

Sevoflurane Anesthesia in Pregnant Mice Induces Neurotoxicity in Fetal and Offspring Mice

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ABSTRACT

Background: Each year, over 75,000 pregnant women in the United States undergo anesthesia care. The authors set out to assess the effects of the anesthetic sevoflurane on neurotoxicity in pregnant mice and on learning and memory in fetal and offspring mice.

Methods: Pregnant mice (gestational day 14) and mouse primary neurons were treated with 2.5% sevoflurane for 2 h and 4.1% sevoflurane for 6 h, respectively. Brain tissues of both fetal and offspring mice (P31) and the primary neurons were harvested and subjected to Western blot and immunohistochemistry to assess interleukin-6, the synaptic markers postsynaptic density-95 and synaptophysin, and caspase-3 levels. Separately, learning and memory function in the offspring mice was determined in the Morris water maze.

Results: Sevoflurane anesthesia in pregnant mice induced caspase-3 activation, increased interleukin-6 levels ($256 \pm 50.98\%$ [mean \pm SD] *vs.* $100 \pm 54.12\%$, $P = 0.026$),

What We Already Know about This Topic

- The effects of maternal exposure to sevoflurane on fetal neurotoxicity and neurobehavioral outcome are controversial

What This Article Tells Us That Is New

- Sevoflurane may induce detrimental effects in fetal and offspring mice, which can be mitigated by environmental enrichment

and reduced postsynaptic density-95 ($61 \pm 13.53\%$ *vs.* $100 \pm 10.08\%$, $P = 0.036$) and synaptophysin levels in fetal and offspring mice. The sevoflurane anesthesia impaired learning and memory in offspring mice at P31. Moreover, interleukin-6 antibody mitigated the sevoflurane-induced reduction in postsynaptic density-95 levels in the neurons. Finally, environmental enrichment attenuated the sevoflurane-induced increases in interleukin-6 levels, reductions of synapse markers, and learning and memory impairment.

Conclusions: These results suggest that sevoflurane may induce detrimental effects in fetal and offspring mice, which can be mitigated by environmental enrichment. These findings should promote more studies to determine the neurotoxicity of anesthesia in the developing brain.

ANESTHESIA neurotoxicity in the developing brain has been investigated in animals and in humans and has become a major health issue of interest to both the medical community¹ and the public.² Anesthesia and surgery may induce neurodevelopmental impairment and cognitive dysfunction in children (reviewed in Sun³). In preclinical studies, anesthesia has been shown to induce neurotoxicity and learning and memory impairment in young animals⁴ (reviewed in Creeley and Olney⁵).

Each year, over 75,000 pregnant women in the United States have nonobstetric surgery and fetal intervention procedures under anesthesia.⁶ Anesthesia neurotoxicity in the developing brain could occur in the fetus because (1) brain development starts as early as the second trimester of pregnancy; (2) anesthesia can induce neurotoxicity in both adult and young mice, and most general anesthetics are lipophilic

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Received from the Department of Anesthesia, Critical Care and Pain Medicine, Massachusetts General Hospital and Harvard Medical School, Boston, Massachusetts. Submitted for publication March 14, 2012. Accepted for publication August 14, 2012. Supported by grants R21AG029856, R21AG038994, and R01 GM088801 from the National Institutes of Health, Bethesda, Maryland; and from Cure Alzheimer's Fund, Wellesley, Massachusetts (to Dr. Xie). The cost of anesthetic sevoflurane was generously provided by the Department of Anesthesia, Critical Care and Pain Medicine, Massachusetts General Hospital and Harvard Medical School, Boston, Massachusetts. Drs. Zheng and Dong contributed equally to this work.

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and thus cross the placenta easily; and (3) uterine exposure to ethanol, valproic acid, and the anesthetic isoflurane have been shown to induce behavioral abnormalities in adulthood⁷ (reviewed in Reitman and Flood⁸). It remains largely to be determined, however, whether anesthesia in pregnant mice can induce (1) neurotoxicity in fetal mice (the developing brain) and (2) neurotoxicity and learning and memory impairment in offspring mice after birth.

Sevoflurane is currently the most commonly used inhalation anesthetic. Previous studies have shown that anesthesia with 2.5% sevoflurane for 2 h can induce neurotoxicity in the brain tissues of adult (5-month-old) mice without statistically significant alteration in the values of blood pressure and blood gas.⁹ We therefore determined whether the same sevoflurane anesthesia in pregnant mice could induce neurotoxicity and learning and memory impairment in fetal and offspring mice. Finally, we investigated whether environmental enrichment (EE), a complex living milieu that has been shown to improve learning and memory,^{10–12} could ameliorate the sevoflurane-induced detrimental effects.

Materials and Methods

Mice Anesthesia

The protocol was approved by the Massachusetts General Hospital Standing Committee (Boston, Massachusetts) on the Use of Animals in Research and Teaching. Three-month-old C57BL/6J female mice (The Jackson Laboratory, Bar Harbor, ME) were mated with male mice. The pregnant mice were identified and then housed individually. The offspring mice were weaned 21 days after birth. Animals were kept in a temperature-controlled (22°–23°C) room under a 12-h light/dark period (light on at 7:00 AM); standard mouse chow and water were available *ad libitum*. At gestational day (G) 14, the pregnant mice were assigned randomly to an anesthesia group or a control group. Mice randomized to the anesthesia group received 2.5% sevoflurane in 100% oxygen for 2 h in an anesthetizing chamber. The control group received 100% oxygen at an identical flow rate for 2 h in an identical chamber as described in our previous studies.⁹ The mice breathed spontaneously, and concentrations of anesthetic and oxygen were measured continuously (Datex-Ohmeda Inc., Tewksbury, MA). The temperature of the anesthetizing chamber was controlled to maintain rectal temperature of the animals at 37° ± 0.5°C. Mean arterial blood pressure was not measured in these mice because the same sevoflurane anesthesia was shown not to alter the values of blood pressure and blood gas in our previous studies.⁹ Anesthesia was terminated by discontinuing sevoflurane and placing the animals in a chamber containing 100% oxygen until 20 min after return of the righting reflex. The anesthesia with 2.5% sevoflurane (approximately 1.1 minimum alveolar concentration) for 2 h in mice was used to demonstrate whether clinically relevant sevoflurane anesthesia in pregnant mice, which had been shown to induce neurotoxicity in adult

mice,⁹ could also induce neurotoxicity in fetal mice and then neurobehavioral deficits in offspring mice. Twenty pregnant mice were included in the experiments, which generated a sufficient number of fetal mice for the biochemistry studies (n = 6 per arm), and offspring mice for the biochemistry (n = 6 per arm) and behavioral studies (n = 15 per arm). Our pilot studies showed a mean difference of 1.5 (3 *vs.* 1.5) in platform crossing times, with an SD of 1.8 in the control group and 1.3 in the anesthesia group. From the pilot study, we also estimated a mean difference of 150% (250% *vs.* 100%) in interleukin (IL)-6 levels in brain tissues, with an SD of 51 in the control group and 54 in the anesthesia group. Assuming this study would have similar effect sizes, a sample size of 6 per arm for the biochemistry studies and a sample size of 15 per arm for the behavioral studies would lead to a 90% or larger power to detect the differences using two-sample Student *t* test with 5% type I error.

