Selective Anesthesia-induced Neuroinflammation in Developing Mouse Brain and Cognitive Impairment

Xia Shen, M.D., Ph.D.,* Yuanlin Dong, M.D., M.S.,† Zhipeng Xu, M.D., Ph.D.,‡ Hui Wang, M.D., Ph.D.,§ Changhong Miao, M.D., Ph.D.,‖ Sulpicio G. Soriano, M.D.,# Dandan Sun, M.D., Ph.D.,** Mark G. Baxter, Ph.D.,†† Yiying Zhang, M.D., M.S.,‡ Zhongcong Xie, M.D., Ph.D.‡‡

What We Already Know about This Topic

- Children with multiple exposures to anesthesia and surgery at an early age may be at increased risk of cognitive impairment

What This Article Tells Us That Is New

- Using an animal model with sevoflurane and desflurane, the authors demonstrated anesthesia-induced cognitive impairment, and it was associated with neuroinflammatory changes. However, development of cognitive impairment was dependent on developmental stage, anesthetic agent, and number of exposures

Abstract

Background: Recent population studies have suggested that children with multiple exposures to anesthesia and surgery at an early age are at an increased risk of cognitive impairment. The authors therefore have established an animal model with single *versus* multiple exposures of anesthetic(s) in young *versus* adult mice, aiming to distinguish the role of different types of anesthesia in cognitive impairment.

Methods: Six- and 60-day-old mice were exposed to various anesthesia regimens. The authors then determined the effects of the anesthesia on learning and memory function, levels of proinflammatory cytokine interleukin-6 and tumor necrosis factor-α in brain tissues, and the amount of ionized calcium-binding adaptor molecule 1–positive cells, the marker of microglia activation, in the hippocampus.

Results: In this article, the authors show that anesthesia with 3% sevoflurane for 2 h daily for 3 days induced cognitive impairment and neuroinflammation (*e.g.*, increased interleukin-6 levels, 151 ± 2.3% [mean ± SD] *vs.* 100 ± 9.0%, $P = 0.035$, n = 6) in young but not in adult mice. Anesthesia with

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* Research Fellow, Geriatric Anesthesia Research Unit, Department of Anesthesia, Critical Care and Pain Medicine, Massachusetts General Hospital and Harvard Medical School, Charlestown, Massachusetts; Attending Anesthesiologist, Shanghai Eye, Ear, Nose, and Throat Hospital, Fudan University, Shanghai, People's Republic of China. † Senior Research Technologist, ‡ Research Fellow, ‡‡ Associate Professor, Geriatric Anesthesia Research Unit, Department of Anesthesia, Critical Care and Pain Medicine, Massachusetts General Hospital and Harvard Medical School, Charlestown, Massachusetts. § Research Fellow, Geriatric Anesthesia Research Unit, Department of Anesthesia, Critical Care and Pain Medicine; Massachusetts General Hospital and Harvard Medical School, Charlestown, Massachusetts; Attending Anesthesiologist, Department of Anesthesia, Beijing Chaoyang Hospital, Capital Medical University, Beijing, People's Republic of China. ‖ Professor, Fudan University Shanghai Cancer Center, Department of Anesthesia, Shanghai Medical College, Fudan University, Shanghai, People's Republic of China. # Professor, Department of Anesthesiology, Perioperative and Pain Medicine, Boston Children's Hospital, Harvard Medical School, Boston, Massachusetts. ** Professor, Department of Neurology, University of Pittsburgh, Pittsburgh, Pennsylvania. †† Professor, Friedman Brain Institute and Department of Neuroscience, Mount Sinai School of Medicine, New York, New York.

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Address correspondence to Dr. Xie: Geriatric Anesthesia Research Unit, Department of Anesthesia, Critical Care and Pain Medicine, Massachusetts General Hospital and Harvard Medical School, 149 13th Street, Room 4310, Charlestown, Massachusetts 02129-2060. zxie@partners.org. Information on purchasing reprints may be found at www.anesthesiology.org or on the masthead page at the beginning of this issue. Anesthesiology's articles are made freely accessible to all readers, for personal use only, 6 months from the cover date of the issue.

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3% sevoflurane for 2 h daily for 1 day and 9% desflurane for 2 h daily for 3 days induced neither cognitive impairment nor neuroinflammation. Finally, an enriched environment and antiinflammatory treatment (ketorolac) ameliorated the sevoflurane-induced cognitive impairment.

Conclusions: Anesthesia-induced cognitive impairment may depend on developmental stage, anesthetic agent, and number of exposures. These findings also suggest the cellular basis and the potential prevention and treatment strategies for anesthesia-induced cognitive impairment, which may ultimately lead to safer anesthesia care and better postoperative outcomes for children.

AN estimated 6 million children undergo surgical care each year in the United States alone.[1] The widespread and prevalent use of anesthesia in children makes its safety a major health issue of interest[2] (reviewed in Sun[3]). Recent population studies have suggested that anesthesia and surgery could be risk factors for subsequent cognitive impairment (reviewed in Sun[3]). Specifically, children who have multiple exposures (e.g., three times) to anesthesia and surgery at an early age (*e.g.*, before age 4) are at an increased risk of developing learning disabilities[4,5] (reviewed in Sun[3]). These data suggest that children may not reach cognitive potentials compared with their peers who have not undergone anesthesia and surgery. These findings have become a major public health issue.[2]

However, not every child develops cognitive impairment after having anesthesia and surgery, and older children may be less susceptible to this phenomenon.[4] Therefore, we have hypothesized that there is a multifactorial model of the cognitive impairment such that the combination of an environmental insult (precipitating factors, *e.g.*, selective anesthesia) with an age vulnerability (predisposing factors, *e.g.*, certain age groups) is needed to cause the cognitive impairment. In the present studies, we have tested this hypothesis by identifying the selective effects of anesthetics (sevoflurane *vs.* desflurane) and anesthesia regimen (one *vs.* three times) on cognitive impairment in different age groups (6 *vs.* 60 days) of mice.

