental devices consisted of two chambers (50 cm × 45 cm × 50 cm) (contexts A and B) whose environments were completely different in texture, lighting, odor, and floor. Both roofs of the chambers were transparent and equipped with a video camera, which was monitored by the AniLab for Conditioned Fear Control Software (Software Technology Co., Ltd. Ningbo, China). Two 2-cm-diameter conduits on opposite sides of the chamber allowed the inflow and outflow of oxygen and sevoflurane. The grid floor used to deliver shocks was composed of 19 stainless steel bars, each 5 mm in diameter; all the bars were spaced 18 mm from each other and these bars were connected to a shock delivery system (SED-251, Software Technology Co., Ltd. Ningbo, China). In addition, another equilibration chamber was used for anesthesia delivery before fear conditioning. In this model, rats were first placed in context A and given 15 foot-shocks, which simulated a stressful or "traumatic" event. The next day, the rats were placed in a different environment, context B, and given a single foot-shock, a reminder of the original stressful event. Over the following 2 days, the rats were tested for their fear to each environment in the absence of shock. During context A, 65 db of beep sound was produced by a squirrel fan, while white noise (65 db) was supplied by a compact disc player in context B.

Drugs administration

LiCl was purchased from Beijing Chemical Plant (Beijing, China) and diluted with normal saline to the final concentration of 100 mg/ml. The rats that received LiCl were injected intraperitoneally with 100 mg/kg LiCl 30 min before the contextual fear conditioning. Sevoflurane was purchased from Shanghai Pharmacological Co., Ltd (Shanghai, China). Different concentrations of sevoflurane were administrated in the air/oxygen ($FiO_2 = 0.3$) via a standard vaporizer at a rate of 2 L/min. The rats breathed spontaneously, and the concentrations of sevoflurane and oxygen were measured continuously (Datex-Ohmeda, Capnomac Ultima, Finland; Register marker, Vamos ARTJ-0062).

Experimental design

Experiment 1: effects of sevoflurane administration during the 15 foot-shocks on SEFL formation in rats

Eighty rats were used in this experiment. On day 1, rats received different concentrations of sevoflurane exposure {0, 0.4, 0.8, and 1.6 %; the minimum alveolar concentration (MAC) was reported to be 2.0 % for adult Sprague–Dawley rats [13]} for 30 min in the equilibration chamber. Thereafter, rats were removed as quickly as possible to the anesthetizing chamber (context A) where the sevoflurane concentration was equivalent to the concentration of the equilibration chamber. Each concentration group of rats was randomly assigned to receive either no or 15 foot-shocks (1-mA, 1-s shock with a variable intershock interval of 240–480 s, producing a total pre-exposure time of 90 min) during context A, then the rats were removed from the chamber and placed back into their home cages.

The following day, the rats received a single foot-shock (1 mA, 1 s) 192 s after placement in context B, and freezing for the 192-s pre-shock period was assessed to provide a baseline prior to foot-shock in context B; the rats were then allowed to stay in the chamber for another 30 s. Freezing was defined as the absence of all movement except for respiration [14].

On day 3, the rats were placed in context B for 512 s for the test of fear conditioning to context B to determine whether there was a SEFL.

Experiment 2: effects of sevoflurane administration immediately after the 15 foot-shocks on SEFL formation in rats

Given that our preliminary study suggested that sevoflurane administration higher than 2.6 % (1.3 MAC) inhibited the spontaneous breathing of the rats while 0.8 % (0.4 MAC) sevoflurane failed to suppress the SEFL when given after the 15 foot-shocks (data not shown), we therefore determined whether sevoflurane concentrations higher than 0.8 % could inhibit SEFL after the 15 foot-shocks. Based on these preliminary results, we finally chose the sevoflurane concentrations (0, 1.4, 2.0, and 2.6 %) studied in experiment 2.