Mouse Primary Neurons

The protocol was approved by the Massachusetts General Hospital Standing Committee on the Use of Animals in Research and Teaching. The harvest of neurons was performed as described in our previous studies.^{13,14} Seven to 10 days after harvesting, the neurons were treated with 4.1% sevoflurane for 6 h as described in our previous studies.⁹ The treatment with 4.1% sevoflurane for 6 h was used to determine whether the sevoflurane anesthesia, which can induce cytotoxicity,⁹ could also reduce levels of postsynaptic density-95 (PSD-95), the marker for synapse. The IL-6 antibody (10 µg/ml) was administered to the neurons 1 h before the sevoflurane treatment. The neurons were harvested at the end of anesthesia and were subjected to Western blot analysis.

Brain Tissue Harvest and Protein Level Quantification

Immediately after the sevoflurane anesthesia, we performed a cesarean section to extract the fetal mice and harvested their brain tissues. We also used decapitation to kill postnatal day (P) 31 offspring mice and harvested their brain tissues. Separate groups of mice were used for the Western blot analysis and the immunohistochemistry studies, respectively. For the Western blot analysis, the harvested brain tissues were homogenized on ice using immunoprecipitation buffer (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 2 mM ethylenediaminetetraacetic acid, and 0.5% Nonidet P-40) plus protease inhibitors (1 µg/ml aprotinin, 1 µg/ml leupeptin, and 1 µg/ml pepstatin A) as described in our previous studies.¹⁵ The lysates were collected, centrifuged at 12,000 rpm for 15 min, and quantified for total proteins with bicinchoninic acid protein assay kit (Pierce Technology Co., Iselin, NJ).¹⁵

Western Blot Analysis

Western blot analysis was performed using the methods described in our previous studies.¹⁵ Whole cerebral hemispheres were used for Western blot analysis because

there would be an insufficient amount of hippocampus tissues from the fetal mice for Western blot analysis. IL-6 antibody (1:1,000 dilution; Abcam, Cambridge, MA) was used to recognize IL-6 (24 kDa). PSD-95 antibody (1:1,000; Cell Signaling Technology, Danvers, MA) was used to detect PSD-95 (95 kDa). A caspase-3 antibody (1:1,000 dilution; Cell Signaling Technology) was used to recognize full-length caspase-3 (35–40 kDa) and caspase-3 fragment (17–20 kDa) resulting from cleavage at aspartate position 175. Antibody anti- β -actin (1:10,000; Sigma, St. Louis, MO) was used to detect β -actin (42 kDa). Western blot quantification was performed as described by Xie *et al.*¹⁶ Briefly, signal intensity was analyzed using a Bio-Rad (Hercules, CA) image program (Quantity One). We quantified the Western blots in two steps. First, we used β -actin levels to normalize (*e.g.*, determining the ratio of IL-6 to β -actin amount) protein levels and control for loading differences in the total protein amount. Second, we presented changes in protein levels in mice or neurons undergoing sevoflurane anesthesia as a percentage of those in the control group. One hundred percent of protein level changes refer to control levels for the purpose of comparison with experimental conditions.

The quantification of Western blot was based not only on the images presented in figures but also on the images not presented in the figures to have adequate effect size (*e.g.*, $n = 6$ in biochemistry studies).¹⁵

Immunohistochemistry

Immunohistochemistry was performed using the methods described in our previous studies.¹⁷ P31 offspring mice were anesthetized with sevoflurane briefly (2.5% sevoflurane for 4 min) and perfused transcardially with heparinized saline followed by 4% paraformaldehyde in 0.1M phosphate buffer at pH 7.4. The anesthesia with 2.5% sevoflurane for 4 min in mice provided adequate anesthesia for the perfusion procedure without causing statistically significant changes in blood pressure and blood gas according to our previous studies.⁹ Mouse brain tissues were removed and kept at 4°C in paraformaldehyde. Five-micron frozen sections from the mouse brain hemispheres were used for the immunohistochemistry staining.¹⁷ The sections were incubated with the primary antibody synaptophysin (1:500; Sigma) dissolved in 1% bovine serum albumin in phosphate-buffered saline at 4°C overnight. The next day, the sections were exposed to secondary antibody (Alexa Fluor 594 goat anti-rabbit IgG [H+L]; Invitrogen, Grand Island, NY). Finally, the sections were wet mounted and viewed immediately using a fluorescence microscope (60 \times). We used the mouse hippocampus in the studies of immunohistochemistry density quantification to determine whether sevoflurane anesthesia can induce neurotoxicity in the hippocampus. The photographs were taken and an investigator who was blind to the experimental design counted the density of synaptophysin using ImageJ version 1.38 (National Institutes of Health, Bethesda, MD).¹⁷

Morris Water Maze

A round steel pool, 150 cm in diameter and 60 cm in height, was filled with water to a height of 1.0 cm above the top of a 10-cm diameter platform. The pool was covered with a black curtain and was located in an isolated room with four visual cues on the wall of the pool. Water was kept at 20°C and opacified with titanium dioxide. The P31 offspring mice were tested in the Morris water maze (MWM) four times per day for 7 days. Each of the mice was put in the pool to search for the platform, and the starting points were random for each mouse. When the mouse found the platform, the mouse was allowed to stay on it for 15 s. If a mouse did not find the platform within a 90-s period, the mouse was gently guided to the platform and allowed to stay on it for 15 s. A video tracking system recorded the swimming motions of the animals, and the data were analyzed using motion-detection software for the MWM (Institute of Materia Medica, Chinese Academy of Medical Sciences & Peking Union Medical College, Beijing, People's Republic of China). At the end of the reference training (P37), the platform was removed from the pool and the mouse was placed in the opposite quadrant. Mice were allowed to swim for 90 s and the times the mouse swam to cross the platform area was recorded (platform crossing times). Mouse body temperature was maintained by active heating as described by Bianchi *et al.*¹⁸ Specifically, after every trial, each mouse was placed in a holding cage under a heat lamp for 1 to 2 min until dry before being returned to its regular cage.

