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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. We set out to assess the effects of anesthetic sevoflurane in pregnant mice on neurotoxicity and learning and memory in fetal and offspring mice.

Methods—Pregnant mice (gestation stage 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 (postnatal day 31), and the primary neurons were harvested and subjected to

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Summary Statement: Sevoflurane anesthesia in pregnant mice induced increases in interleukin-6 levels, reductions in synaptic marker postsynaptic density-95 and synaptophysin levels, caspase-3 activation, and learning and memory impairment in fetal and offspring mice.

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Western blot and immunhistochemistry to assess interleukin-6, 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% ± 50.98 (mean ± SD) *vs.* 100% ± 54.12, $P = 0.026$], and reduced postsynaptic density-95 (61% ± 13.53 *vs.* 100% ± 10.08, $P = 0.036$) and synaptophysin levels in fetal and offspring mice. The sevoflurane anesthesia impaired learning and memory in offspring mice at postnatal day 31. 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.

Conclusion—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.

Introduction

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 community[1] and the public[2]. Anesthesia and surgery may induce neurodevelopment impairment and cognitive dysfunction in children [reviewed in[3]]. In preclinical studies, anesthesia has been shown to induce neurotoxicity and learning and memory impairment in young animals [[4], reviewed in[5]].

Each year over 75,000 pregnant women in the United States have non-obstetric surgery and fetal intervention procedures under anesthesia[6]. Anesthesia neurotoxicity in the developing brain could happen 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 and thus cross placenta easily; (3) moreover, uterine exposure to ethanol, valproic acid, and anesthetic isoflurane have been shown to induce behavioral abnormalities in adulthood [[7], reviewed in[8]]. It remains largely to be determined, however, whether anesthesia in pregnant mice can induce neurotoxicity in fetal mice (the developing brain), and 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 anesthesia-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 gestation stage day 14 (G14), the pregnant mice were randomly assigned to an anesthesia or 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 (Ohmeda, Tewksbury, MA). 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 (about 1.1 minimum alveolar concentration) for two hours in mice was employed 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, an standard deviation (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 IL-6 levels in brain tissues, 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 90% or larger power to detect the differences using two-sample *t*-test with 5% type 1 error.

Mouse primary neurons

The protocol was approved by the Massachusetts General Hospital Standing Committee (Boston, Massachusetts) 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 interleukin (IL)-6 antibody (10 μg/ml) was administrated to the neurons one hour before the sevoflurane treatment. The neurons were harvested at the end of the anesthesia and were subjected to Western blot analysis.

Brain tissue harvest and protein level quantification

Immediately following 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, 0.5% Nonidet P-40) plus protease inhibitors (1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin A) as described in our previous studies[15]. The lysates were collected, centrifuged at 12,000 rapid per minute for 15 min, and quantified for total proteins with bicinchoninic acid protein assay kit (Pierce, 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, Danvers, MA) was used to detect PSD-95 (95 kDa). A caspase-3 antibody (1:1000 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 using 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. 100% of protein level changes refer to control levels for the purpose of comparison to experimental conditions.

The quantification of Western blot was based not only on the images presented in figures, but also the images not presented in the figures in order 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 μm frozen sections from the mouse brain hemispheres were used for the immunohistochemistry staining[17]. The sections were incubated with the primary antibody synaptophysin (Sigma, 1:500) 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 X). We used the mouse hippocampus in the studies of immunohistochemistry density quantification to determine whether sevoflurane anesthesia can induce neurotoxicity in the hippocampus. The photos were taken and an investigator who was blind to the experimental design counted the density of synaptophysin using Image J Version 1.38 (National Institutes of Health, Bethesda, MD)[17].

Morris Water Maze (MWM)

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 15-centimeter 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 pool. Water was kept at 20 °C and opacified with titanium dioxide. The P31 offspring mice were tested in the MWM four trials 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, P.R. 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 cross 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 returning to its regular cage.

Environmental enrichment

The EE in the current experiment was created in a large cage (70 × 70 × 46 centimeter) that included 5 – 6 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 everyday for two hours before delivery. The pregnant mice delivered offspring mice at G21. Then, the mother and the babies were put in the EE again everyday for two hours from P4 to P30. The objects were changed two to three times a week to provide newness and challenge.

Statistics

The nature of the hypothesis testing was two-tailed. Data were expressed as mean ± SD. The data for platform crossing time were not normally distributed, thus were expressed as median and interquartile range (IQR). The number of samples varied from 6 to 15, and the samples were normally distributed except 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 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 Bonferroni method (with 7 tests, and 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. 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 test was used to determine the difference between the sevoflurane and control conditions on platform crossing times. *P* values less than 0.05 (*, # and ^) and 0.01 (**, ## and ^^) were considered statistically significant. SAS software version 9.2 (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 gestation stage day 14 (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 non-significant difference in the platform crossing times between the control condition and the sevoflurane anesthesia (fig. 1B, $P = 0.051$, Mann-Whitney test,