Eighty rats were used in this experiment. On day 1, rats received either 0 %, 1.4 % (0.7 MAC), 2.0 % (1.0 MAC), or 2.6 % (1.3 MAC) sevoflurane for 2 h (the duration was the same as experiment 1) immediately after no or 15 foot-shocks (1-mA, 1-s shock with a variable intershock interval of 240–480 s, a total of 90 min) in context A, then they were transferred to their home cages and treated the same as in experiment 1 on the subsequent 2 days.

Experiment 3: molecular mechanisms underlying the inhibitory effects of sevoflurane administration on the SEFL of rats

One hundred and fifty-two rats were randomly divided into the following four groups ($n = 38$): control group (Control group); 0 % sevoflurane group (0 % Sev group); 0.8 % sevoflurane group (0.8 % Sev group); and 0.8 % sevoflurane + LiCl group (0.8 % Sev + LiCl group). The control group received no foot-shocks while the other groups received 15 foot-shocks on day 1 in context A. Rats in the 0 % Sev group received the 15 foot-shocks in context A without sevoflurane administration, whereas rats in the 0.8 % Sev group received 0.8 % sevoflurane for a total of 2 h from 30 min before entering context A. In the 0.8 % Sev + LiCl group, rats received LiCl (100 mg/kg) intraperitoneally 30 min before entering context A, then this group was treated the same as the 0.8 % Sev group. Ten rats in each group were used to assess freezing behavior in context B, as described above. The remaining rats in each group were randomly decapitated at 0, 0.5, 1, and 2 h after training in context A on day 1 and the hippocampal tissues were stored in liquid nitrogen for biochemistry studies.

Western blot analysis

Hippocampal protein extraction was prepared in Radio-Immunoprecipitation Assay Buffer as described previously [15]. Briefly, 40 μg of detergent-soluble protein samples were separated on 10 % acrylamide gels and transferred onto 0.45-mm nitrocellulose membrane (Bio-Rad, USA) for 1 h at 350 mA in transfer buffer (25 mM Tris and 192 mM glycine, pH 8.3). Membrane blots were blocked for 1 h in blocking solution [Tris-buffered saline with Tween (TBST) with 4 % non-fat milk] at 24 °C, followed by incubation of primary antibodies diluted in blocking solution overnight at 4 °C. The following day, the membranes were washed three times with TBST (0.1 % Tween-20), and incubated in secondary antibody (goat anti-rabbit HRP, 1:1000, Santa Cruz, USA) diluted in TBST with 4 % non-fat milk for 1 h. Membranes were visualized with electrochemiluminescence plus chemiluminescent substrate (GE Healthcare, USA) and protein expression levels were quantified by densitometric analysis using UN-SCAN-IT gel 6.1 software (National Institutes of Health, Bethesda, MD, USA). Primary antibodies used in this study were polyclonal rabbit anti-phosphorylated GSK-3β ser 9 (1:1000; Cell Signaling Technology, Boston, MA, USA), polyclonal rabbit anti-GSK-3β (1:2000; Cell Signaling Technology), polyclonal rabbit anti-Akt (1:2000; Cell Signaling Technology), and polyclonal rabbit-anti-phosphorylated Akt Ser 437 (1:1000; Cell Signaling Technology); secondary antibodies used in this study were goat anti-rabbit HRP (1:1000; Santa Cruz, USA).

Enzyme-linked immunosorbent assay (ELISA)

The level of BDNF was measured using an ELISA kit (Cusabio Biotech Co., Ltd., Newark, DE, USA), BDNF Emax Immuno Assay System (Millipore, USA), according to the manufacturer's instructions. Brain tissues were homogenized in modified protein extraction buffer, followed by bicinchoninic acid quantification assay to determine protein concentration. To measure BDNF levels, 96-well immunoplates were coated with 100 ml per well of monoclonal anti-rat-BDNF antibody (1:2000). After an overnight incubation at 4 °C, plates were washed three times with wash buffer and the protein samples (100 ml) were incubated in coated wells for 2 h at 24 °C. Immobilized antigen was incubated with an anti-human BDNF antibody for 2 h at room temperature. The plates were then incubated with an anti-IgY horseradish peroxidase for 1 h at room temperature followed by tetramethyl benzidine/peroxidase substrate solution and 1 M HCl (100 ml/well). The colorimetric reaction product was measured at 450 nm using a microplate reader. BDNF concentration was determined based on linear regression of the BDNF standards that were incubated under similar conditions in each assay. All samples were assayed in duplicate.