Environmental Enrichment

The EE in the current experiment was created in a large cage (70 \times 70 \times 46 cm) that included five or six toys (*e.g.*, wheels, ladders, and small mazes) as described in previous studies, with modification.^{10,11} The pregnant mice were put in the EE every day for 2 h before delivery. The pregnant mice delivered offspring mice at G21. Then, the mother and the babies were put in the EE again every day for 2 h from P4 to P30. The objects were changed two to three times per week to provide newness and challenge.

Statistical Analysis

The nature of the hypothesis testing was two-tailed. Data were expressed as mean \pm SD. The data for platform crossing time were not distributed normally and thus were expressed as median and interquartile range (IQR). The number of samples varied from 6–15, and the samples were distributed normally, with the exception of platform crossing time (tested by normality test, data not shown). Two-way ANOVA was used to determine the interaction of IL-6 antibody and sevoflurane treatment, and the interaction of EE and sevoflurane anesthesia. Interaction between time and group factors in a two-way ANOVA with repeated measurements was used to analyze the difference of learning curves (based on escape latency) between mice in the control group and mice treated with anesthesia in the

MWM. Multiple comparisons in escape latency of MWM were adjusted using the Bonferroni method (with seven tests and a threshold of $0.05/7 = 0.0071$). There were no missing data for the variables of MWM (escape latency and platform crossing time) during the data analysis. The Student two-sample t test was used to determine the difference between the sevoflurane and control conditions on levels of IL-6, PSD-95, and synaptophysin. Finally, the Mann–Whitney U test was used to determine the difference between the sevoflurane and control conditions on platform crossing times. Values of $P < 0.05$ were considered statistically significant. SAS software version 9.2 (SAS Institute Inc., Cary, NC) was used to analyze the data.

Results

Sevoflurane Anesthesia in Pregnant Mice Induced Learning and Memory Impairment in Offspring Mice

The pregnant mice were either treated with 2.5% sevoflurane anesthesia for 2 h or under the control condition at G14. The mice delivered offspring mice at G21, and the offspring mice were tested in the MWM from P31 to P37. Comparison of the time that each mouse took to reach a platform during reference training (escape latency) showed that there was a statistically significant interaction between time and group based on escape latency in the MWM between mice following the control condition and mice that were given sevoflurane anesthesia (fig. 1A) ($P = 0.012$, two-way ANOVA with repeated measurement). Comparison of the number of times that each mouse crossed the location of an absent platform at the end of reference training (platform crossing times) indicated that there was a nonsignificant difference in the platform crossing times between the control condition (median, 2; IQR, 2–4.5) and the sevoflurane anesthesia (median, 1; IQR, 1–3) ($P = 0.051$, Mann–Whitney U test) (fig. 1B). There was no statistically significant difference in mouse swimming speed between the sevoflurane anesthesia and the control group (data not shown). Considered together, these data suggest that sevoflurane anesthesia in pregnant mice may induce learning and memory impairment in offspring mice.

Sevoflurane Anesthesia in Pregnant Mice Induced Neurotoxicity in Fetal Mice

Given that the sevoflurane anesthesia in pregnant mice can induce learning and memory impairment in offspring mice, we assessed the effects of sevoflurane anesthesia on the levels of proinflammatory cytokine IL-6, PSD-95, and caspase-3 activation, the neurotoxicity which may represent underlying mechanisms of learning and memory impairment.^{19–28} The pregnant mice received anesthesia with 2.5% sevoflurane for 2 h or the control condition at G14. We harvested the brain tissues of the fetal mice at the end of the experiment, and these tissues were subjected to Western blot analysis. Immunoblotting of IL-6 showed that the sevoflurane

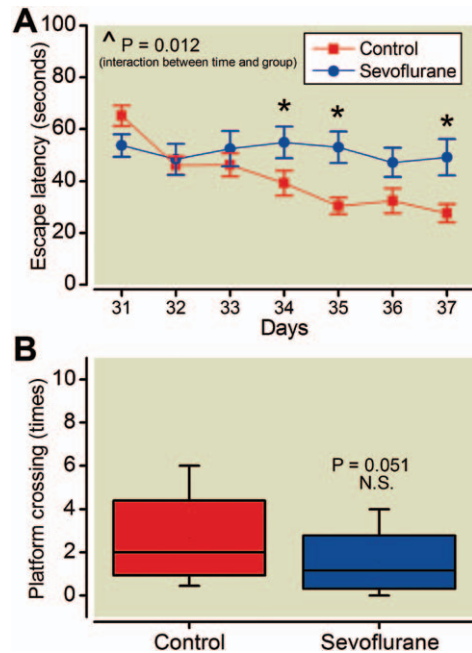


Fig. 1. Anesthesia with 2.5% sevoflurane for 2 h in pregnant mice at G14 induces learning and memory impairment in offspring mice tested at P31. (A) Sevoflurane anesthesia increases escape latency time of mice swimming in the Morris water maze (MWM) as compared with the control condition. Two-way ANOVA with repeated measurement analysis shows that there is a statistically significant interaction between time and group based on escape latency between mice following the control condition and mice following the sevoflurane anesthesia in the MWM ($\wedge P = 0.012$). Asterisk indicates that there is a statistically significant difference in the escape latency between the control group and the sevoflurane group. (B) Sevoflurane anesthesia reduces the platform crossing times of mice swimming in the MWM (median, 1; IQR, 1–3) as compared with the control condition (median, 2; IQR, 2–4.5; $P = 0.051$, Mann–Whitney U test). IQR = interquartile range ($n = 15$ per arm).

anesthesia induced more visible bands representing IL-6 as compared with the control condition (fig. 2A). There was no significant difference in β -actin levels between the control condition and the sevoflurane anesthesia. Quantification of the Western blot showed that the sevoflurane anesthesia increased IL-6 levels in the brain tissues of fetal mice as compared with the control condition ($256 \pm 50.98\%$ vs. $100 \pm 54.12\%$, $P = 0.026$) (fig. 2B).