sevoflurane: median = 1 and IQR = 1 to 3 *versus* control: median = 2 and IQR = 2 to 4.5). There was no statistically significant difference in mouse swimming speed between the sevoflurane anesthesia and the control group (data not shown). Taken 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 of 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 anesthesia induced more visible bands representing IL-6 as compared to the control condition (fig. 2A). There was no statistically 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 to the control condition: 256% ± 50.98 *versus* 100% ± 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 to 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 to the control condition: 61% ± 13.53 *versus* 100% ± 10.08, * $P = 0.036$ (fig. 2D). Finally, we assessed effects of the sevoflurane anesthesia in pregnant mice 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 FL 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 FL-caspase-3, revealed that the sevoflurane anesthesia in pregnant mice induced caspase-3 activation as compared to control condition (fig. 2F): ** $P = 0.0075$, 198% ± 35 *versus* 100% ± 21.

Taken 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.

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 IL-6 and 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% ± 14.00 *versus* 100% ± 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 compared to the control condition (fig. 4A). The treatment with sevoflurane reduced PSD-95 levels as compared to 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% ± 4.58 *vs.* 100% ± 19, ** $P = 0.001$) and IL-6 antibody mitigated the sevoflurane anesthesia-induced reduction in PSD-95 levels: 36% ± 8.33 *versus* 20% ± 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. 4A and 4B).

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 anesthesia-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 anesthesia-induced increases in escape latency of mice swimming in the MWM (^^ $P = 0.0004$, fig. 5A). Sevoflurane anesthesia plus EE also increased the platform crossing times of mice in the MWM as compared to sevoflurane anesthesia plus SE (** $P = 0.003$, Mann-Whitney test, fig. 5B, sevoflurane plus EE: median = 4 and IQR = 3.75 to 4.25 versus sevoflurane plus SE: median = 1 and IQR = 1 to 3). EE alone did not alter escape latency nor platform crossing times of mice swimming in the MWM (fig. 5C and 5D). 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 standard environment (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 ($P = 0.009$), figure 5C.

Finally, the mice swimming speed in the MWM among all of these conditions were not different (data not shown). Taken 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 EE can ameliorate the sevoflurane anesthesia-induced learning and memory impairment, and synapse loss is the pathological finding closely associated with cognitive dysfunction and dementia[24], we determined the effects of EE on the sevoflurane anesthesia-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 EE mitigated the effects (fig. 6A). The quantification of the Western blot illustrated that the sevoflurane anesthesia increased IL-6 levels: 250% ± 77 *versus* 100% ± 25, * $P = 0.0032$. EE mitigated the sevoflurane-induced increase in IL-6 levels: 89% ± 17 *versus* 250% ± 77, # $P = 0.016$ (fig. 6B). There was no statistically significant difference in IL-6 levels between the control condition plus SE and control condition plus EE (fig. 6C). 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 anesthesia-induced reduction in PSD-95 levels in the brain tissues of offspring mice examined at P31 (fig. 6D and 6E, ## $P = 0.0046$): 102 ± 3.23 (sevoflurane plus EE) *versus* 38% ± 19.39 (sevoflurane plus SE) *versus* 100% ± 20.6 (control plus SE). There was a higher level of PSD-95 in the control plus EE as compared to 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 to the control condition (fig. 6G and 6H, ** $P = 0.00003$): 77% ± 17 *versus* 100% ± 21, EE mitigated the sevoflurane anesthesia-induced reduction in synaptophysin levels in the brain tissues of offspring mice at P31 (fig. 6G and 6H, ## $P = 0.000001$): 77% ± 17 (sevoflurane plus SE) *versus* 141% ± 36.44 (Sevoflurane plus EE). Collectively, these results suggest that EE may rescue the sevoflurane anesthesia-induced neuroinflammation and synaptic loss, leading to amelioration of the sevoflurane anesthesia-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 interest [[1], reviewed in[3]]. This has become a matter of even greater concern with the evidence that anesthesia and surgery may induce neurodevelopment impairment in children, and that anesthetics are neurotoxic in young animals [reviewed in[3]]. 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 impairment (MCI)[37] 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 AD-associated dementia and impairment of learning and memory [[23,24], reviewed in[25]]. 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. Taken 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 antiinflammation medicine(s) can rescue the sevoflurane anesthesia-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. 4A and 4B). This could be due 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 nuclear factor-κB signaling pathway[46–49]. Activated nuclear factor-κB translocates to the nucleus where it binds to the promoter region of multiple genes, including cytokine genes[46–50]. Thus, the 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 nuclear factor-κB 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 factor[54] after brain injury. EE has also been shown to improve learning and memory function[10–12]. We found that EE ameliorated the sevoflurane anesthesia-induced learning and memory impairment, and mitigated the sevoflurane anesthesia-induced increase in IL-6 levels and reduction in synaptic markers (fig. 5 and 6). These results suggest that EE may rescue the sevoflurane anesthesia-induced neuroinflammation and synaptic loss, leading to improvement of the sevoflurane anesthesia-induced impairment of learning and memory.