Statistical analysis

Data analysis was performed using SPSS for Windows software (Version 16.0; SPSS, Chicago, IL, USA). Data were presented as mean ± standard error of mean (SEM). Differences

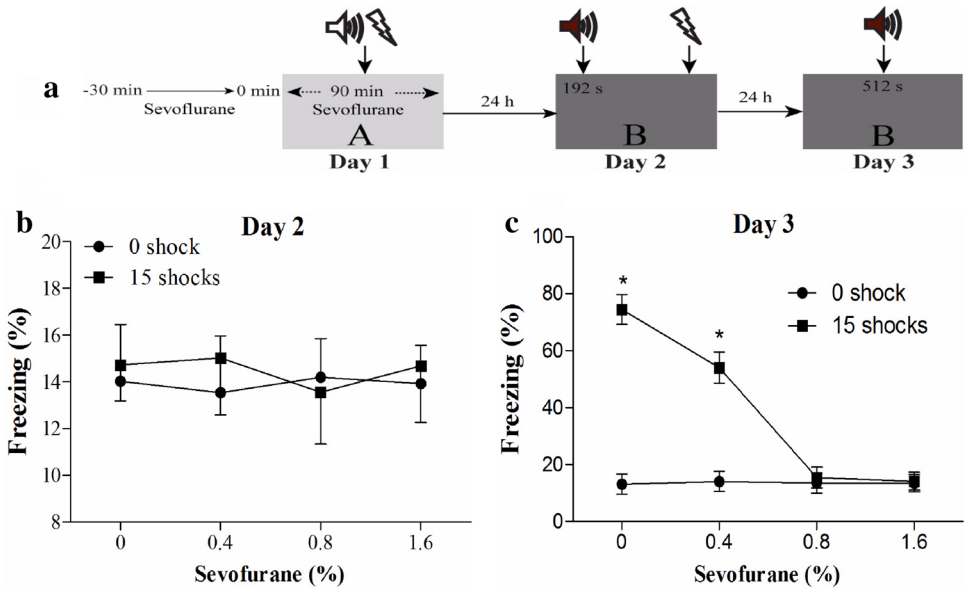

Fig. 1 **a** Schematic of the procedure used in experiment 1. **b** Freezing measured during the baseline period before the single foot-shock in context B. All concentrations of sevoflurane had no significant influence on baseline freezing rate in context B. **c** Rats pre-exposed to 15 foot-shocks breathing 0 % or 0.4 % sevoflurane showed SEFL, whereas 0.8 % sevoflurane and higher concentrations did not show enhanced freezing behavior compared with the nonpreshocked rats in context B on day 3. Data are presented as mean ± SEM ($n = 10$); *$P < 0.05$ vs. no shock at the corresponding concentration