Neuroinflammation, including microglia activation and increases in the levels of proinflammatory cytokines in the brain, may lead to cognitive impairment.[6–10] Specifically, proinflammatory cytokines, particularly tumor necrosis factor (TNF)-α and interleukin (IL)-6, can be released by the microglia during its activation, fueling brain inflammation and leading to cognitive impairment in humans[11–14] and in animals.[15–17] We therefore assessed the effects of different anesthetics and anesthesia regimens on the levels of IL-6 and TNF-α, and ionized calcium-binding adaptor molecule 1 (IBA1), the marker of microglia activation,[18,19] in the brain tissues of mice.

Finally, enriched environment (EE) has been shown to improve brain function. Delayed EE mitigates anesthesia-induced learning and memory impairment in rats.[20] We therefore determined whether EE, which occurred immediately after the anesthesia, could attenuate the anesthesia-induced cognitive impairment in mice in the present experiments.

Materials and Methods

Mice Anesthesia and Treatment

The animal protocol was approved by the Standing Committee on Animals at Massachusetts General Hospital, Boston, Massachusetts. Postnatal day (P) 6 or P60 C57BL/6J (The Jackson Laboratory, Bar Harbor, ME) mice (both male and female) received either anesthetic sevoflurane or desflurane plus 60% oxygen (balanced with nitrogen) to maintain sufficient partial pressure of oxygen (P_{O_2}) levels during anesthesia, as performed in our previous studies.[21] Control groups received 60% oxygen at an identical flow rate in similar chambers. There was no significant difference in learning and memory function between the mice that received 60% oxygen and the mice that received 21% oxygen (data not shown). The anesthetic and oxygen concentrations were measured continuously (GE Datex-Ohmeda, Tewksbury, MA). The temperature of the anesthetizing chamber was controlled to maintain a 37° ± 0.5°C rectal temperature in the mice. We determined pH, and partial pressure of oxygen (P_{O_2}) and carbon dioxide (P_{CO_2}) in the neonatal mice using the methods described by Satomoto *et al.*[22] Specifically, the young mice had a quick arterial blood sampling from the femoral artery at the end of 2 h of anesthesia, and the samples were transferred into heparinized glass capillary tubes. A single sample (100 μl) was analyzed immediately after blood collection by using a blood gas analyzer (ITC, Edison, NJ). Anesthesia with 3% sevoflurane[21,22] for 2 h did not significantly change the values of pH, P_{O_2}, or P_{CO_2} as compared with the control group. Anesthesia with 9% desflurane for 2 h did not significantly change the values of pH, P_{O_2}, or P_{CO_2} as compared with the control group (pH, 7.33 ± 0.05 *vs.* 7.41 ± 0.14 for control *vs.* desflurane anesthesia; P_{O_2}, 174 ± 12.4 mmHg *vs.* 142 ± 27.0 mmHg for control *vs.* desflurane anesthesia; and P_{CO_2}, 48 ± 5.1 mmHg *vs.* 41 ± 9.9 mmHg for control *vs.* desflurane anesthesia). Furthermore, as compared with the control mice, the anesthetized mice did not show significant changes in behavior (*e.g.*, eating, drinking, general activity, and body weight) after the anesthesia. Mortality rate of mice in these studies was less than 1%. For the intervention studies, ketorolac (1 mg/kg),[23] one of the nonsteroidal antiinflammatory drugs, was given to mice 30 min by means of intraperitoneal injection before each of the 3-day sevoflurane anesthesia sessions.

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 P30 or P84 mice were tested in the Morris water maze (MWM) four times per day for 7 days. Each mouse was placed in the pool to search for the platform. The starting points were random for each mouse. Mice that found the platform were allowed to stay on it for 15 s. If a mouse did not find the platform within a 90-s period, it 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 (P36 or P90), the platform was removed from the pool and the mouse was placed in the opposite quadrant. Each mouse was allowed to swim for 90 s, and the number of times the mouse swam across the platform area was recorded (platform crossing times). Mouse body temperature was maintained by active heating as described by Bianchi *et al.*[24] Specifically, after every trial, each mouse was placed in a holding cage under a heat lamp for 1–2 min to dry before being returned to its regular cage.

Brain Tissue Harvest and Protein Level Quantification

Different groups of mice under both the control and anesthesia conditions were used for biochemistry studies. Immediately after the anesthesia, the mice were killed by decapitation (for Western blot analysis) or transcardial perfusion (for immunohistochemistry studies). Separate groups of mice were used for the Western blot analysis and the immunohistochemistry studies. 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 EDTA, and 0.5% Nonidet P-40) plus protease inhibitors (1 μg/ml aprotinin, 1 μg/ml leupeptin, and 1 μg/ml pepstatin A). The lysates were collected, centrifuged at 12,000 rpm for 10 min, and quantified for total protein with the bicinchoninic acid protein assay kit (Pierce Technology Co., Iselin, NJ).

Western Blot Analysis

IL-6 antibody (1:1,000 dilution; Abcam, Cambridge, MA) was used to recognize IL-6 (24 kDa). TNF-α antibody (1:1,000 dilution; Abcam) was used to recognize TNF-α (17 kDa). Western blot quantification was performed as described by Xie *et al.*[25] Briefly, signal intensity was analyzed using the image analysis program Quantity One (Bio-Rad Laboratories, Hercules, CA). We quantified the Western blots in two steps. First, we used β-actin levels to normalize protein levels (*e.g.*, determining the ratio of IL-6 to β-actin amount) and control for loading differences in the total protein amount. Second, we presented protein level changes in mice undergoing 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.