The studies have several limitations. First, we did not determine the long-term (*e.g.*, 3 to 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 only focused on 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, which need to be investigated in future studies.

It is unknown whether the anesthesia itself contributes to the clinically observed cognitive impairment, or the need for anesthesia/surgery is a marker for other unidentified factors that contribute. In order 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 anesthetic 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 anesthetic-induced neurotoxicity and neurobehavioral deficits[55]. The 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, 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 the 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 anesthesia-induced learning and memory impairment by mitigating the sevoflurane anesthesia-induced synaptic loss and neuroinflammation. These findings will promote more research in anesthesia neurotoxicity in the developing brain, especially mechanistic studies.

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Final Boxed Summary Statement

What we know about this topic

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

What new information this study provides

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

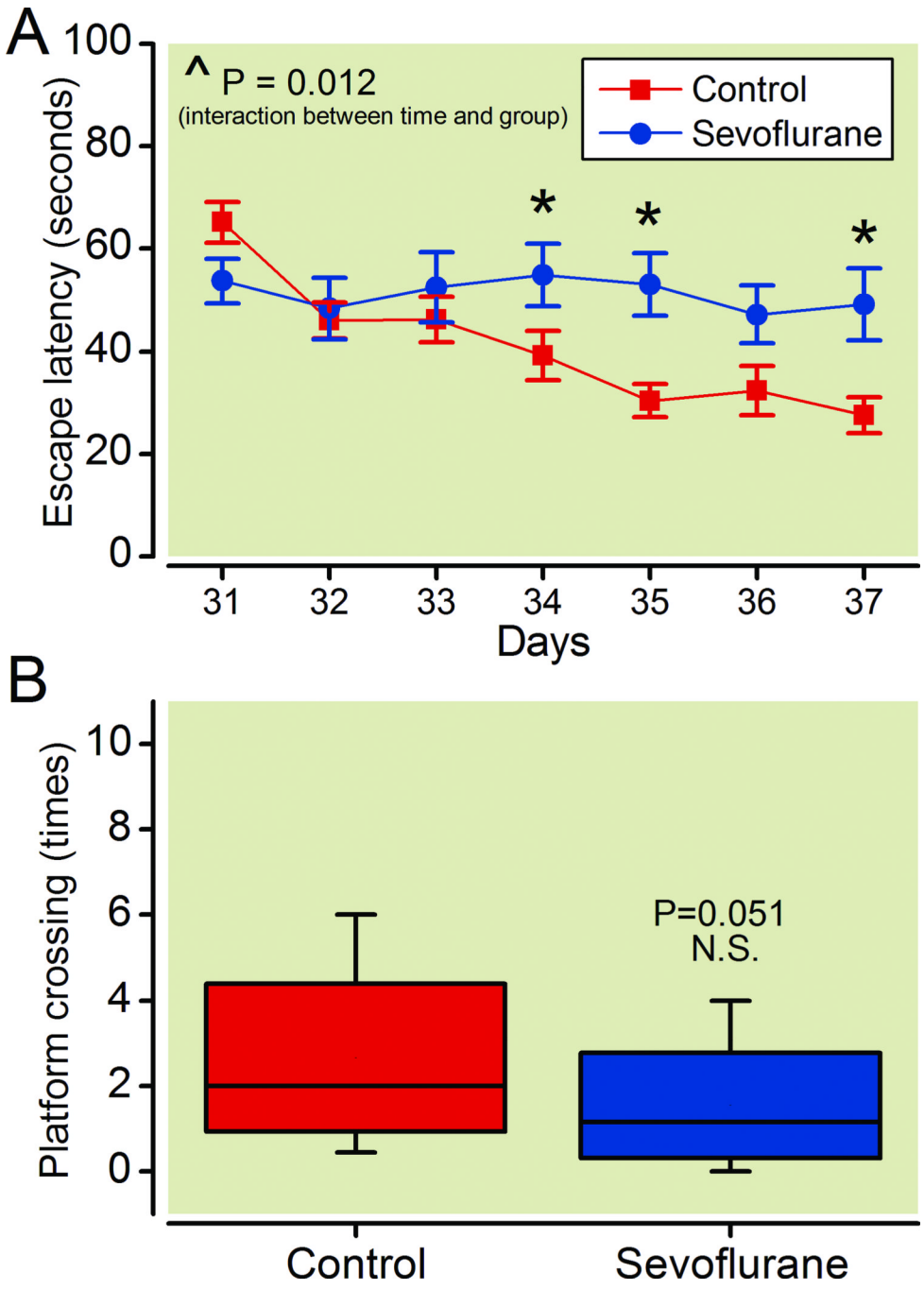

Figure 1. Anesthesia with 2.5% sevoflurane for two hours in pregnant mice at gestation stage day 14 (G14) induces learning and memory impairment in offspring mice tested at postnatal day (P)31

A. Sevoflurane anesthesia increases escape latency time of mice swimming in the Morris Water Maze (MWM) as compared to the control condition. Two way ANOVA with repeated measurement analysis shows that there is 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 (^ P = 0.012). * 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 as compared to the control condition (P = 0.051, Mann-Whitney test, median = 1 and IQR = 1 to 3 versus control: median = 2 and IQR = 2 to 4.5). G, gestation stage day; P, postnatal day; MWM, Morris Water Maze; ANOVA, analysis of variance; IQR, interquartile range. n = 15.