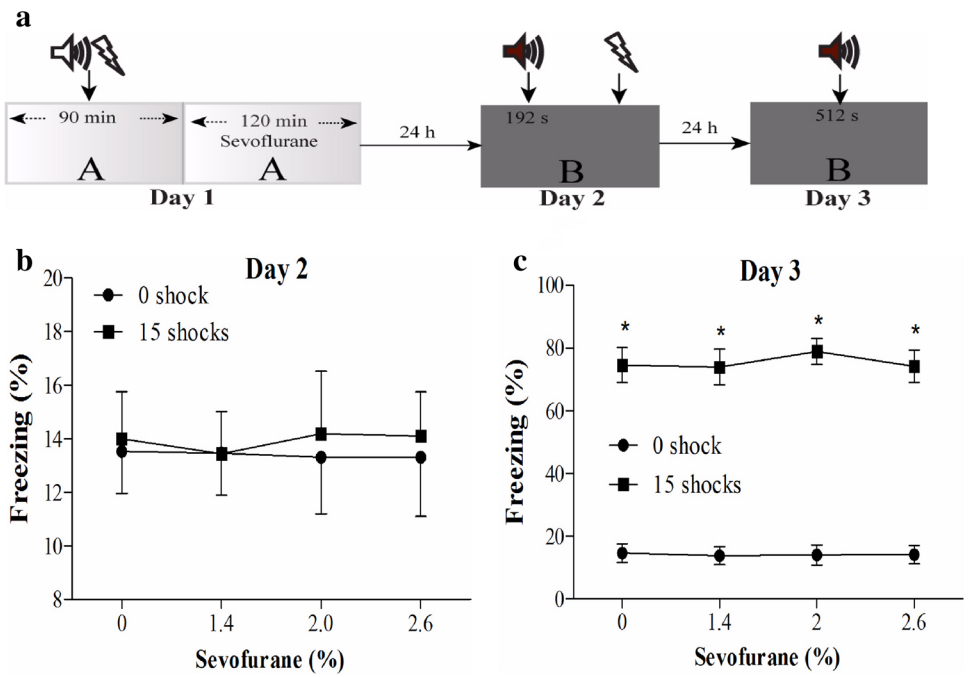

Fig. 2 **a** Schematic of the procedure used in experiment 2. **b** Freezing measured during the baseline period before the single foot-shock in context B. Sevoflurane administration did not affect this measurement. **c** Increasing concentrations of sevoflurane had no effect on the freezing behavior on nonpreshocked or preshocked rats on day 3 in context B. Data are presented as mean ± SEM ($n = 10$); *$P < 0.05$ vs. no shock at the corresponding concentration

between multiple means were assessed by one-way or two-way analysis of variance (ANOVA) followed by a Tukey test if appropriate. $P < 0.05$ was considered statistically significant.

Results

Experiment 1

On day 2 in context B, there was no difference in freezing behavior during the 192-s preshocked period, indicating that the rats were able to differentiate between context A and context B ($F = 0.34$, $P = 0.933$, Fig. 1b).

On day 3 in context B, increasing concentrations of sevoflurane suppressed SEFL in the preshocked rats but did not affect nonpreshocked rats. Furthermore, rats pre-exposed to the 15foot-shocks breathing 0 or 0.4 % sevoflurane showed SEFL, whereas 0.8 % sevoflurane and higher concentrations did not show enhanced freezing behavior compared with the nonpreshocked rats ($F = 9.795$, $P < 0.05$, Fig. 1c). Based on these results, 0.8 % sevoflurane (0.4 MAC) was used to explore the

underlying mechanism involved in its suppressive effects on SEFL in experiment 3.

Experiment 2

There was no difference in freezing time between preshocked and nonpreshocked rats in context B during the 192-s preshocked period on day 2 ($F = 0.15$, $P = 0.993$, Fig. 2b). Surprisingly, increasing concentrations of sevoflurane, administered immediately after foot-shock, had no effect on freezing behavior in nonpreshocked or preshocked rats. Furthermore, all concentrations of sevoflurane did not affect SEFL on the rats pre-exposed to the 15 foot-shocks compared with their counterparts on day 3 in context B ($F = 17.55$, $P < 0.05$, Fig. 2c).

Experiment 3

As shown in Fig. 3b, the freezing time in the 0 % sevoflurane group on day 3 was significantly higher than that on day 2, suggesting there was SEFL. However, 0.8 % sevoflurane administration during the stressor suppressed SEFL in the preshocked rats, and this effect could be reversed by intraperitoneal administration of LiCl ($F = 6.469$, $P < 0.05$).

The level of p-GSK-3β was increased and reached a stable level from 1 h after the training conditioned to context A ($F = 15.347$, $P < 0.05$, Fig. 4a). As shown in Fig. 4b, stress induced by 15 foot-shocks significantly increased the p-GSK-3β levels compared with the control group. However, 0.8 % sevoflurane inhibited p-GSK-3β in the hippocampus at 1 h after the training conditioned to context A, whereas LiCl administration reversed the decrease in p-GSK-3β ($F = 7.024$, $P < 0.05$, Fig. 4b).

