Long-term sevoflurane exposure relieves stress-enhanced fear learning and anxiety in PTSD mice

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Research Article

**Keywords:** PTSD, sevoflurane, stress, memory impairment

**Posted Date:** May 4th, 2023

**DOI:** https://doi.org/10.21203/rs.3.rs-2450625/v1

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Abstract

Objectives: Post-traumatic stress disorder (PTSD) is characterized by recurrent episodes of severe anxiety after exposure to traumatic events. It is believed that these episodes are triggered at least in part by environmental stimuli associated with the precipitating trauma through classical conditioning, termed conditioned fear. However, traditional methods of conditioned fear memory extinction are frequently ineffective for PTSD treatment due to the contribution of non-associative sensitization caused by trauma. Anesthetics have shown promise for treating various psychiatric diseases such as depression.

Methods: In this study, we examined if the inhaled anesthetic sevoflurane can suppress stress-enhanced fear learning (SEFL) in PTSD model mice. Model mice exposed to 2.4% sevoflurane for 6 h exhibited reduced freezing time and behavioral anxiety compared to sham-treated model mice. To explore the underlying mechanisms, we evaluated the regional expression levels of glucocorticoid receptors (GRs), cannabinoid CB1 receptors (CB1Rs), D1 dopamine receptors (D1Rs), and D2 dopamine receptors (D2Rs).

Results: We verified that both GR and CB1R were significantly upregulated in the hippocampus, amygdaloid nucleus, and prefrontal cortex (PFC) of model mice, while D1R and D2R were downregulated. All of these expression changes were partially normalized in the PFC by 6-h but not 2-h sevoflurane exposure.

Conclusions: These results showed that Sevoflurane exposure following traumatic events may be a safe and effective treatment for PTSD.

1. Introduction

Post-traumatic stress disorder (PTSD) is a chronic and debilitating psychiatric illness induced by the experience of life-threatening events or catastrophic psychological trauma. It is characterized by recurrent episodes of severe fear and anxiety that interfere with daily life and are frequently difficult to treat. Both associative learning from the pairing of stimuli during the precipitating event and non-associative sensitization are implicated in PTSD pathogenesis. Extinction-based therapies can ameliorate the associative fear, but are often of limited efficacy for reducing non-associative symptoms produced by trauma. Thus, non-associative fear memories could be a significant source of refractory symptoms, necessitating the development of alternative treatments.

Anesthetics and sedatives have demonstrated promise for the treatment of other neurological and psychiatric disorders, suggesting potential utility for the non-associative symptoms of PTSD. Sevoflurane is a widely applied inhaled anesthetic that induces amnesia, unconsciousness, and immobility, and several inhaled anesthetics including sevoflurane have shown cytoprotective efficacy at low doses. For instance, it has been reported that sevoflurane can protect against ischemia/reperfusion injury and immune-related tissue injury by enhancing antioxidant capacity and immune modulation. However, there have been few studies examining the potential application of sevoflurane for psychiatric diseases.
In our previous study, we reported that sevoflurane exposure for several hours promoted the extinction of fear memory in mice exposed to repeated pairing of sound (the conditioned stimulus) with shock (an unconditioned stimulus)\(^1\), suggesting potential therapeutic value for PTSD. Given that extinction training is ineffective in many PTSD patients, we hypothesized that sevoflurane may also diminish non-associative sensitization. Therefore, in the current study we established a stress-enhanced fear learning (SEFL) model of PTSD and re-evaluated the effects of sevoflurane administration.

2. Materials and methods

2.1. Animals

All experiments were conducted consistent with the applicable laws and guidelines on animal care. All procedures were approved by the Nanjing University Institutional Animal Care and Use Committee. All processing methods were in line with Directive 2010/63/EU. 8–10 weeks of male C57BL/6 mice, weighted of 20–25 g, were used in the experiment. All mice were housed with a controlled 12-hour light/dark cycle (lights turned on at 8:00) and were accessible to food and water for at least two weeks before any interference was performed. The room temperature was kept at 20–22°C, and the room humidity was maintained 50–60%.