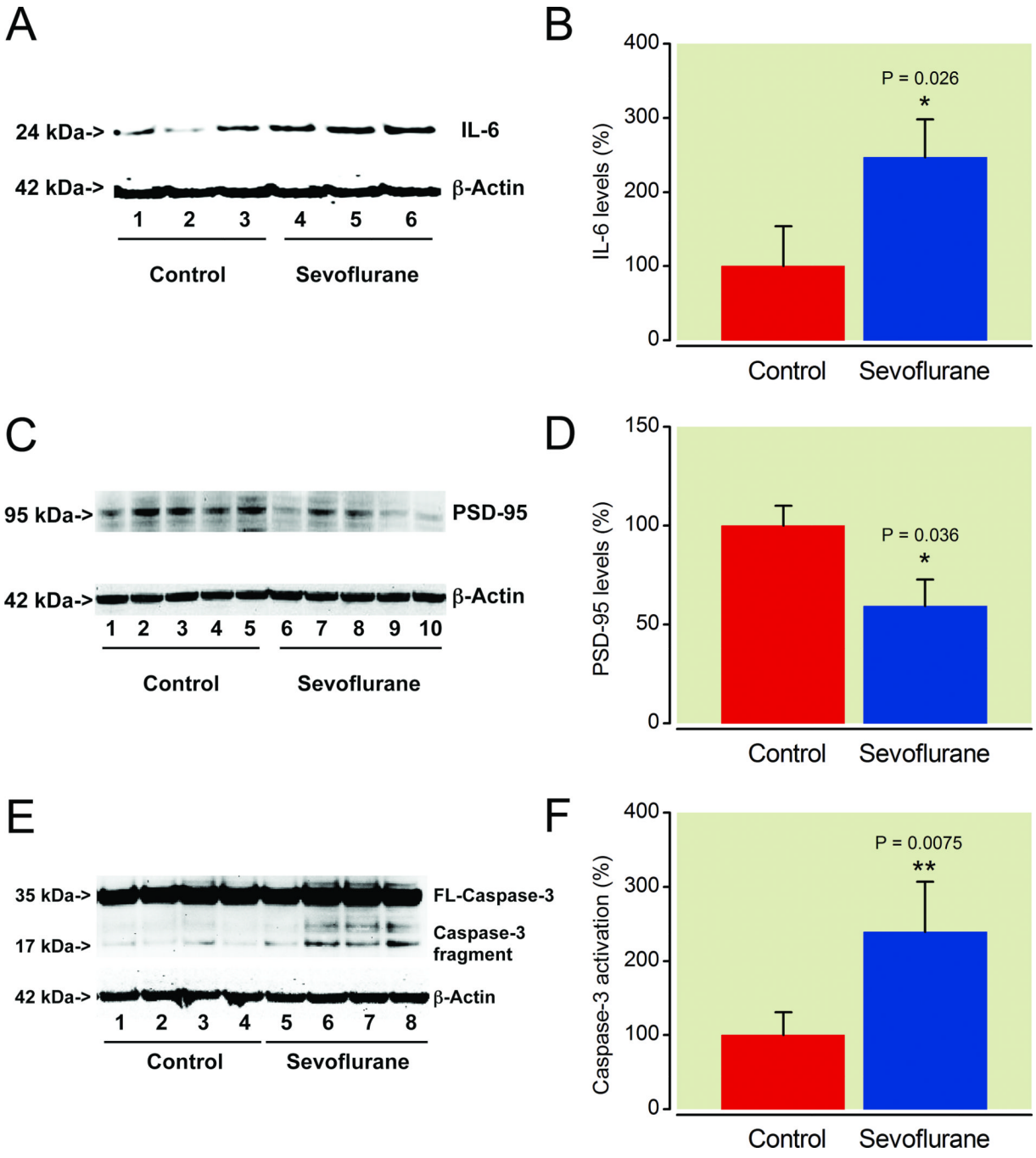

Figure 2. Anesthesia with 2.5% sevoflurane for two hours in pregnant mice at G14 increases IL-6 levels, decreases PSD-95 levels and induce caspase-3 activation in the brain tissues of fetal mice

A. Sevoflurane anesthesia increases IL-6 levels in the brain tissues of fetal mice as compared to the control condition in 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 to the control condition (* P = 0.026). **C**. The sevoflurane anesthesia reduces PSD-95 levels in the brain tissues of fetal mice as compared to the control condition in 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 to the control condition (* P = 0.036). **E.** The sevoflurane anesthesia induces caspase-3 activation in the brain tissues of fetal mice as compared to the control condition in 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. **F.** Quantification of the Western blot shows that sevoflurane anesthesia induces caspase-3 activation in the mouse brain tissues as compared to the control condition (** P = 0.0075). G, gestation stage day; IL-6, interleukin-6; PSD, postsynaptic density-95; FL, full length. n = 6.