As indicated in Fig. 5a, the hippocampal p-Akt expression peaked at 2 h after the training conditioned to context A ($F = 7.514$, $P < 0.05$). As shown in Fig. 5b, 15 foot-shocks significantly increased the p-Akt levels compared with the control group. Again, sevoflurane administration during the stressor inhibited hippocampal p-Akt expression at 1 h, whereas lithium reversed the decreased p-Akt expression after the training conditioned to context A ($F = 9.434$, $P < 0.05$).

As shown in Fig. 6a, the level of BDNF peaked at 1 h after the training conditioned to context A in the 0 % Sev group ($F = 7.821$, $P < 0.05$). As shown in Fig. 6b, stress induced by 15 foot-shocks significantly increased the BDNF levels compared with the control group. However, sevoflurane administration during the stressor reduced the level of BDNF in the hippocampus at 1 h while LiCl administration reversed the decreased level of BDNF ($F = 8.978$, $P < 0.05$).

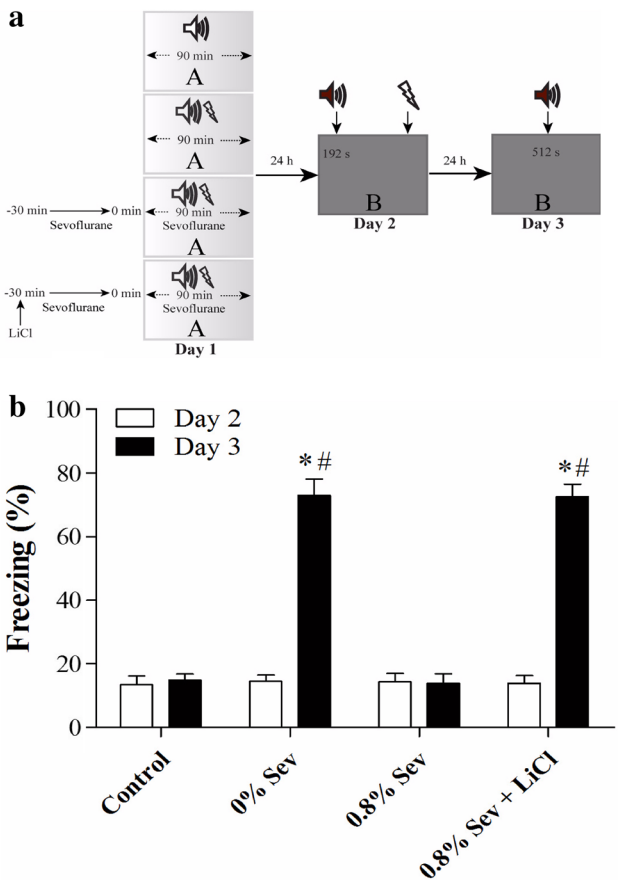

Fig. 3 **a** Schematic of the procedure used in experiment 3. **b** The freezing time in the 0 % sevoflurane group on day 3 was significantly higher than that on day 2, suggesting there was SEFL. Administration of 0.8 % sevoflurane during the stressor suppressed SEFL in the preshocked rats, and this effect could be reversed by the intraperitoneal administration of lithium chloride (LiCl). Data are presented as mean ± SEM ($n = 10$); $^{*}P < 0.05$ vs. day 2; $^{\#}P < 0.05$ vs. 0.8 % Sev group on day 3

Discussion

Using this animal model of PTSD [16, 17], our data demonstrated that rats showed a robust conditioned fear on day 3, which confirms the traumatic nature of shock exposure. Notably, we reported that 0.8 % or higher concentrations of sevoflurane administration during but not after the stressor attenuates SEFL, which may be attributed to the reduction of BDNF levels and the inhibition of the Akt/GSK-3β signaling pathway.