Immunohistochemistry

Mice were anesthetized with isoflurane briefly (1.4% isoflurane for 5 min) and perfused transcardially with heparinized saline followed by 4% paraformaldehyde in 0.1 M phosphate buffer at pH 7.4. The anesthesia with 1.4% isoflurane for 5 min in mice provided adequate anesthesia for the perfusion procedure without causing significant changes in blood pressure and blood gas according to our previous studies.[25] 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. The sections were incubated with IBA1 antibody (ab5076, 1:100; Abcam) and secondary antibody (Alexa 568, 1:500; Invitrogen, Carlsbad, CA). Finally, the sections were analyzed in mounting medium under a confocal microscope with a 20× objective lens and photographs of the sections were taken. An investigator who was blind to the experimental design counted the number of IBA1-positive cells using ImageJ version 1.38 (National Institutes of Health, Bethesda, MD).

Enriched Environment

The EE in the current experiment was performed as described in previous studies with modifications.[26–28] Specifically, an EE was created in a large cage (70 × 70 × 46 cm) that included five or six toys (*e.g.*, wheels, ladders, and small mazes). The mice were put in the EE every day for 2 h from P8–P30. The objects were changed two to three times per week to provide a novel and challenging environment.

Statistical Analysis

Data regarding biochemistry changes were expressed as mean ± SD. Data in changes of escape latency were expressed as mean ± SEM. The data for platform crossing time were not distributed normally and thus are expressed as median and interquartile range. The number of samples varied from 6–16, and the samples were distributed normally with the exception of platform crossing time (tested by normality test, data not shown). 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. The *post hoc* Bonferroni test was used to compare the difference in escape latency between the control and anesthesia groups in each day of the MWM. The Mann–Whitney *U* test was used to determine the difference between the sevoflurane and control conditions on platform crossing times. There were no missing data for the variables of MWM (escape latency and platform crossing time) during the data analysis. Finally, a Student two-sample *t* test was used to determine the difference between the anesthesia and control groups in levels of IL-6, TNF-α, and

IBA1 positive cells. 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

Multiple Exposures of Sevoflurane in Young Mice Induced Cognitive Impairment and Accumulation of Brain IL-6 and TNF-α in Mice

Given the clinical observation that three exposures, but not one exposure, to anesthesia and surgery increased the risk of cognitive impairment in children,[4,5] we have established an animal model in which mice were treated with sevoflurane for 2 h daily for 1 or 3 days. This animal model conceptually mimics the single *versus* multiple exposures of anesthesia and allows us to study the anesthesia-induced developmental neurotoxicity.

The mice were treated with 3% sevoflurane anesthesia for 2 h daily for 3 days from P6–P8. The mice were tested in the MWM from P30–P36. A comparison of the time that each mouse took to reach the platform during reference training (escape latency) showed that there was a statistically significant interaction of time and group based on escape latency in the MWM between mice following the control condition and mice following the sevoflurane anesthesia (fig. 1A) ($P = 0.0063$, two-way ANOVA with