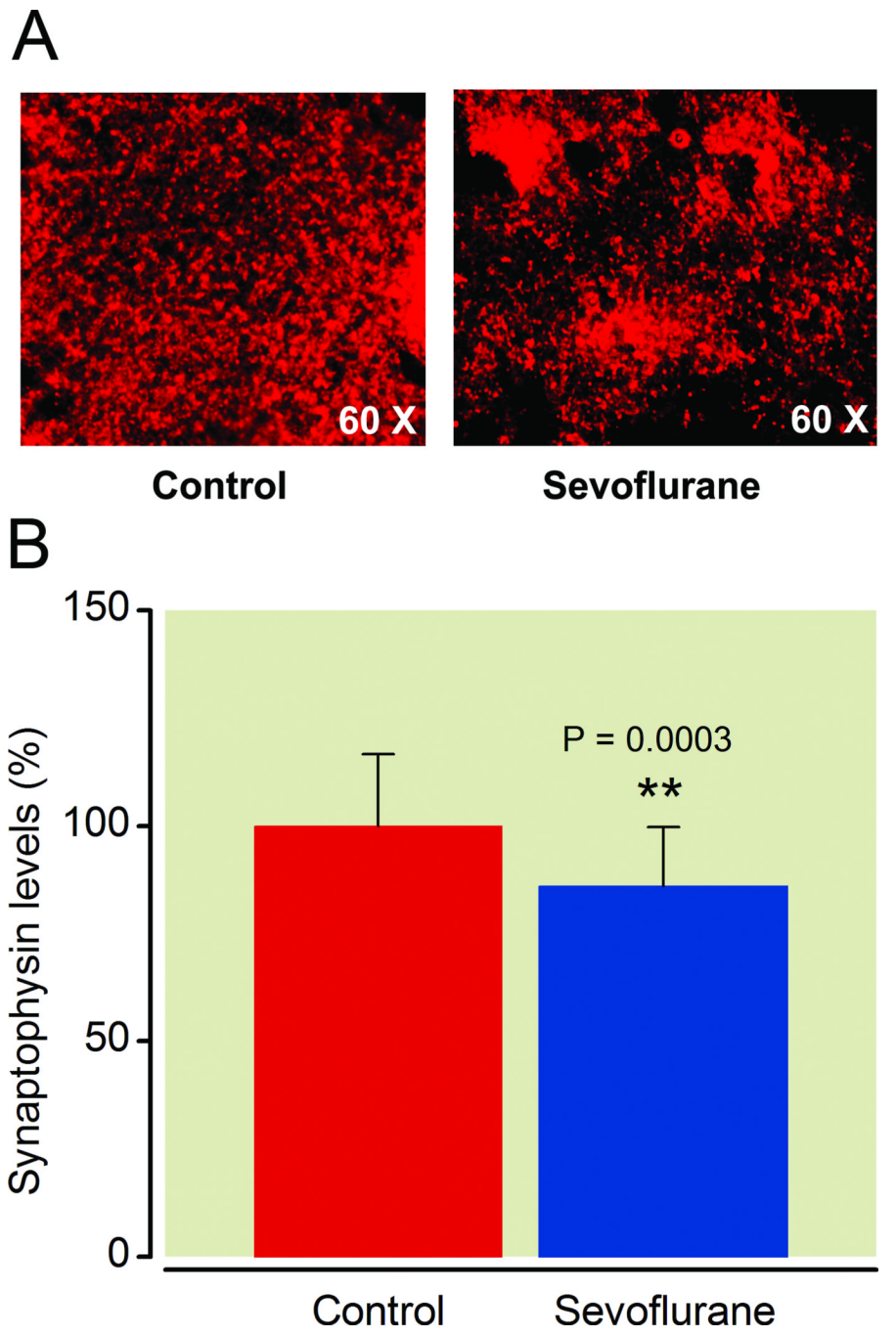

Figure 3. Anesthesia with 2.5% sevoflurane for two hours in pregnant mice at G14 decreases synaptophysin levels in the hippocampus of offspring mice examined at P31

A. Sevoflurane anesthesia decreases synaptophysin levels in the brain tissues of offspring mice as compared to the control condition in immunohistochemistry analysis. **B**. Quantification of the immunohistochemistry image shows that sevoflurane anesthesia decreases synaptophysin levels in the mouse brain tissues as compared to the control condition (** P = 0.0003). G, gestation stage day; P, postnatal day. n = 6.