It has been suggested that 0.3 MAC of inhaled anesthetics may impair the process of learning and memory in human [11]. Considering species differences in inhaled anesthetics, we observed freezing behavior by using different concentrations of sevoflurane administration to determine the proper depth of anesthesia that could inhibit SEFL. As our result of experiment 1 revealed,

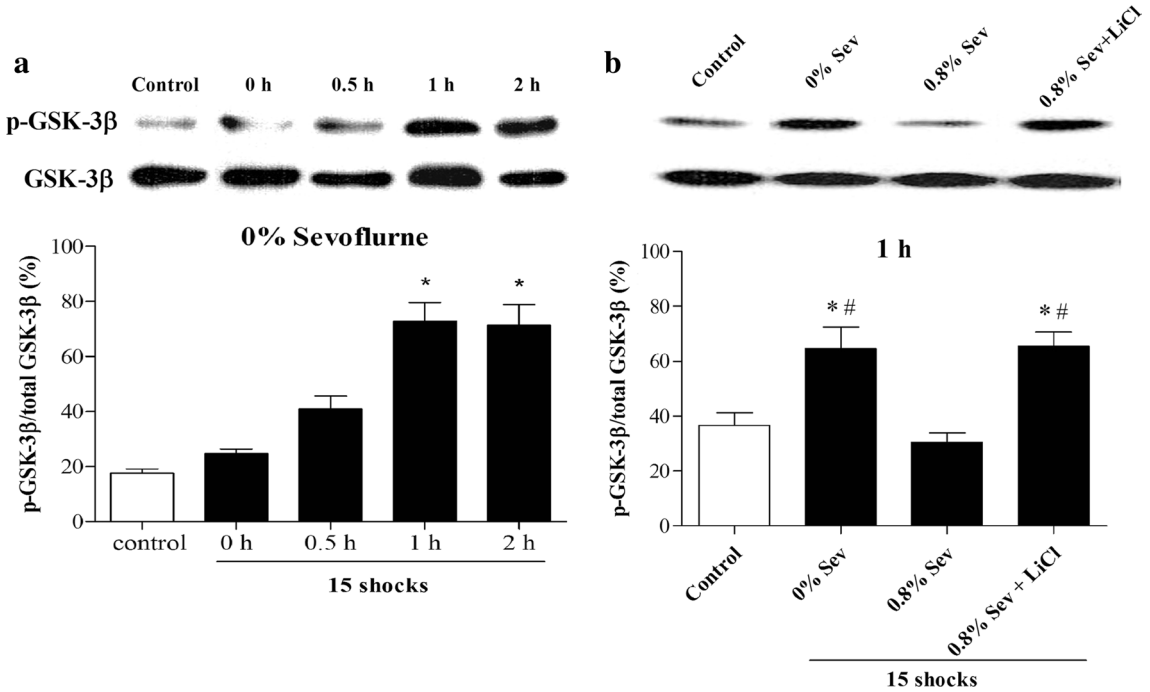

Fig. 4 **a** The expression of p-GSK-3β gradually increased and reached a stable level at 1 h after the training conditioned to context A. Data are presented as mean ± SEM ($n = 4$); *$P < 0.05$ vs. Control group. **b** Stress induced by 15 foot-shocks significantly increased p-GSK-3β levels compared with the control group. The expression of p-GSK-3β in the 0 % Sev group and 0.8 % Sev + LiCl group were significantly increased compared to the 0.8 % Sev group at 1 h after the training conditioned to context A. Data are presented as mean ± SEM ($n = 4$); *$P < 0.05$ vs. Control group; #$P < 0.05$ vs. 0.8 % Sev group