Next, we investigated the effects of the sevoflurane anesthesia in pregnant mice on levels of PSD-95, the marker of synapse, in the brain tissues of the fetal mice. Immunoblotting of PSD-95 showed that the sevoflurane anesthesia in pregnant mice produced less visible bands representing PSD-95 in the Western blot as compared with the control condition (fig. 2C). Quantification of the Western blot showed that the sevoflurane anesthesia in pregnant mice reduced PSD-95 levels in the brain tissues of fetal mice as compared with the control condition ($61 \pm 13.53\%$ vs. $100 \pm 10.08\%$, $P = 0.036$) (fig. 2D). Finally, we assessed the effects of sevoflurane anesthesia in pregnant mice

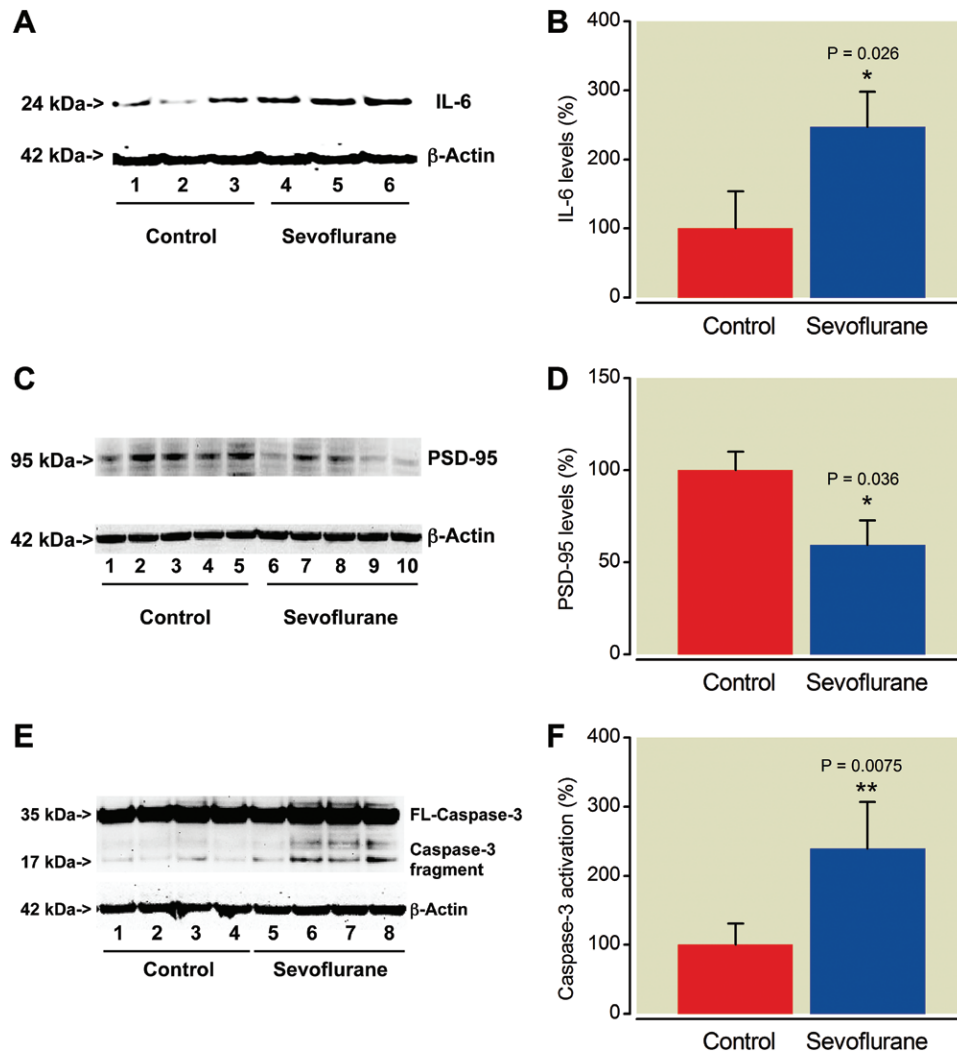


Fig. 2. Anesthesia with 2.5% sevoflurane for 2 h in pregnant mice at G14 increases IL-6 levels, decreases PSD-95 levels, and induces caspase-3 activation in the brain tissues of fetal mice. (A) Sevoflurane anesthesia increases IL-6 levels in the brain tissues of fetal mice as compared with the control condition on Western blot analysis. There is no statistically significant difference in the amounts of β -actin in the mouse brain tissues following the sevoflurane anesthesia or control condition. (B) Quantification of the Western blot shows that sevoflurane anesthesia increases IL-6 levels in the mouse brain tissues as compared with the control condition (* $P = 0.026$). (C) The sevoflurane anesthesia reduces PSD-95 levels in the brain tissues of fetal mice as compared with the control condition on Western blot analysis. There is no statistically significant difference in the amounts of β -actin in the mouse brain tissues following the sevoflurane anesthesia or control condition. (D) Quantification of the Western blot shows that sevoflurane anesthesia reduces PSD-95 levels in the mouse brain tissues as compared with the control condition (* $P = 0.036$). (E) The sevoflurane anesthesia induces caspase-3 activation in the brain tissues of fetal mice as compared with the control condition on Western blot analysis. There is no significant difference in the amounts of β -actin in the mouse brain tissues following the sevoflurane anesthesia or control condition. (F) Quantification of the Western blot shows that sevoflurane anesthesia induces caspase-3 activation in the mouse brain tissues as compared with the control condition (** $P = 0.0075$). FL = full length ($n = 6$ per arm); IL-6 = interleukin-6; PSD = postsynaptic density-95.

on caspase-3 activation in the brain tissues of fetal mice. Caspase-3 immunoblotting showed that the sevoflurane anesthesia in pregnant mice increased levels of caspase-3 fragment without statistically significant changes in the levels of full-length caspase-3 in the brain tissues of fetal mice (fig. 2E). The quantification of the Western blot, based on the ratio of caspase-3 fragment to full-length caspase-3, revealed that the sevoflurane anesthesia in pregnant mice

induced caspase-3 activation as compared with the control condition ($198 \pm 35\%$ vs. $100 \pm 21\%$, $P = 0.0075$) (fig. 2F). Considered together, these results suggest that anesthesia with 2.5% sevoflurane for 2 h in pregnant mice may induce neurotoxicity, including increases in proinflammatory cytokine levels, a reduction in synapse marker numbers, and caspase-3 activation in fetal mice, which may then lead to learning and memory impairment.

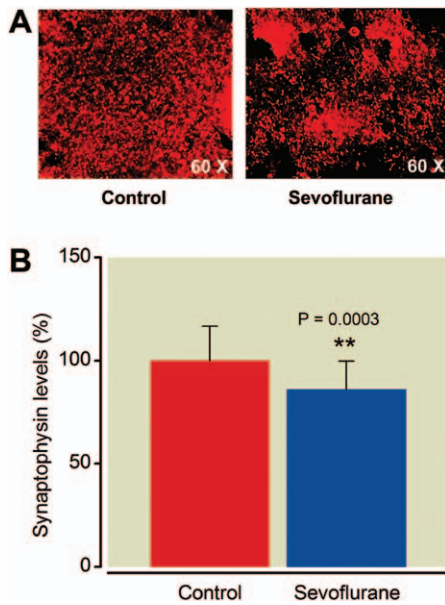


Fig. 3. Anesthesia with 2.5% sevoflurane for 2 h in pregnant mice at G14 decreases synaptophysin levels in the hippocampus of offspring mice examined at P31. (A) Sevoflurane anesthesia decreases synaptophysin levels in the brain tissues of offspring mice as compared with the control condition on immunohistochemistry analysis. (B) Quantification of the immunohistochemistry image shows that sevoflurane anesthesia decreases synaptophysin levels in the mouse brain tissues as compared with the control condition (** $P = 0.0003$) ($n = 6$ per arm).