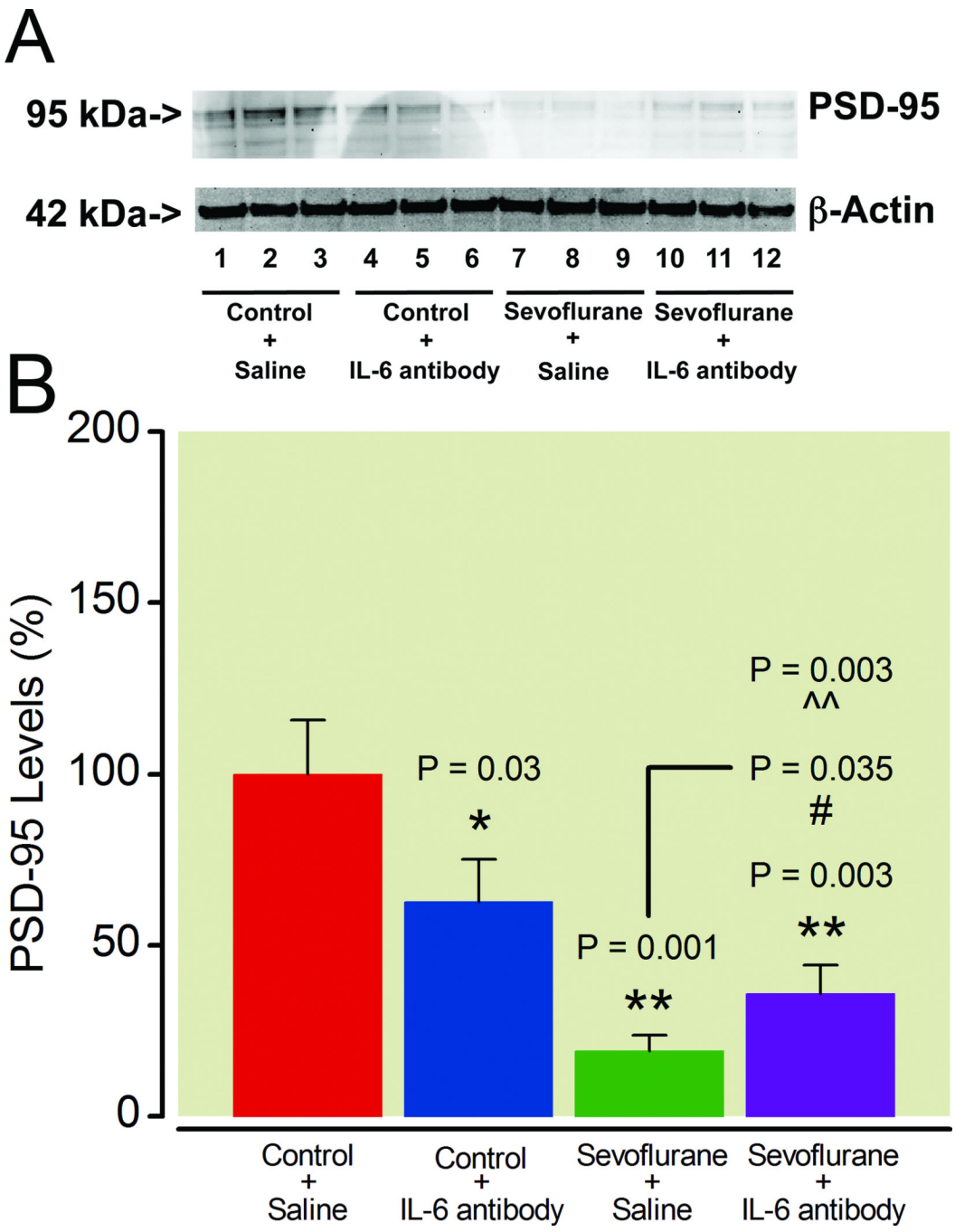

Figure 4. IL-6 antibody mitigates the sevoflurane-induced reduction in PSD-95 levels in mouse primary neurons

A. Treatment with 4.1% sevoflurane for six hours (lanes 7 to 9) reduces PSD-95 levels as compared to the control condition (lanes 1 to 3). The treatment of IL-6 antibody (lanes 10 – 12) mitigates the sevoflurane-induced reduction in PSD-95 levels. There is no statistically 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 to 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 to treatment with sevoflurane plus

saline (# P = 0.035). 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 (^^ P = 0.003). IL-6, interleukin-6; PSD-95, postsynaptic density-95; ANOVA, analysis of variance. n = 6.