2.2. PTSD model

The PTSD model was derived from Rau’s previous study with minor alteration. This experiment was conducted using a fear conditioning experimental system (Panlab, Spain). On the first day, mice were placed in a new context (A) and then were given 6 foot-shocks (0.8 mV), which simulated a stressful or “traumatic” event. The next day, we put mice in a different context (B), and they were given a single foot-shock, which referred to a reminder of the original stressful event. On the third day, mice were returned to context B for 512s to test their fear to context B in the absence of shock. The fear memory was estimated as freezing time and the PTSD mice should increase significantly in freezing time compared with the control group. Context A and context B were distinguished by different coarseness, flavor, and brightness. Mice were tested for their fear of context B in the absence of shock. During context A, a single high-frequency sound (4000 Hz, 80 dB) was produced while white noise (80 dB) was supplied in context B. At the end of the experiment, the mice were returned to the cage. The test box was wiped with 75% alcohol at the end of each test to avoid the impact of other mouse odors on the experiment.

2.3. Anesthesia

The anesthesia box air inlet was connected to the anesthesia machine (Remain, China) gas evaporation tank, consisting of an air connection with an anesthesia monitor (Drager, Germany) for monitoring the sevoflurane concentrations (Jiangsu Xinchen Pharmaceutical Co., Ltd., China).

A 1.0 MAC sevoflurane anesthesia was chosen in our study. All mice were placed in an anesthesia chamber and exposed to a mixture of 2.4% sevoflurane in 100% oxygen for 6h, at a flow rate of 2 L/min.
A heated sheet was used to maintain the body temperature of mice at 37°C during anesthesia. Respiratory frequency and skin color were observed every 30 minutes to keep animals healthy. Mice in the control group were placed into the container and were exposed to air only.

2.4. Open field test

The open field test was conducted in an uncovered plastic chamber (50 × 50 × 40 cm). All the animals were placed in the center of the floor to make sure they had free access to explore the chamber. Each mouse explored freely in 10 min. Activity time of the center area and the periphery area were respectively recorded by an automated video-tracking system (Biobserve, Bonn, Germany). The percentage of time in the center was defined as percent time spent in the central 30 × 30 cm area of the open field. Total distance was calculated as centimeters traveled in 10 min.

2.5. Forced swimming test

The forced swimming test was conducted in a cylindrical container with water (23 ± 1°C). The diameter and height of this container were 10cm and 25cm. The water was up to 19cm bottom to make mice swim freely in it. All the mice had to swim for 10 minutes and only the last 6 minutes were calculated because the majority of mice seemed to be very active at the start of the test which could have inconclusive effects on the experiment. Mice were considered as freezing when it stops swimming and only keeps its head above the water and appears to float on water. The percentage of freezing time was calculated and analyzed among groups.

2.6. Western Blot

The samples were homogenized in radioimmunoprecipitation assay lysis buffer (Beyotime, Shanghai, China) with a mixture of protease and phosphatase inhibitor cocktail (Abcam, Cambridge, UK). Proteins (20 µg) were separated on a sodium dodecyl sulfate-polyacrylamide gel electrophoresis gel and transferred to polyvinylidene difluoride membrane filters (Millipore, Burlington, MA). The blots were blocked with 5% bovine serum albumin and incubated with primary antibodies overnight at 4°C. The following primary antibodies were used: anti-β-actin (1:1000 dilution; Abcam), anti-GADPH (1:1000 dilution; Abcam), anti-CB1R (1:1000 dilution; Abcam), anti-D1R (1:1000 dilution; Abcam), anti-D2R (1:1000 dilution; Abcam), and anti-GR (1:1000 dilution; Abcam). The membranes were washed in Tris-buffered saline with Tween 20 buffer and incubated with anti-mouse horseradish peroxidase (HRP)-conjugated secondary antibody (1:10,000 dilution; Abcam) or anti-rabbit HRP-conjugated secondary antibody (1:10,000 dilution; Abcam) for 2 h at room temperature. Protein bands were visualized with a Western Chemiluminescent HRP Substrate kit (Millipore) using a Tanon 5200 (Tanon Science & Technology, Shanghai, China). Relative intensities of the specific protein bands normalized to β-actin or GADPH were quantified by ImageJ software (National Institute of Health, Bethesda, MD).

2.7. Statistical Analysis

All data are presented as the mean ± SD. Statistical analyses were performed using a computerized statistical package (SPSS 22.0) and GraphPad Prism software version 7.0 (GraphPad Software, Inc., San
Diego, CA, USA). One-way ANOVA followed by Bonferroni post hoc tests was used to evaluate the quantitative differences and the behavioral data. Differences were deemed statistically significant at probability values (p) of < 0.05.