Sevoflurane Anesthesia in Pregnant Mice Reduced Synaptophysin Levels in the Hippocampus of Offspring Mice

Given that sevoflurane anesthesia may cause acute neurotoxicity in fetal mice and learning and memory impairment in offspring mice at a later time (e.g., P31), we assessed the effects of the sevoflurane anesthesia on levels of synapse markers in the hippocampus of P31 mice. Immunohistochemistry analysis showed that the sevoflurane anesthesia reduced levels of synaptophysin, the synapse marker,²⁹ in the hippocampus of P31 mice (fig. 3A). Quantification of the immunohistochemistry image showed that the sevoflurane anesthesia decreased levels of synaptophysin ($77 \pm 14.00\%$ vs. $100 \pm 16.73\%$, $P = 0.0003$) (fig. 3B). These results suggest that the sevoflurane anesthesia in pregnant mice may induce synaptic loss at a later time (e.g., P31), leading to learning and memory impairment.

The Sevoflurane-induced Reduction in PSD-95 Level Was Dependent on the Sevoflurane-induced Increases in IL-6 Level

Given that the sevoflurane anesthesia increased IL-6 levels and decreased PSD-95 levels in brain tissues of fetal mice at G14, we then determined their potential association in mouse primary neurons. Treatment with 4.1% sevoflurane for 6 h reduced PSD-95 levels in mouse primary neurons as

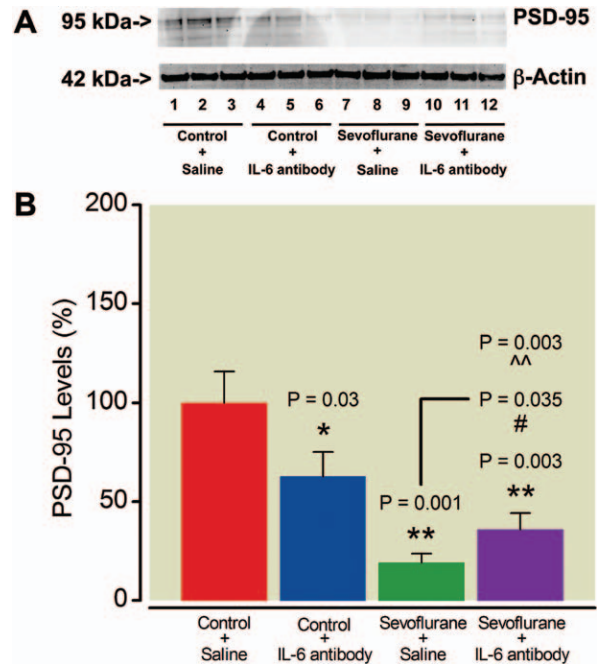


Fig. 4. IL-6 antibody mitigates the sevoflurane-induced reduction in PSD-95 levels in mouse primary neurons. (A) Treatment with 4.1% sevoflurane for 6 h (lanes 7–9) reduces PSD-95 levels as compared with the control condition (lanes 1–3). The treatment of IL-6 antibody (lanes 10–12) mitigates the sevoflurane-induced reduction in PSD-95 levels. There is no significant difference in the amounts of β -actin in the mouse primary neurons following the treatments of sevoflurane, IL-6 antibody, or control condition. (B) Quantification of the Western blot shows that sevoflurane treatment decreases PSD-95 levels as compared with the control condition (** $P = 0.001$). Treatment with sevoflurane plus IL-6 antibody leads to a lesser degree of reduction in PSD-95 levels as compared with treatment with sevoflurane plus saline ($\#P = 0.035$). IL-6 antibody itself also decreases PSD-95 levels ($*P = 0.03$). Two-way ANOVA shows that there is an interaction of IL-6 antibody and sevoflurane, and that IL-6 antibody mitigates the sevoflurane-induced reduction in PSD-95 levels ($\wedge\wedge P = 0.003$) ($n = 6$ per arm). IL-6 = interleukin-6; PSD-95 = postsynaptic density-95.

compared with the control condition (fig. 4A). The treatment with sevoflurane reduced PSD-95 levels as compared with the control condition, but IL-6 antibody mitigated the sevoflurane-induced reduction in PSD-95 levels, evidenced by more visible bands representing PSD-95 following the treatment of sevoflurane plus IL-6 antibody than following the treatment of sevoflurane plus saline (fig. 4A). Quantification of the Western blot showed that the sevoflurane treatment reduced PSD-95 levels ($20 \pm 4.58\%$ vs. $100 \pm 19\%$, $P = 0.001$) and IL-6 antibody mitigated the sevoflurane-induced reduction in PSD-95 levels ($36 \pm 8.33\%$ vs. $20 \pm 4.58\%$, $P = 0.035$) (fig. 4B). Two-way ANOVA indicated that there was an interaction between IL-6 antibody and sevoflurane, and that IL-6 antibody mitigated the sevoflurane-induced reduction in PSD-95 levels ($P = 0.003$) (fig. 4B). These results suggest that the sevoflurane-induced reduction in PSD-95 level may be dependent on the sevoflurane-induced increases in

IL-6 level. Interestingly, IL-6 antibody also reduced PSD-95 levels in the primary neurons (fig. 4, A and B).

EE Attenuated the Sevoflurane Anesthesia-induced Learning and Memory Impairment in Offspring Mice

EE has been shown to improve learning and memory,^{30,31} and we therefore assessed whether EE can ameliorate the sevoflurane-induced learning and memory impairment. Two-way ANOVA with repeated measurement analysis showed that there was a statistically significant interaction between time and group based on escape latency between mice following sevoflurane anesthesia plus standard environment (SE) and sevoflurane anesthesia plus EE, and EE mitigated the sevoflurane-induced increases in escape latency of mice swimming in the MWM ($P = 0.0004$) (fig. 5A). Sevoflurane anesthesia plus EE (median, 4; IQR, 3.75–4.25) also increased the platform crossing times of mice in the MWM as compared with sevoflurane anesthesia plus SE (median, 1; IQR, 1–3; $P = 0.003$, Mann–Whitney U test) (fig. 5B). EE alone did not alter escape latency or platform crossing times of mice swimming in the MWM (fig. 5, C and D). Two-way ANOVA with repeated measurement analysis showed that

there was no statistically significant interaction between time and group based on escape latency between mice following the control condition plus SE and control condition plus EE in the MWM ($P = 0.345$) (fig. 5C), although there was a statistically significant group main effect based on escape latency between mice following the control condition plus SE and control condition plus EE ($P = 0.009$) (fig. 5C).