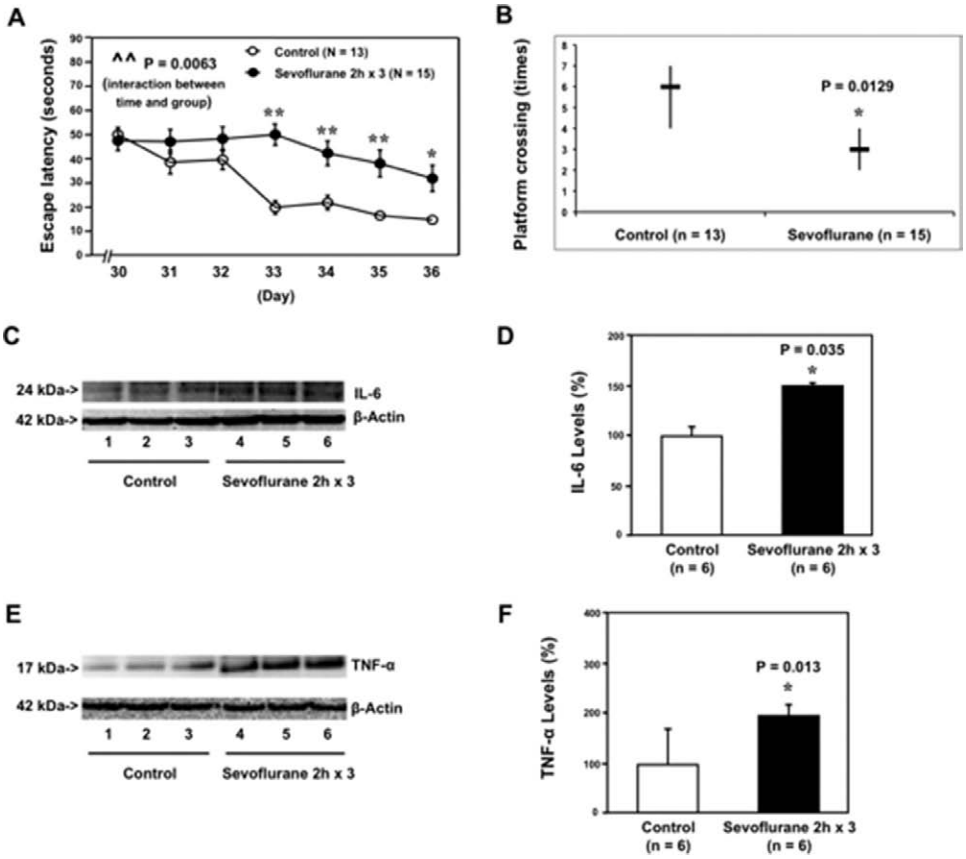

Fig. 1. Anesthesia with 3% sevoflurane for 2 h daily for 3 days in P6 mice induces cognitive impairment in the mice tested from P30–P36, and increases IL-6 and TNF-α levels in the brain tissues of the mice. (*A*) Anesthesia with 3% sevoflurane for 2 h daily for 3 days in P6 mice increases the escape latency of mice swimming in the MWM as compared with the control condition (tested from P30–P36) (control, n = 13; sevoflurane, n = 15). Two-way ANOVA with repeated measurement analysis shows that there is a statistically significant interaction of time and group based on escape latency of MWM between mice following the control condition and mice following the sevoflurane anesthesia in the MWM. Specifically, mice that received the sevoflurane anesthesia had a longer escape latency time at P33, P34, P35, and P36 as compared with the control condition. (*B*) Anesthesia with 3% sevoflurane for 2 h daily for 3 days in P6 mice reduces the platform crossing times of mice swimming in the MWM as compared with the control condition tested at P36 (control, n = 13; sevoflurane, n = 15). (*C*) The sevoflurane anesthesia increases IL-6 levels in the brain tissues of the mice as compared with the control condition. There is no significant difference in β-actin levels in the brain tissues of the mice between the sevoflurane anesthesia and control conditions. (*D*) Quantification of the Western blot (n = 6) shows that the sevoflurane anesthesia increases IL-6 levels in the brain tissues of the mice as compared with the control condition. (*E*) Sevoflurane anesthesia increases TNF-α levels in the brain tissues of the mice as compared with the control condition. There is no significant difference in β-actin levels in brain tissues of the mice between the sevoflurane anesthesia and control groups. (*F*) Quantification of the Western blot (n = 6) shows that the sevoflurane anesthesia increases TNF-α levels in the brain tissues of the mice as compared with the control group. IL = interleukin; MWM = Morris water maze; TNF = tumor necrosis factor.

repeated measurement). The *post hoc* Bonferroni test showed that the mice that received the sevoflurane anesthesia had a longer escape latency than the mice following the control condition on P33, P34, P35, and P36. A comparison of the number of times that each mouse crossed the location of the absent platform at the end of reference training (platform crossing times) indicated that the sevoflurane (n = 15) anesthesia (median, 3; interquartile range, 2–4) decreased the platform crossing times as compared with the control (n = 13) condition (median, 6; interquartile range, 4–7; $P = 0.0129$, Mann–Whitney U test) (fig. 1B). There was no significant difference in mouse swimming speed between the mice in the sevoflurane anesthesia group and the mice in the control group (data not shown). These data suggest that multiple exposures of sevoflurane in young mice may induce cognitive impairment in the mice approximately 1 month after anesthesia.

Given the findings that multiple exposures to sevoflurane in young mice might induce cognitive impairment, we next investigated the underlying mechanisms. Proinflammatory cytokines, particularly IL-6 and TNF-α, are associated with cognitive impairment.[11–17] We therefore assessed the effects of the sevoflurane anesthesia on the levels of IL-6 and TNF-α in brain tissues of the mice. The brain tissues of the mice were harvested at the end of the anesthesia (P8) and subjected to Western blot analysis to determine levels of IL-6 and TNF-α. Immunoblotting of IL-6 showed that the sevoflurane anesthesia led to a more visible band representing IL-6 as compared with the control condition (fig. 1C). There was no significant difference in β-actin levels between the mice that received the sevoflurane anesthesia and the mice with the control condition (fig. 1C). The quantification of the Western blot illustrated that the sevoflurane anesthesia increased IL-6 levels in brain tissues of the mice (151 ± 2.3% *vs.* 100 ± 9.0 %, n = 6, $P = 0.035$) (fig. 1D). Immunoblotting of TNF-α showed that the sevoflurane anesthesia caused a more visible band representing TNF-α as compared with the control group (fig. 1E). There was no significant difference in β-actin levels between the mice that received the sevoflurane anesthesia and the mice with the control condition (fig. 1E). The quantification of the Western blot revealed that the sevoflurane anesthesia increased TNF-α levels in the brain tissues of the mice (178 ± 24% *vs.* 100 ± 72.2%, n = 6, $P = 0.013$) (fig. 1F). Considered together, these results suggest that multiple exposures of sevoflurane in young mice may increase proinflammatory cytokine levels in brain tissues, ultimately leading to cognitive impairment.

Single Exposure of Sevoflurane in Young Mice Induced neither Cognitive Impairment nor Accumulation of Brain IL-6 and TNF-α in Mice

Next, we asked whether a single exposure to sevoflurane anesthesia in young (P6) mice could induce cognitive impairment and accumulation of brain IL-6 and TNF-α in mice. The 6-day-old mice were treated with 3% sevoflurane anesthesia for 2 h at P6 only. The mice were tested in the MWM from P30–P36. Although there was a statistically significant interaction of time and group based on escape latency in the MWM between the mice in the control group and the mice in the sevoflurane anesthesia group, the mice that received sevoflurane anesthesia had faster, rather than slower, escape latency than the mice with the control condition on P31 (fig. 2A). There was no significant difference in the platform crossing times between the mice in the control group and the mice in the sevoflurane anesthesia group (fig. 2B). Western blot analysis showed that the anesthesia with 3% sevoflurane for 2 h daily for 1 day at P6 did not increase levels of IL-6 (fig. 2, C and D) or TNF-α (fig. 2, E and F) in the brain tissues of the mice. These data suggest that a single exposure of sevoflurane anesthesia in young mice may not induce neuroinflammation or cognitive impairment in the mice.