3.Results

3.1.Long-term sevoflurane suppressed SEFL in the PTSD mice.

In test 1 on day 3, PTSD mice showed significantly enhanced freezing time. (68.12 ± 16.2%, p < 0.001 vs. Ctrl and Sevo (untreated), Fig. 1A, 1B) which indicated successful modeling of mice shocked six times seriously in context A on day 1. After exposed to sevoflurane for 6 h, mice in the PTSD + Sevo group exhibited decreased freezing time compared with PTSD (32.59 ± 8.35% vs. 60.74 ± 20.84, p < 0.001, Fig. 1A, 1C) in test 2 which designed for evaluating therapeutic effects of sevoflurane on PTSD mice and our results give a positive answer on it. To further explore the effective time of sevoflurane, we repeated test 2 after 7 days and the result was the same as before (28.03 ± 10.08% vs. 55.62 ± 14.33, p < 0.01 vs. PTSD, Fig. 1A, 1D). In addition, we performed test 3 in order to exclude the influence of memory generalization in this PTSD model. It can be observed that all groups showed a high degree of activity without fear in the context c (p > 0.05, Fig. 1A, 1E).

3.2.Long-term sevoflurane ameliorated anxiety in PTSD mice.

To further investigate the effects of sevoflurane on anxiety in PTSD mice, we conducted the open field test on days 12 (7 days after anesthesia). PTSD mice showed reduced time spent in the center of the arena compared with other groups (70.58 ± 16.28, p < 0.001 vs. Ctrl, Sevo and PTSD + Sevo, Fig. 2A). Similar data could be seen in the total distance of PTSD mice (37.72 ± 2.98, p < 0.001 vs. Ctrl, Sevo and PTSD + Sevo, Fig. 2B). In the forced swimming test, the freezing time was similar among all groups (p > 0.05, Fig. 2C).

3.3.Short-term sevoflurane improved SEFL transiently in the PTSD mice

For further investigating whether short-term sevoflurane inhaled could induce SELF either, we repeated experiment 1 expects that the duration of anesthesia was shortened to 2h. In test 1, the data showed obviously enhanced freezing time in the PTSD group. (66.93 ± 19.78%, p < 0.001 vs. Ctrl and Sevo (untreated), Fig. 3A, 3B). After exposed to sevoflurane for 2 h, mice in the PTSD + Sevo group exhibited decreased freezing time compared with PTSD (35.24 ± 11.12% vs. 62.41 ± 18.28, p < 0.001, Fig. 3A, 3C) in test 2. We repeated test 2 after 7 days and the result was modified. The PTSD mice still showed higher freeing time than the control group (54.50 ± 14.34% vs. 23.21 ± 7.30, p < 0.01, Fig. 3A, 3D), while mice
exposure to sevoflurane after 7 days could not reverse the effect (45.34 ± 11.75%, p > 0.05 vs. PTSD, p < 0.05 vs. Ctrl and Sevo, Fig. 3A, 3D).

### 3.4. GR and CB1R expressions were increased in brain areas in PTSD mice and sevoflurane normalized this effect.

We explored the level of relative GR and CB1R expressions in the hippocampus, amygdala and prefrontal cortex (PFC) in all groups. In the PTSD group, the expression of GR was upregulated in the hippocampus (2.02 ± 0.30), amygdala (2.20 ± 0.35) and PFC (2.11 ± 0.26) compared with the control and Sevo group (p < 0.01 vs. Ctrl and Sevo). Sevoflurane administered for 6 h modified these alterations in the three brain areas (hippocampus: 1.36 ± 0.19, amygdala 1.23 ± 0.22, PFC: 1.22 ± 0.30, p < 0.05 vs. PTSD, Fig. 4A-B). Similar changes were seen in CB1R relative expression. Higher expression of CB1R was found in the PTSD group in the hippocampus (2.22 ± 0.22), amygdala (3.03 ± 0.63) and PFC (2.33 ± 0.56) compared with the control and Sevo group (p < 0.01 vs. Ctrl and Sevo). 6 h of sevoflurane treated normalized the alterations in the three brain areas (hippocampus: 1.41 ± 0.15, amygdala 1.60 ± 0.47, PFC: 1.16 ± 0.39, p < 0.05 vs. PTSD, Fig. 4A, C).