Finally, the swimming speeds of the mice in the MWM between all of these conditions were not different (data not shown). Considered together, these data suggest that EE may ameliorate the learning and memory impairment in the offspring mice that is caused by the sevoflurane anesthesia in the pregnant mice. These results are consistent with the findings that EE ameliorates cognitive deficits.^{10,11}

EE Mitigated the Sevoflurane-induced Increase in IL-6 Levels and Reduction in Levels of PSD-95 and Synaptophysin in the Brain Tissues of Offspring Mice

Given that EE can ameliorate the sevoflurane-induced learning and memory impairment, and synapse loss is the pathologic finding closely associated with cognitive dysfunction and dementia,²⁴ we determined the effects of EE

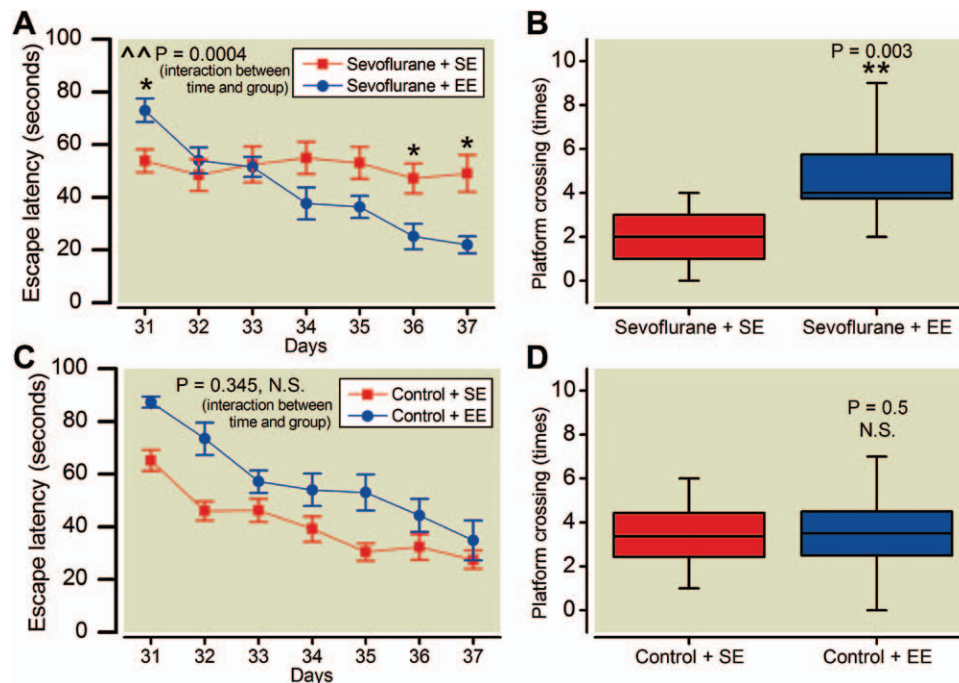


Fig. 5. Environmental enrichment (EE) attenuates the sevoflurane-induced learning and memory impairment in offspring mice. (A) Two-way ANOVA with repeated measurement analysis shows that there is a statistically significant interaction between time and group based on escape latency between mice following sevoflurane anesthesia plus SE and sevoflurane anesthesia plus EE ($^{^^}P = 0.0004$). Asterisk indicates that there is a statistically significant difference in the escape latency between the sevoflurane plus SE group and the sevoflurane plus EE group. (B) Mann–Whitney U test shows that the platform crossing time of mice swimming in the MWM following the sevoflurane anesthesia plus EE (median, 4; IQR, 3.75–4.25) is more than that of mice following the sevoflurane anesthesia plus SE (median, 1; IQR, 1–3; $^{**}P = 0.003$). (C) ANOVA shows that there is no statistically significant interaction between time and group based on escape latency of mice swimming in the MWM between the control condition plus SE and the control condition plus EE ($P = 0.345$, N.S.). (D) Mann–Whitney U test shows that there is no statistically significant difference in platform crossing time of mice swimming in the MWM between the control condition plus SE (median, 3; IQR, 2–4) and the control condition plus EE (median, 2; IQR, 2–4.25; $P = 0.499$, N.S.) ($n = 15$ per arm). EE = environmental enrichment; IQR = interquartile range; MWM = Morris water maze; N.S. = nonsignificant; SE = standard environment.

on the sevoflurane-induced alterations of IL-6 and synapse marker PSD-95 and synaptophysin levels in the brain tissues of offspring mice. IL-6 immunoblotting showed that sevoflurane anesthesia in pregnant mice increased IL-6 levels in the brain tissues of P31 offspring mice and that EE mitigated the effects (fig. 6A). The quantification of

the Western blot illustrated that the sevoflurane anesthesia increased IL-6 levels ($250 \pm 77\%$ vs. $100 \pm 25\%$, $P = 0.032$). EE mitigated the sevoflurane-induced increase in IL-6 levels ($89 \pm 17\%$ vs. $250 \pm 77\%$, $P = 0.016$) (fig. 6B). There was no significant difference in IL-6 levels between the control condition plus SE and control condition plus EE (fig. 6C).