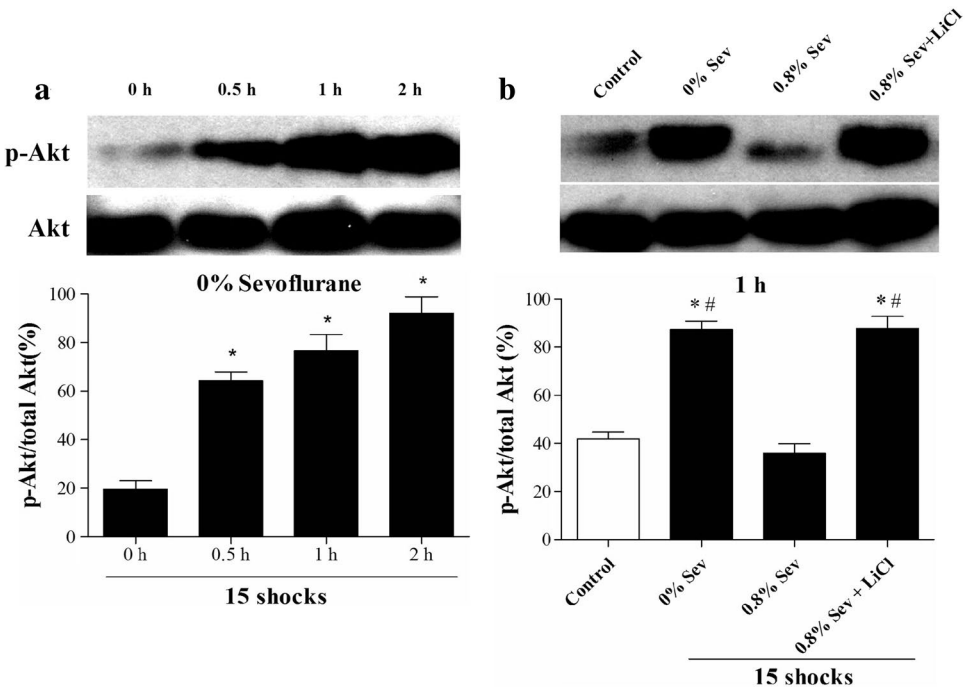

Fig. 5 **a** The expression of p-Akt peaked at 2 h after the training conditioned to context A. Data are presented as mean ± SEM ($n = 4$), *$P < 0.05$ vs. 0 h in 0 % Sev group. **b** Stress induced by 15 foot-shocks significantly increased p-Akt levels compared with the control group. The expressions of p-Akt in the 0 % Sev group and 0.8 % Sev + LiCl groups were significantly increased compared to the 0.8 % Sev group at 1 h after the training conditioned to context A. Data are presented as mean ± SEM ($n = 4$); *$P < 0.05$ vs. Control group; #$P < 0.05$ vs. 0.8 % Sev group

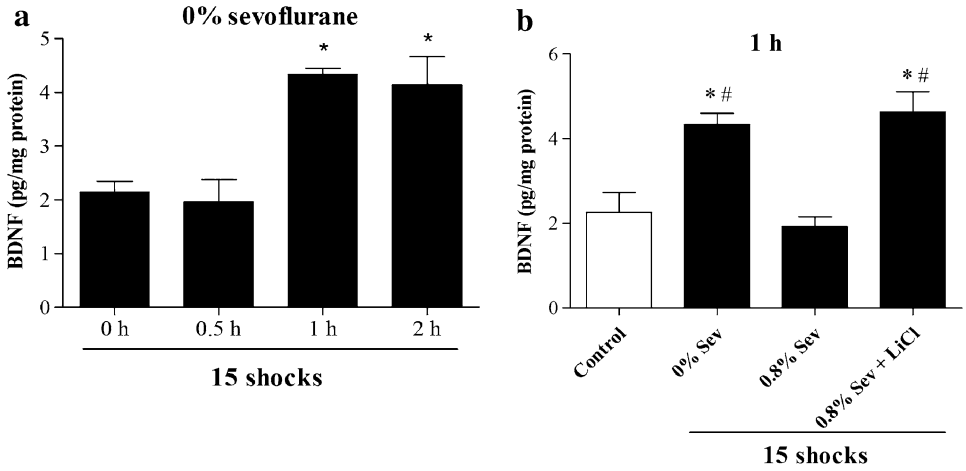

Fig. 6 **a** The expression of BDNF peaked at 1 h after the training conditioned to context A in the 0 % Sev group compared with 0 h. Data are presented as mean ± SEM ($n = 6$); $^{*}P < 0.05$ vs. 0 h. **b** Stress induced by 15 foot-shocks significantly increased BDNF levels compared with the control group. Compared with the 0.8 % Sev group, the expressions of BDNF in the 0 % SEV and 0.8 % Sev + LiCl groups were significantly increased at 1 h after the training conditioned to context A. Data are presented as mean ± SEM ($n = 6$); $^{*}P < 0.05$ vs. Control group; $^{\#}P < 0.05$ vs. 0.8 % Sev group