### 3.5. The expression of D1/2 dopamine receptors decreased after modeling and sevoflurane normalized this change partly in the PFC.

We next investigated the expression of D1R and D2R in the hippocampus, amygdala and PFC in all groups. No alterations in D1R were observed in the hippocampus (1.11 ± 0.25) or amygdala (1.05 ± 0.16) of PTSD mice (p > 0.05 vs. all groups, Fig. 5A, B). In the PFC, a significant group effect was found for D1R in the PTSD group (0.55 ± 0.15, p < 0.05 vs. Ctrl, Sevo) and the sevoflurane inhaled increased the level (0.94 ± 0.13, p < 0.05 vs. PTSD, Fig. 5A, B). While when it came to D2R, it changed obviously. The level of D2R was decreased in the hippocampus (0.52 ± 0.14), amygdala (0.42 ± 0.10) and PFC (0.44 ± 0.14) in the PTSD mice (p < 0.01 vs. Ctrl and Sevo). Sevoflurane did not reverse the downregulation very well. Only in the PFC could sevoflurane improve the level of D2R (1.13 ± 0.27, p < 0.05 vs. PTSD, Fig. 5A, C). No differences were observed in the hippocampus (0.56 ± 0.10), amygdala (0.50 ± 0.15) compared with the PTSD group (p > 0.05, Fig. 5A, C).

### 4. Discussion

The SEFL animal model of PTSD demonstrated robust fear in context A on day 3 post-induction, confirming that shock exposure (trauma) can induce a non-associative fear response. Notably, 2.4% sevoflurane administration for 6 h on day 4 after modeling significantly attenuated SEFL and anxiety for at least 7 days, while 2 h of exposure had only a transient suppressive effect. This study suggests that a single exposure to sevoflurane several days after trauma can partially ameliorate PTSD symptoms, presumably by normalizing the changes in transmitter signaling triggered by physiological responses and memories of the traumatic event.
The anesthetic ketamine was recently approved by the United States Food and Drug Administration (FDA) for the treatment of major depression, and there is growing interest in the use of other anesthetics in clinical psychology and psychiatry, especially for memory-related diseases such as PTSD\textsuperscript{13, 14}. Sevoflurane is generally considered to have milder effects on cognitive function than isoflurane\textsuperscript{15–17}, suggesting greater safety and tolerability as a potential treatment for PTSD. Indeed, the SEFL model examined in this study demonstrated persistent PTSD-like symptoms that were suppressed by a sufficient isoflurane dose administered several days after the traumatic event (exposure to repeated electrical shocks). A previous study reported that sevoflurane inhalation during trauma reduced ensuing SEFL in mice\textsuperscript{18}, but such preventative use is impractical as treatment, so our demonstration that sevoflurane can be used to reverse the deleterious effects of exacerbated non-associative fear memory has potential clinical significance. This salutary effect was dose-dependent, as a 2-h administration significantly improved SEFL only on the first day post-treatment while the treatment effect of the 6-h administration lasted at least 7 days. This sustained effect is also critical because repeated administration of inhaled anesthetics can induce brain damage in mice and humans\textsuperscript{19, 20}. However, we only tested two dose regimens (2 h and 6 h at a minimum alveolar concentration of 1.0), so other dose regimens should be tested to identify the most suitable starting parameters for subsequent studies.

Exposure to stress upregulates the expression of GR in brain regions implicated in fear memory such as the hippocampus, amygdala, and PFC, while administration of GR antagonists can prevent the neuronal damage caused by stress\textsuperscript{21, 22}. In accord with previous studies, GD expression was elevated in PTSD model mice, while inhalation of sevoflurane reversed this upregulation but had no effect on basal GR expression in control mice. In addition to dysregulation of GR expression, PTSD is reported to alter expression of CB1R, a receptor widely expressed in both limbic structures and hypothalamic nuclei where it can modulate GR signaling\textsuperscript{23, 24}. In the current study, the traumatic experience upregulated CB1R expression, and this response was reversed by sevoflurane. In contrast, a previous study reported that CB1R was downregulated during protracted contextual fear memory and that a CB1R agonist suppressed fear memory\textsuperscript{25, 26}. This discrepancy may result from the different modeling parameters used in these studies, such as the stress-induction stimulus, intensity, and repetition. For instance, the six shocks in context A actually reduced durable sensitization among PTSD model mice in the aforementioned study.