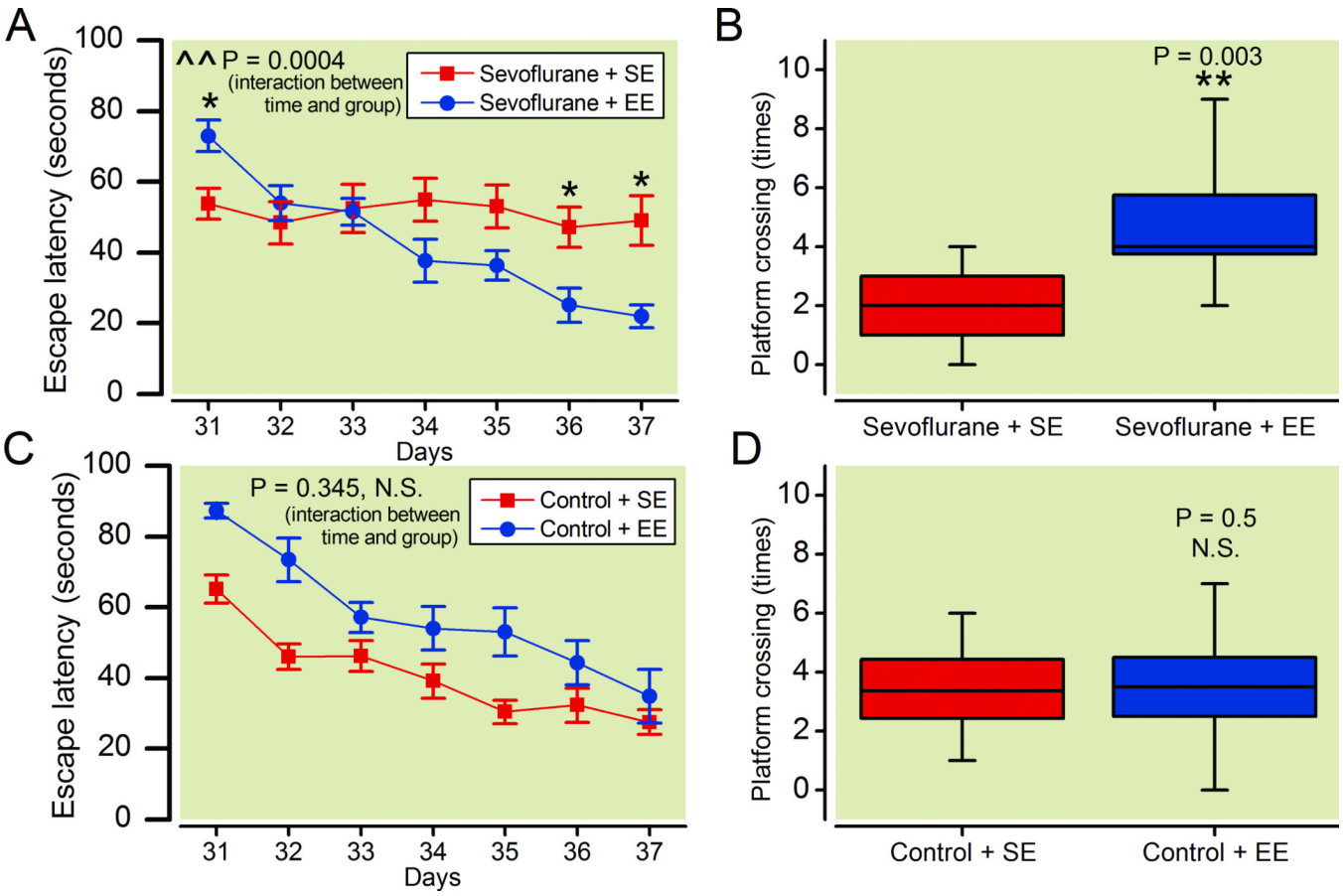

Figure 5. Environmental enrichment (EE) attenuates the sevoflurane-induced learning and memory impairment in offspring mice

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). * 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 test shows that the platform crossing time of mice swimming in the MWM following the sevoflurane anesthesia plus EE is longer than that of mice following the sevoflurane anesthesia plus SE (** P = 0.003, sevoflurane plus EE: median = 4 and IQR = 3.75 to 4.25 versus sevoflurane plus SE: median = 1 and IQR = 1 to 3). **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 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 and the control condition plus EE (P = 0.499, N.S., control condition plus EE: median = 3 and IQR = 2 to 4 versus control condition plus SE: median = 2 and IQR = 2 to 4.25). MWM, Morris Water Maze; ANOVA, analysis of variance; IQR, interquartile range; SE, standard environment; EE, environmental enrichment. n = 15.