Multiple Exposures of Desflurane in Young Mice Induced neither Cognitive Impairment nor Accumulation of Brain IL-6 and TNF-α in Mice

It has been reported that sevoflurane can induce apoptosis and Aβ accumulation,[21,22,29] whereas desflurane does not.[30–32] Therefore, we assessed the effects of multiple exposures of 9% desflurane (minimum alveolar concentration equivalent to 3% sevoflurane) on the function of learning and memory and brain levels of proinflammatory cytokines in mice. The 6-day-old mice were treated with 9% desflurane anesthesia for 2 h daily for 3 days from P6–P8. The mice were tested in the MWM from P30–P36. MWM studies showed that the desflurane anesthesia did not increase escape latency (fig. 3A) and did not reduce platform crossing times as compared with the control group (fig. 3B). Moreover, the desflurane anesthesia did not increase IL-6 (fig. 3, C and D) or TNF-α (fig. 3, E and F) levels in the brain tissues of the mice. These data suggest that desflurane may not lead to cognitive impairment and neuroinflammation in the developing brain.

Multiple Exposures of Sevoflurane in Adult Mice Induced neither Cognitive Impairment nor Neuroinflammation in the Mice

There is a critical period of vulnerability for the developing brain, also known as the brain growth spurt period, in humans (up to 36 months) and in rodents (up to 3 weeks).[33,34] In this period, the brain is susceptible to developing acute neural injuries. Moreover, older children may have less risk of developing the cognitive impairment following anesthesia and surgery.[4] We therefore asked whether the same multiple exposures of sevoflurane anesthesia could induce cognitive impairment and neuroinflammation in adult mice. The 60-day-old mice were treated with 3% sevoflurane for 2 h daily for 3 days at P60. The mice were tested in the MWM approximately 1 month after anesthesia (from P84–P90). MWM studies showed that the sevoflurane anesthesia did not increase escape latency (fig. 4A) and did not reduce

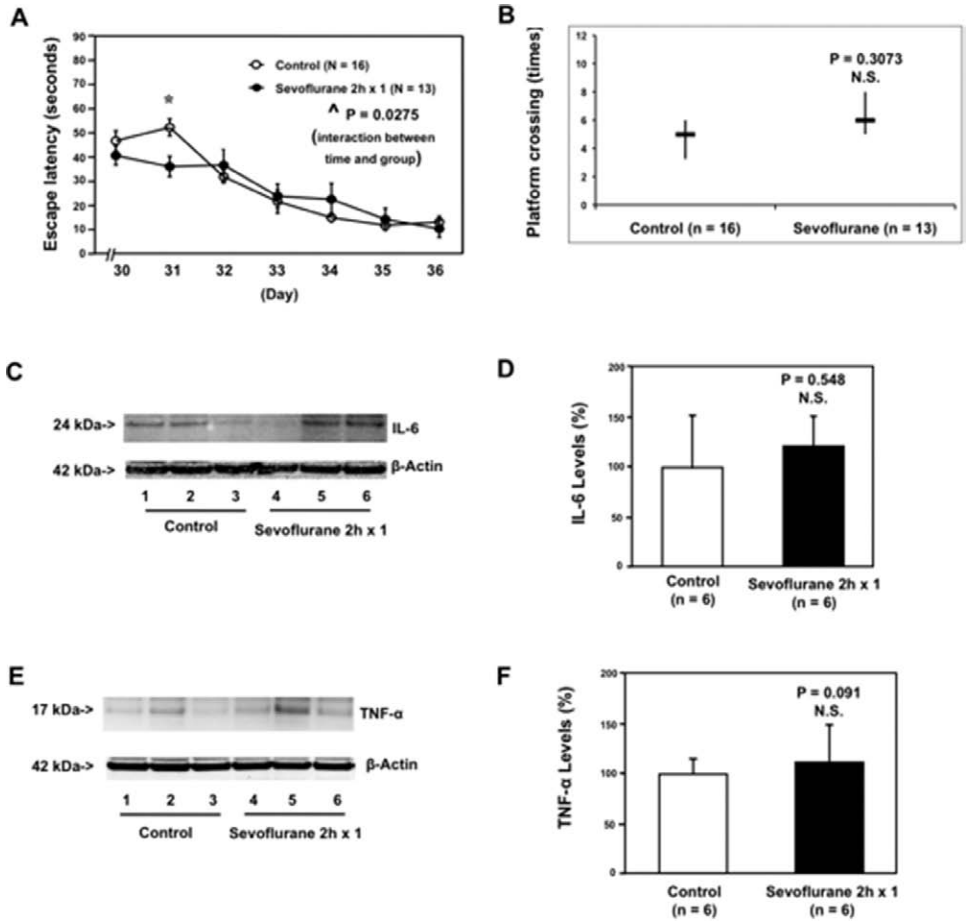

Fig. 2. Anesthesia with 3% sevoflurane for 2 h daily for 1 day in P6 mice does not induce cognitive impairment in the mice tested from P30–P36, and does not increase IL-6 and TNF-α levels in the brain tissues of the mice. (*A*) Anesthesia with 3% sevoflurane for 2 h daily for 1 day in P6 mice does not increase escape latency of mice swimming in the MWM as compared with the control condition (tested from P30–P36). Two-way ANOVA with repeated measurement analysis shows that there is a statistically significant interaction of time and group based on escape latency of MWM between the mice following the control condition and mice following the sevoflurane anesthesia in the MWM (control, n = 16; sevoflurane, n = 13). Specifically, mice that received the sevoflurane anesthesia have a shorter escape latency time at P31 as compared with the control condition. (*B*) Anesthesia with 3% sevoflurane for 2 h daily for 1 day in P6 mice does not reduce the platform crossing times of mice swimming in the MWM as compared with the control condition tested at P36 (control, n = 16; sevoflurane, n = 13). (*C*) The sevoflurane anesthesia does not increase IL-6 levels in the brain tissues of the mice as compared with the control group. There is no significant difference in β-actin levels in the brain tissues of the mice between the sevoflurane anesthesia and control groups. (*D*) quantification of the Western blot (n = 6) shows that the sevoflurane anesthesia does not increase IL-6 levels in the brain tissues of the mice as compared with the control group. (*E*) The sevoflurane anesthesia does not increase TNF-α levels in the brain tissues of the mice as compared with the control condition. There is no significant difference in β-actin levels in the brain tissues of the mice between the sevoflurane anesthesia and control conditions. (*F*) The quantification of the Western blot (n = 6) shows that the sevoflurane anesthesia does not increase TNF-α levels in the brain tissues of the mice as compared with the control condition. IL = interleukin; MWM = Morris water maze; TNF = tumor necrosis factor.

platform crossing times (fig. 4B) in the adult (P60) mice. Moreover, the sevoflurane anesthesia did not increase IL-6 (fig. 4, C and D) or TNF-α (fig. 4, E and F) levels in the brain tissues of the adult mice.