0.8 % (0.4 MAC) or higher concentrations of sevoflurane administration could suppress SEFL, which was consistent with previous results showing that 0.4 % isoflurane (0.4 MAC) and greater concentrations suppressed contextual fear conditioned using a similar paradigm [16]. However, one previous study showed that animals still showed enhanced conditioned fear in the single foot-shock context when fear in the 15 foot-shocks was extinguished with repeated presentations to the context or when its acquisition was blocked pharmacologically [4]. Since at least two processes, including associative learning (i.e. fear conditioning) and non-associative sensitization (i.e. SEFL), are involved in the process of PTSD, our results, together with previous findings, support the idea that SEFL relies on non-associative changes in fear conditioning circuits. Furthermore, we determined whether sevoflurane administration after pre-exposure to 15 foot-shocks could suppress SEFL once PTSD had been established. As the result of the second experiment revealed, sevoflurane given after the 15 foot-shocks in context A did not suppress freezing during context B, indicating that it should be given during the stressor to suppress SEFL.

Next, we explored the molecular mechanisms underlying the inhibitory effect of 0.8 % sevoflurane administration on SEFL during the stressor in the rats. The activity of GSK-3β has been reported to play a key role during the induction of long-term depression, indicating that GSK-3β activity may contribute to the control of synaptic plasticity and memory function [18]. Indeed, it has been suggested that alterations in GSK-3β are implicated in the pathogenesis of Alzheimer disease [19], the consolidation of fear memory [13], and the severity of PTSD-like symptoms [20]. In line with these results, our data revealed that the expression of p-GSK-3β increased and reached a stable level from 1 h after the training conditioned to context A. Importantly, inhibition of GSK-3β activity as a result of acute administration of LiCl reversed the inhibitory effects of 0.8 % sevoflurane on SEFL and the levels of p-GSK-3β. Indeed, it has previously been reported that the enhancement of GSK-3β in the hippocampus is involved in sevoflurane-induced impairment of memory consolidation [13]. In addition, Akt is a major upstream modulator of GSK-3β that directly phosphorylates and inhibits the glycogen synthase kinase GSK-3β [20]. It is reported that trophic factors promote neuronal survival largely through the phosphatidylinositol 3-kinase/Akt signaling pathway [21–23]. On the other hand, BDNF is a crucial regulator of neuronal survival and neuroplasticity in the brain. BDNF-tropomyosin receptor kinase B (TrkB) signaling has been found to be important for long-term potentiation in the hippocampus [13], hippocampal memory consolidation [24], and normal morphology of neurons [25]. Most importantly, animal models of BDNF and TrkB heterozygous knockouts demonstrated decreases in contextual fear conditioning [26] while overexpression of TrkB in mice led to improved memory, contextual fear conditioning, and overall reduced anxiety [27]. Paradoxically, we observed an acute increase in BDNF expression after the training conditioned to context A. Our results are supported by the view that the level of BDNF increases in the acute phase but decreases in the late phase after exposure to a severe traumatic event [28, 29]. Together, our data suggested that sevoflurane administration during the stressor may inhibit SEFL through

regulating hippocampal BDNF expression and the Akt/GSK-3β signaling pathway.

In summary, 0.8 % and higher concentrations of sevoflurane administration during but not after the stressor can attenuate freezing behavior in a rat model of PTSD, which may be due, at least in part, to the regulation of hippocampal BDNF levels and the Akt/GSK-3β signaling pathway. In addition, our study emphasizes that adequate depth of anesthesia is required throughout surgery to prevent PTSD as a result of accidental intraoperative awareness.

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Conflict of interest The authors have no potential conflicts of interest to disclose.

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