In addition to effects on glucocorticoid signaling, CB1R has also been reported to modulate the expression of dopamine receptors, which in turn can regulate fear memory\textsuperscript{27}. Genes related to D2 receptor signaling have also been linked to an increased risk of PTSD\textsuperscript{28}. Further, the amygdala–hippocampal–cortical pathway is responsible for processing and storing fear-related memories and for coordinating fear-related behaviors\textsuperscript{29}. Thus, we measured the levels of D1R and D2R in these brain regions. While D1R expression was relatively stable, D2R expression was substantially reduced in model mice. Sevoflurane reversed this effect in PFC but not in hippocampus and amygdala, possibly because the PFC is involved in the storage of long-term fear memory\textsuperscript{30} and expression was not examined until 13 days after the trauma. Moreover, the exacerbated reaction to shock-related cues may last for months, suggesting marked changes in the PFC\textsuperscript{31}. Additional studies are required to fully elucidate the
spatiotemporal changes in neural D2R, GR, and CB1R expression levels following trauma and various post-traumatic treatments.

5. Conclusion

The present study provides support for the effectiveness of sevoflurane as a suppressor of SEFL and anxiety in PTSD model mice. This sevoflurane treatment model may also help unravel the neurocellular and molecular mechanisms underlying PTSD.

Declarations

Ethics approval and consent to participate

This study was approved by the Nanjing University Institutional Animal Care and Use Committee, in accordance with international regulations. The study is reported in accordance with ARRIVE guidelines.

Consent for publication

Not applicable

Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Acknowledgements

We are very grateful to Dr Lizhi Xu, Dr. Huan Dou and Dr. Jie Ding (Medical School, Nanjing University) for their technical support.

Funding

This work was supported by grants from the National Natural Science Foundation of China (Grant Number: 81730033, 82171193), the Key Talent Project for Strengthening Health during the 13th Five-Year Plan Period (Grant Number: ZDRCA2016069), the National Key R&D Program of China (Grant Number: 2018YFC2001901) and Qing Lan Project.

Competing interests

The authors declare that they have no known conflicts of interest that could influence the work reported in this paper.

Author Contributions

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References


Figures
Figure 1

Effects of long-term sevoflurane exposure on the fear performance of PTSD mice.

(A) The flow chart. (B) The freezing time in test 1 after PTSD modeling. (C) The freezing time in test 2 after 6h sevoflurane inhaling. (D) The freezing time in test 3 after 7 days of test 2. (D) The activity time in test 4 of memory generalization. Data are expressed as the mean ± SD, N=8. a P<0.05 vs. control group, b P<0.05 vs. PTSD group, c P<0.05 vs. sevoflurane group.
Figure 2

Effects of long-term sevoflurane exposure on the anxiety of PTSD mice.

(A) Time of mice spent in center space in the open field test. (B) total distance of mice run in the open field test. (C) The freezing time of mice in the forced swimming test. Data are expressed as the mean ± SD, N=8. a P<0.05 vs. control group, b P<0.05 vs. PTSD group.

Figure 3

Effects of short-term sevoflurane exposure on the fear performance of PTSD mice.
(A) The flow chart. (B) The freezing time in test 1 after PTSD modeling. (C) The freezing time in test 2 after 2h sevoflurane inhaling. (D) The freezing time in test 3 after 7 days of test 2. Data are expressed as the mean ± SD, N=8. a P<0.05 vs. control group, b P<0.05 vs. PTSD group, c P<0.05 vs. sevoflurane group.

Figure 4

Changes of GR and CB1R expression in the hippocampus, amygdala and prefrontal cortex.

(A) Representative blots of each protein. (B-C) Statistical analysis of the relative protein expression. Data are expressed as the mean ± SD, N=3. a P<0.05 vs. control group, b P<0.05 vs. PTSD group.
Figure 5

Changes of D1R and D2R expression in the hippocampus, amygdala and prefrontal cortex.

(A) Representative blots of each protein. (B-C) Statistical analysis of the relative protein expression. Data are expressed as the mean ± SD, N=3. a P<0.05 vs. control group, b P<0.05 vs. PTSD group.