Neuroinflammation includes an increase in proinflammatory cytokines and microglia activation.[6–10] We therefore assessed and compared the effects of the multiple exposures of sevoflurane on microglia activation in the hippocampus between young and adult mice. Immuonhistochemistry image analysis showed that the anesthesia with 3% sevoflurane for 2 h daily for 3 days increased the density of IBA1-positive cells, the marker of microglia activation,[18,19] as compared with the control group in the hippocampus of young (P8) but not adult (P62) mice (fig. 5A). Quantification of the images illustrated that the sevoflurane anesthesia increased the amount of IBA1-positive cells in the hippocampus of young (128 ± 16.6% *vs.* 100 ± 13.5%, n = 6, $P = 0.036$) (fig. 5B) but not adult mice (95 ± 13.9% *vs.* 100 ± 12.7%, n = 6, $P = 0.378$, not significant). Two-way ANOVA demonstrated that there was a significant interaction and that young age

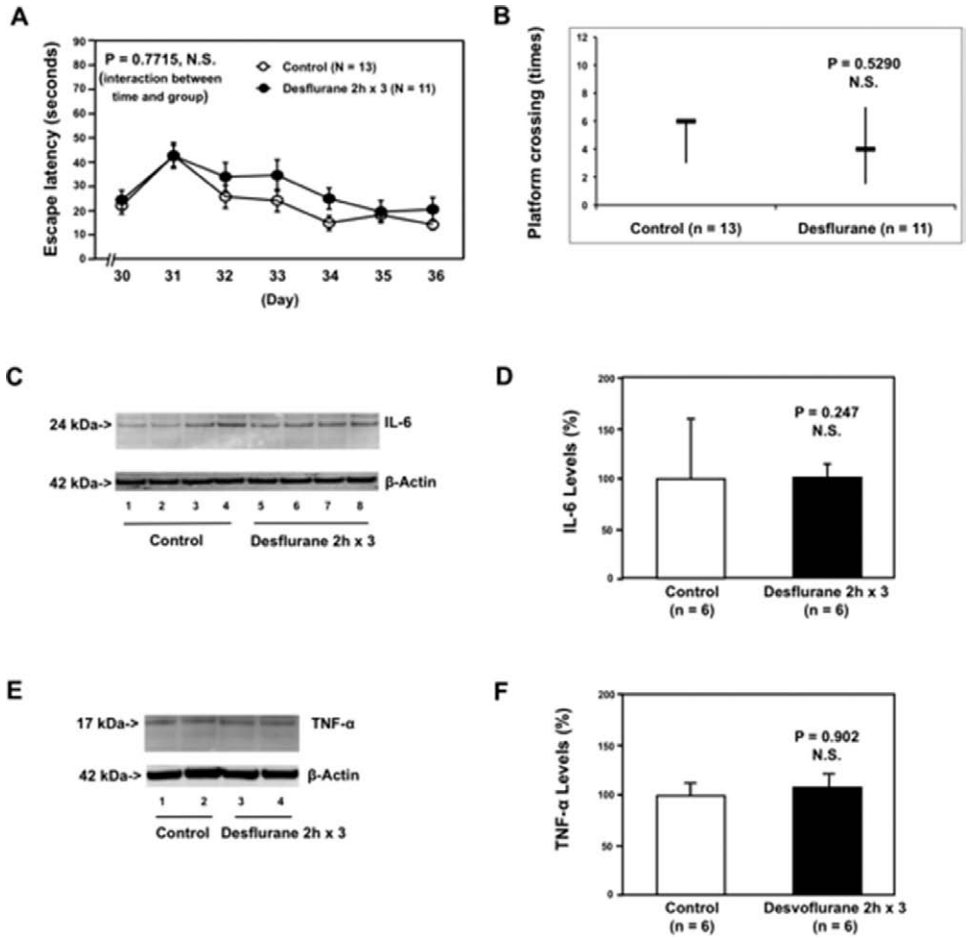

Fig. 3. Anesthesia with 9% desflurane for 2 h daily for 3 days in P6 mice does not induce cognitive impairment in the mice tested from P30–P36, and does not increase IL-6 and TNF-α levels in the brain tissues of the mice. (*A*) Anesthesia with 9% desflurane for 2 h daily for 3 days in P6 mice does not increase escape latency of mice swimming in the MWM as compared with the control condition (tested from P30–P36). Two-way ANOVA with repeated measurement analysis shows that there is no statistically significant interaction of time and group based on escape latency of MWM between mice following the control condition and mice following the desflurane anesthesia in the MWM (control, n = 13; sevoflurane, n = 11). (*B*) Anesthesia with 9% desflurane for 2 h daily for 3 days in P6 mice does not reduce the platform crossing times of mice swimming in the MWM as compared with the control condition tested at P36 (control, n = 13; sevoflurane, n = 11). (*C*) The desflurane anesthesia does not increase IL-6 levels in the brain tissues of the mice as compared with the control condition. There is no significant difference in β-actin levels in the brain tissues of the mice between the desflurane anesthesia and control groups. (*D*) Quantification of the Western blot (n = 6) shows that the desflurane anesthesia does not increase IL-6 levels in the brain tissues of the mice as compared with the control condition. (*E*) The desflurane anesthesia does not increase TNF-α levels in the brain tissues of the mice as compared with the control condition. There is no significant difference in β-actin levels in the brain tissues of the mice between the desflurane anesthesia and control groups. (*F*) Quantification of the Western blot (n = 6) shows that the desflurane anesthesia does not increase TNF-α levels in the brain tissues of the mice as compared with the control condition. IL = interleukin; MWM = Morris water maze; TNF = tumor necrosis factor.