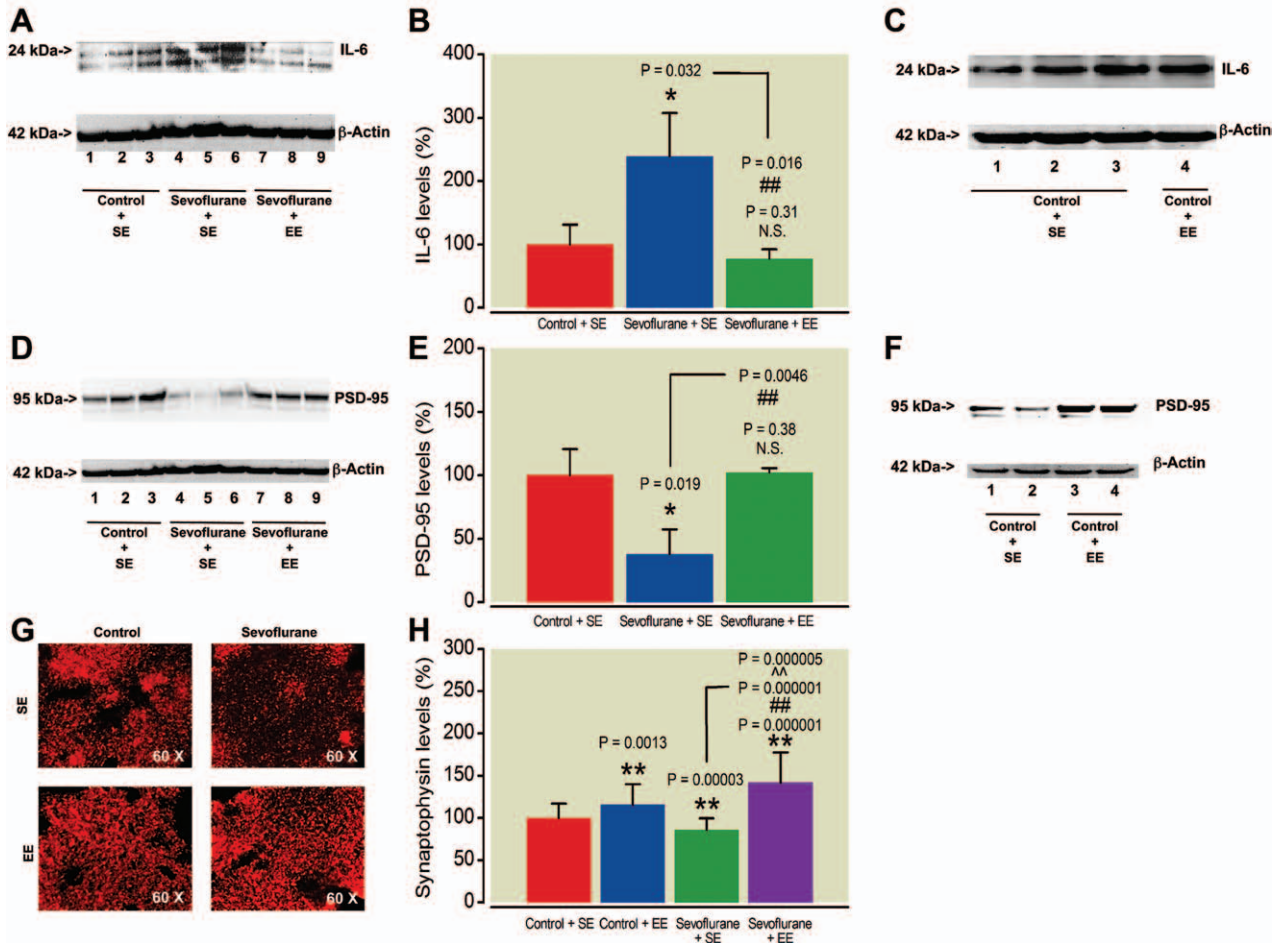


Fig. 6. EE mitigates the sevoflurane-induced increase in IL-6 levels, and reduction in levels of PSD-95 and synaptophysin in mouse brain tissues. (A) Sevoflurane anesthesia plus SE increases IL-6 levels as compared with the control condition plus SE. Sevoflurane anesthesia plus EE leads to lower levels of IL-6 as compared with the sevoflurane anesthesia plus SE. There is no significant difference in β -actin levels between the above treatments. (B) Quantification of the Western blot shows that the sevoflurane anesthesia plus SE increases IL-6 levels as compared with the control condition (* $P = 0.032$), and EE mitigates the sevoflurane-induced increase in IL-6 levels (## $P = 0.016$). (C) There is no significant difference in IL-6 levels between control plus EE and control plus SE. (D) Sevoflurane anesthesia plus SE reduces PSD-95 levels as compared with the control condition plus SE. Sevoflurane anesthesia plus EE leads to higher levels of PSD-95 as compared with the sevoflurane anesthesia plus SE. There is no significant difference in β -actin levels between the above treatments. (E) Quantification of the Western blot shows that the sevoflurane anesthesia plus SE reduces PSD-95 levels as compared with the control condition (* $P = 0.019$), and EE mitigates the sevoflurane anesthesia-induced reduction in PSD-95 levels (## $P = 0.0046$). (F) The PSD-95 level increases following control plus EE as compared with control plus SE. (G) Sevoflurane anesthesia plus SE leads to a reduction in synaptophysin levels in the brain tissues of offspring mice as compared with the control condition on the immunohistochemistry analysis. EE mitigates the sevoflurane-induced reduction in synaptophysin levels. The synaptophysin level increases following control plus EE as compared with control plus SE. (H) Quantification of the immunohistochemistry image shows that sevoflurane anesthesia plus SE (green bar) leads to a reduction in synaptophysin levels in the brain tissues of offspring mice as compared with the control condition plus SE (red bar, ** $P = 0.00003$). Both EE plus control condition (blue bar, ** $P = 0.0013$) and sevoflurane (purple bar, ** $P = 0.000001$) cause higher synaptophysin levels in the brain tissues of offspring mice as compared with the control condition (red bar). Finally, there is an interaction between EE and sevoflurane anesthesia that EE mitigates the sevoflurane-induced reduction in synaptophysin levels in the hippocampus of offspring mice (^^ $P = 0.00005$) ($n = 6$ per arm). EE = environmental enrichment; IL-6 = interleukin-6; PSD-95 = postsynaptic density-95; SE = standard environment.

Immunoblotting of PSD-95 showed that sevoflurane anesthesia in pregnant mice decreased PSD-95 levels in the brain tissues of P31 offspring mice, and EE mitigated the sevoflurane-induced reduction in PSD-95 levels in the brain tissues of offspring mice examined at P31 (fig. 6, D and E) ($102 \pm 3.23\%$ for sevoflurane plus EE *vs.* $38 \pm 19.39\%$ for sevoflurane plus SE *vs.* $100 \pm 20.6\%$ for control plus SE, $P = 0.0046$). There was a higher level of PSD-95 in the control plus EE as compared with the control plus SE (fig. 6F). Immunohistochemistry staining showed that sevoflurane anesthesia in pregnant mice decreased synaptophysin levels in the brain tissues of P31 offspring mice as compared with the control condition (fig. 6, G and H) ($77 \pm 17\%$ *vs.* $100 \pm 21\%$, $P = 0.00003$), and EE mitigated the sevoflurane-induced reduction in synaptophysin levels in the brain tissues of offspring mice at P31 (fig. 6, G and H) ($77 \pm 17\%$ for sevoflurane plus SE *vs.* $141 \pm 36.44\%$ for sevoflurane plus EE, $P = 0.000001$). Collectively, these results suggest that EE may rescue the sevoflurane-induced neuroinflammation and synaptic loss, leading to amelioration of the sevoflurane-induced learning and memory impairment.