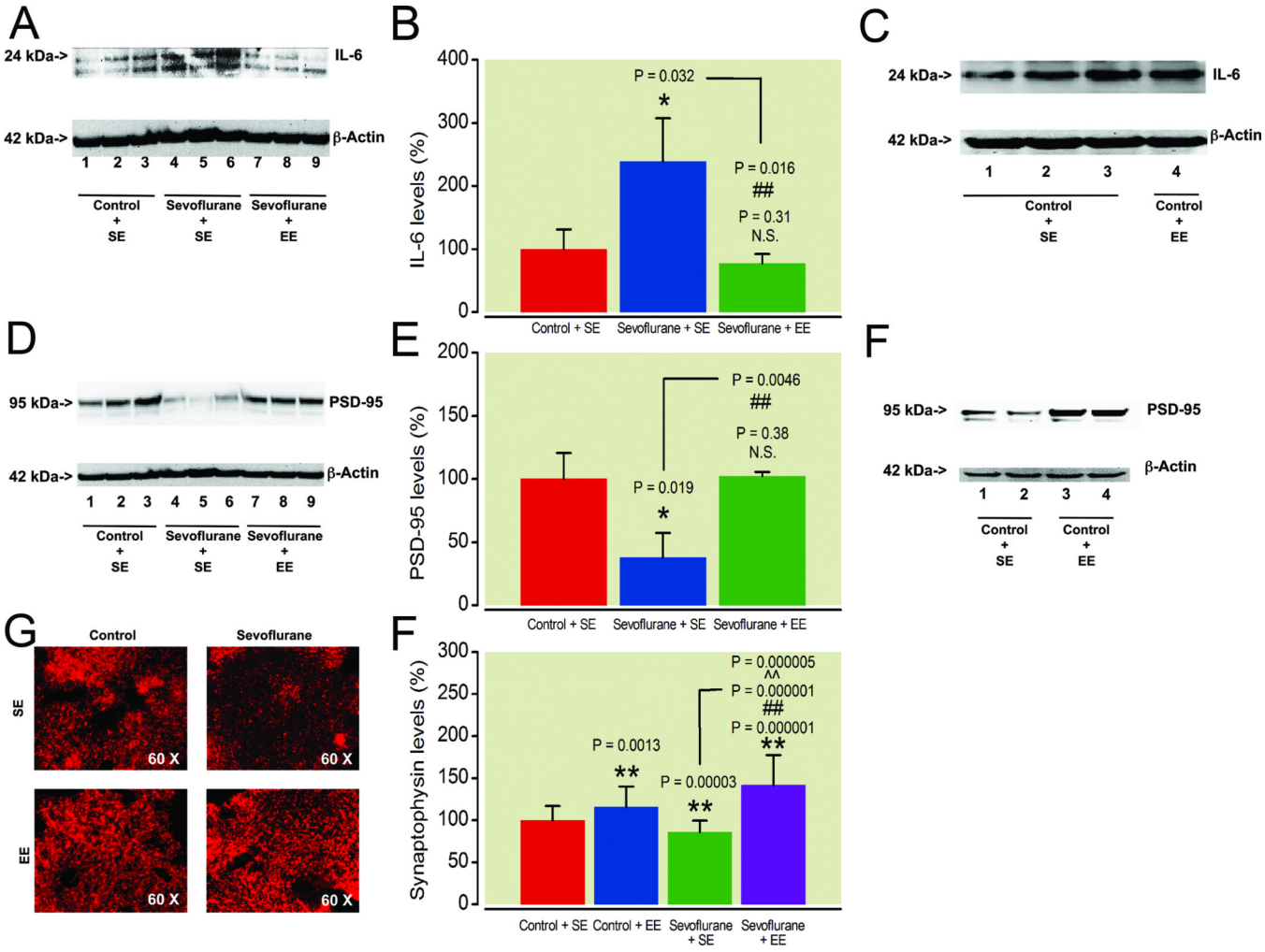

Figure 6. EE mitigates the sevoflurane-induced increase in IL-6 levels, and reduction in levels of PSD-95 and synaptophysin in mouse brain tissues

A. Sevoflurane anesthesia plus SE increases IL-6 levels as compared to the control condition plus SE. Sevoflurane anesthesia plus EE leads to lower levels of IL-6 as compared to the sevoflurane anesthesia plus SE. There is no statistically significant difference in β-Actin levels among the above treatments **B.** Quantification of the Western blot shows that the sevoflurane anesthesia plus SE increases IL-6 levels as compared to the control condition (* P = 0.032), and EE mitigates the sevoflurane anesthesia-induced increase in IL-6 levels (## P = 0.016). **C.** There is no statistically 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 to the control condition plus SE. Sevoflurane anesthesia plus EE leads to higher levels of PSD-95 as compared to the sevoflurane anesthesia plus SE. There is no statistically significant difference in β-Actin levels among the above treatments **E.** Quantification of the Western blot shows that the sevoflurane anesthesia plus SE reduces PSD-95 levels as compared to 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 to control plus SE. **G.** Sevoflurane anesthesia plus SE leads to a reduction in synaptophysin levels in the brain tissues of offspring mice as compared to the control condition in the immunohistochemistry analysis. EE mitigates the sevoflurane anesthesia-induced reduction in synaptophysin levels. The

synaptophysin level increases following control plus EE as compared to control plus SE. **H.** Quantification of the immunohistochemistry image shows that sevoflurane anesthesia plus SE leads to a reduction in synaptophysin levels in the brain tissues of offspring mice as compared to the control condition plus SE (black bar, **P = 0.00003). Both EE plus control condition (net bar, ** P = 0.0013) or sevoflurane (gray bar, ** P = 0.000001) cause higher synaptophysin levels in the brain tissues of offspring mice as compared to the control condition (white bar). Finally, there is an interaction between EE and sevoflurane anesthesia that EE mitigates the sevoflurane anesthesia-induced reduction in synaptophysin levels in the hippocampus of offspring mice (^^ P = 0.00005). PSD-95, postsynaptic density-95; IL-6, interleukin-6; SE, standard environment; EE, environmental enrichment. n = 6.