potentiated the sevoflurane anesthesia–induced increases in the amount of IBA1-positive cells ($P = 0.027$). Collectively, these data suggest that multiple exposures of sevoflurane may induce neuroinflammation, which includes increases in the levels of proinflammatory cytokines and microglia activation, leading to cognitive impairment in young mice (P6, developing brain) but not adult mice (P60). However, the increases in the amount of IBA1-positive cells disappeared at approximately 1 month after anesthesia in the hippocampus of young mice (detected at P30, data not shown). Considered together, these data suggest that the sevoflurane-induced neuroinflammation may trigger other neuropathologic events, ultimately leading to cognitive impairment.

Enriched Environment and Anti-inflammatory Treatment Attenuated the Sevoflurane-induced Cognitive Impairment

EE has been shown to improve brain function.[26–28] We therefore asked whether EE could ameliorate the sevoflurane-induced cognitive impairment in young mice. The mice were treated with 3% sevoflurane for 2 h daily for 3 days

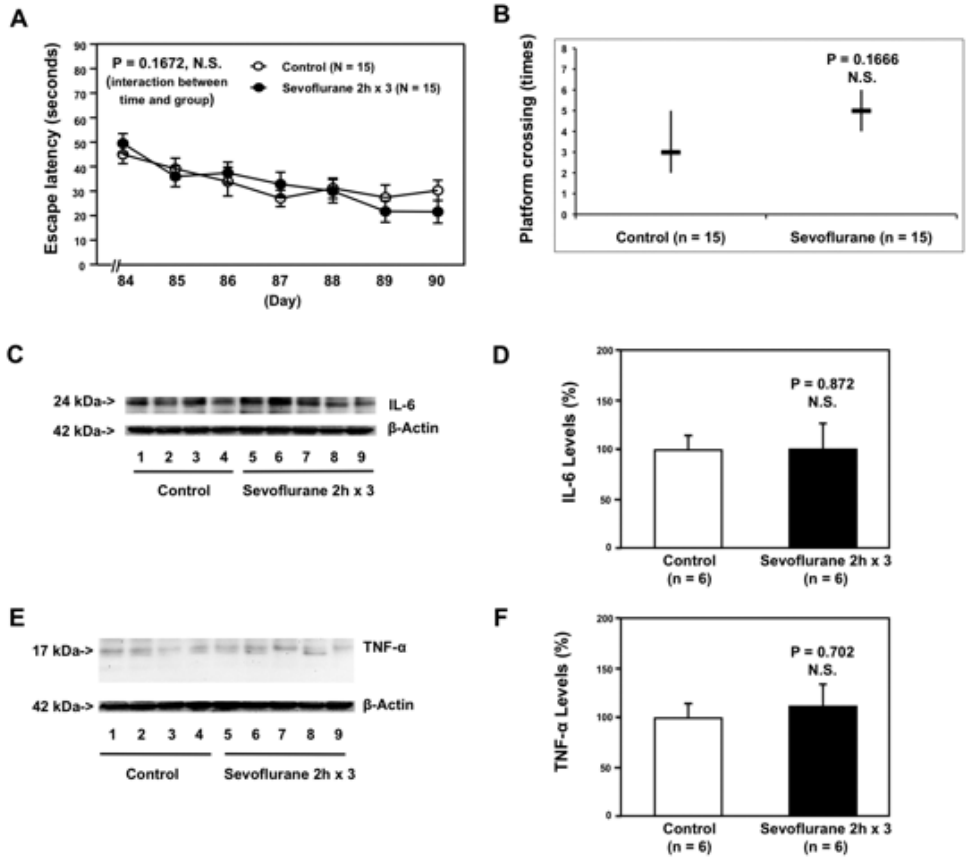

Fig. 4. Anesthesia with 3% sevoflurane for 2 h daily for 3 days in P60 mice induces neither cognitive impairment in the mice tested from P84–P90 nor increases in levels of IL-6 and TNF-α in the brain tissues of the mice. (*A*) Anesthesia with 3% sevoflurane for 2 h daily for 3 days in P60 mice does not increase escape latency of mice swimming in the MWM as compared with the control condition (tested from P84–P90). Two-way ANOVA with repeated measurement analysis (control, n = 15; sevoflurane, n = 15) shows that there is no significant difference in the learning curve based on escape latency between mice following the control condition and mice following the sevoflurane anesthesia in the MWM. (*B*) Anesthesia with 3% sevoflurane for 2 h daily for 3 days in P60 mice does not reduce the platform crossing times of mice swimming in the MWM as compared with the control condition tested at P90 (control, n = 15; sevoflurane, n = 15). (*C*) The sevoflurane anesthesia does not increase IL-6 levels in the brain tissues of the mice as compared with the control condition. There is no significant difference in β-actin levels in the brain tissues of the mice between the sevoflurane anesthesia and control groups. (*D*) Quantification of the Western blot (n = 6) shows that the sevoflurane anesthesia does not increase IL-6 levels in the brain tissues of the mice as compared with the control condition. (*E*) The sevoflurane anesthesia does not increase TNF-α levels in the brain tissues of the mice as compared with the control condition. There is no significant difference in β-actin levels in the brain tissues of the mice between the sevoflurane anesthesia and control conditions. (*F*) Quantification of the Western blot (n = 6) shows that the sevoflurane anesthesia does not increase TNF-α levels in the brain tissues of the mice as compared with the control group. IL = interleukin; MWM = Morris water maze; P = postnatal day; TNF = tumor necrosis factor.