Discussion

The widespread and growing use of anesthesia in the developing brain makes its safety a major health issue of interest¹ (reviewed in Sun³). This has become a matter of even greater concern with the evidence that anesthesia and surgery may induce neurodevelopmental impairment in children and that anesthetics are neurotoxic in young animals (reviewed in Sun³). Many pregnant women in the United States have nonobstetric surgery and fetal intervention procedures under anesthesia each year.^{6,32} We therefore determined whether anesthesia with sevoflurane in pregnant mice could induce detrimental effects in fetal mice and offspring mice. We chose sevoflurane in the studies because sevoflurane is currently the most commonly used inhalation anesthetic, although sevoflurane might be less toxic than isoflurane.³³ Moreover, the effects of isoflurane in pregnant mice on behavioral changes in offspring mice have been determined.⁷

Sevoflurane anesthesia in pregnant mice induced learning and memory impairment in offspring mice at P31 (fig. 1). The same sevoflurane anesthesia induced acute neurotoxicity as evidenced by the increased levels of proinflammatory cytokine IL-6, reduced levels of synapse marker PSD-95, and caspase-3 activation in the brain tissues of fetal mice (fig. 2). The sevoflurane anesthesia in pregnant mice also increased IL-6 levels and decreased levels of PSD-95 and synaptophysin in the brain tissues of P31 offspring mice (fig. 6). Proinflammatory cytokine IL-6 can be released by the microglia cells during their activation, fueling neuroinflammation and leading to cognitive dysfunction^{34–36} and mild cognitive impairment³⁷ in medical and surgical patients.³⁸ PSD-95 is a postsynaptic marker.^{39,40} The reduction of PSD-95 has been shown to be associated with decreases in synapse number or

synaptic loss, a part of the mechanisms underlying Alzheimer's disease-associated dementia and impairment of learning and memory^{23,24} (reviewed in Querfurth and LaFerla²⁵). In the *in vitro* studies, IL-6 antibody attenuated the sevoflurane-induced reduction in PSD-95 levels, which suggests that the sevoflurane-induced increase in IL-6 levels may lead to reduction in PSD-95 levels. Considered together, these data suggest that sevoflurane may increase neuroinflammation (*e.g.*, increase in IL-6 levels), which causes a reduction in synapse number, leading to learning and memory impairment. Future studies, including determination of whether anti-inflammatory medicine(s) can rescue the sevoflurane-induced synaptic loss and impairment of learning and memory, are warranted to further test this hypothesis.

IL-6 antibody itself reduced PSD-95 levels in the primary neurons (fig. 4, A and B). This could be attributable to IL-6 antibody only mitigating the effects associated with IL-6 accumulation (*e.g.*, mitigating a reduction in PSD-95 levels). In the absence of IL-6 accumulation, however, the IL-6 antibody may have nonspecific effects. The exact mechanisms of these effects remain to be determined.

The sevoflurane anesthesia induced caspase-3 activation, increases in IL-6 levels, and a reduction in PSD-95 levels 2 h after the anesthesia in the brain tissues of fetal mice, which occurred more rapidly than in the brain tissues of adult mice (6 h).⁹ These data suggest that fetal mice might be more vulnerable to neurotoxicity than adult mice.

The mechanisms by which anesthetics induce neuroinflammation remain to be determined. Anesthetics have been shown to increase cytosolic calcium levels.^{41–44} The elevation of cytosolic calcium is associated with increased levels of proinflammatory cytokines,⁴⁵ potentially through activation of the nuclear factor- κ B signaling pathway.^{46–49} Activated nuclear factor- κ B translocates to the nucleus, where it binds to the promoter region of multiple genes, including cytokine genes.^{46–50} Thus, future studies will include determining whether anesthetics can increase calcium levels in neurons and microglia cells to trigger generation of proinflammatory cytokine (*e.g.*, IL-6) through the nuclear factor- κ B signaling pathway.

EE, consisting of social interaction and novel stimulation, may result in various neuroplastic changes, including increased hippocampal neurons,⁵¹ improved spatial abilities and enhanced dendritic growth,⁵² increased neurogenesis,⁵³ and increased nerve growth factor⁵⁴ after brain injury. EE has also been shown to improve learning and memory function.^{10–12} We found that EE ameliorated the sevoflurane-induced learning and memory impairment, and mitigated the sevoflurane-induced increase in IL-6 levels and reduction in synaptic markers (figs. 5 and 6). These results suggest that EE may rescue the sevoflurane-induced neuroinflammation and synaptic loss, leading to improvement of the sevoflurane-induced impairment of learning and memory.

This study has several limitations. First, we did not determine the long-term (*e.g.*, 3–6 months) effects of sevoflurane

anesthesia on learning and memory function; however, the current findings were able to illustrate the effects of sevoflurane anesthesia on behavioral changes (*e.g.*, spatial learning and memory impairment) and the potential underlying cellular mechanisms (*e.g.*, caspase activation, increases in IL-6 levels, and synaptic loss). Second, we focused on only one proinflammatory cytokine, IL-6, in the experiments because IL-6 has been shown to contribute to learning and memory impairment. Sevoflurane anesthesia in pregnant mice may also induce other changes (*e.g.*, microglia activation) in the brain tissues of fetal mice consistent with neuroinflammation and need to be investigated in future studies.

It is unknown whether the anesthesia itself contributes to the clinically observed cognitive impairment, or whether the need for anesthesia/surgery is a marker for other unidentified factors that contribute. To either rule in or rule out the contribution of anesthesia, we will determine whether anesthesia alone can induce neuroinflammation and learning and memory in young mice. Our established preclinical mouse model will be used to determine whether anesthesia alone can induce detrimental effects (*e.g.*, learning and memory impairment, and neuroinflammation) in young animals (developing brain), to reveal the underlying mechanisms and to explore targeted interventions. Moreover, nociceptive stimuli such as surgical incision and pain with formalin have been shown to potentiate the anesthesia-induced neurotoxicity and neurobehavioral deficits.⁵⁵ Future studies may also include assessing whether other perioperative factors (*e.g.*, hypothermia and hypotension) can potentiate anesthesia-induced neurotoxicity and neurobehavioral deficits.

In conclusion, clinically relevant sevoflurane anesthesia in pregnant mice can induce acute neurotoxicity, including increases in IL-6 levels and reductions in synapse marker PSD-95 and caspase-3 activation, in the brain tissues of fetal mice. The same sevoflurane anesthesia in pregnant mice also induced long-term detrimental effects, including reductions in synapse marker PSD-95 and synaptophysin, and impairment of learning and memory in offspring mice at 31 days after birth. These results suggest that sevoflurane anesthesia in pregnant mice may induce neuroinflammation, caspase activation, and synaptic loss, leading to learning and memory impairment. Finally, EE may be able to rescue the sevoflurane-induced learning and memory impairment by mitigating the sevoflurane-induced synaptic loss and neuroinflammation. These findings will promote more research in anesthesia neurotoxicity in the developing brain, especially mechanistic studies.

The authors thank Hui Zheng, Ph.D. (Assistant Professor of Medicine, Biostatistics Center, Massachusetts General Hospital and Harvard Medical School, Boston, Massachusetts), for advice and help in the data analysis of the studies.

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