(from P6–P8). Then, the mice were exposed to either EE or standard environment (SE) from P8–P30 (the weaning started at P26) (fig. 6A). Finally, the mice were tested in the MWM from P30–P36. Two-way ANOVA with repeated measurement showed that there was a statistically significant interaction of time and group based on escape latency of MWM between mice following the sevoflurane anesthesia plus SE and those following the sevoflurane anesthesia plus EE (fig. 6B) ($P = 0.001$). The *post hoc* Bonferroni test showed that the mice that received sevoflurane plus EE had faster escape latency as compared with the mice that received sevoflurane plus SE at P32 and P33. There was no significant difference in platform crossing times between the mice that received sevoflurane plus SE and the mice that received sevoflurane plus EE (fig. 6C). Two-way ANOVA with repeated measurement showed that there was no statistically significant interaction of time and group based on escape latency of MWM between mice following the control condition plus SE and those following the control condition plus EE (fig. 6D) ($P = 0.0524$, not significant). There was no significant difference in platform crossing times between the mice following the control condition plus SE and the mice following the control condition plus EE (fig. 6E). Finally, EE did not affect the amount of IBA1-positive cells in the mouse hippocampus (data not shown). These results suggest that EE may ameliorate the sevoflurane-induced cognitive

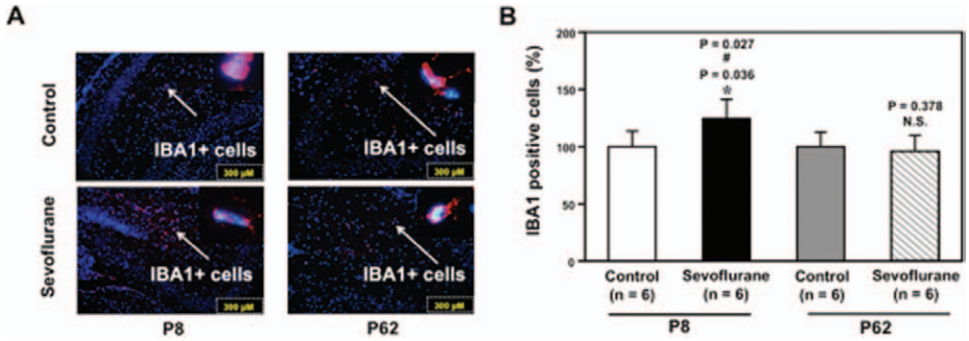

Fig. 5. Anesthesia with 3% sevoflurane for 2 h daily for 3 days increases the amount of IBA1-positive cells in the hippocampus of P6 but not P60 mice. (*A*) The sevoflurane anesthesia increases the amount of IBA1-positive cells in the hippocampus of P6 mice (*left column*) but not P60 mice (*right column*), harvested at the end of the 3-day anesthesia. (*B*) Quantification of the immunohistochemistry image (n = 6) shows that the sevoflurane anesthesia increases the amount of IBA1-positive cells in the hippocampus of P6 mice (*black bar vs. white bar*), but not in P60 mice (*net bar vs. gray bar*). Two-way ANOVA shows that there is a significant interaction and that young age potentiates the sevoflurane anesthesia–induced increases in the amount of IBA1-positive cells. IBA1, ionized calcium-binding adaptor molecule 1.

impairment in young mice, which is supported by the results from a recent study that a delayed EE (4 weeks after anesthesia) can attenuate the sevoflurane-induced learning and memory impairment in rats tested at 8 weeks after anesthesia.[20]

Moreover, the nonsteroidal anti-inflammatory drug ketorolac also ameliorated the sevoflurane-induced cognitive impairment (fig. 7). Specifically, the sevoflurane anesthesia did not increase the escape latency of the MWM (fig. 7A) and did not reduce platform crossing times (fig. 7B) as compared with control condition in the mice pretreated with ketorolac. These data potentially suggest the causal relationship of the sevoflurane-induced neuroinflammation and sevoflurane-induced cognitive impairment.

Discussion

Sevoflurane is the most commonly used anesthetic in children. We first found that anesthesia with 3% sevoflurane for 2 h daily for 3 days in young (P6) mice induced cognitive impairment detected at a later time (P30–P36) (fig. 1). Moreover, we found that anesthesia with 3% sevoflurane for 2 h daily for only 1 day in young (P6) mice did not induce cognitive impairment (fig. 2). These results suggest the selectivity of anesthesia-induced cognitive impairment such that only specific anesthesia regimen(s) (*e.g.*, multiple exposures) may induce detrimental effects. These findings would support the clinical observation that three exposures, but not one exposure, to anesthesia and surgery increased the risk of cognitive impairment in children,[4,5] and suggest that anesthesia may contribute to the clinically observed cognitive impairment in children following anesthesia and surgery.

In addition, anesthesia with 9% desflurane (equivalent minimal alveolar concentration with 3% sevoflurane) for 2 h daily for 3 days in young (P6) mice did not induce cognitive impairment in the mice (fig. 3). These findings may suggest that desflurane could induce a lesser degree of insult in the developing brain, which would promote clinical studies to further determine whether desflurane is a safer anesthetic for children. The findings have also established a system to investigate the difference between sevoflurane and desflurane in brain function.

Brain in the growth spurt period (up to 36 months in humans and up to 3 weeks in rodents)[33,34] is susceptible to acute neural injuries, including those caused by anesthetics.[35] We therefore compared the effects of the same sevoflurane anesthesia on cognitive function between young (P6) and adult (P60) mice, and found that the anesthesia with 3% sevoflurane for 2 h daily for 3 days did not lead to cognitive impairment in adult (P60) mice. These findings are supported by the results from the clinical studies that early exposure to anesthesia/surgery may lead to an increased risk of developing cognitive impairment in children,[4,5] and from the preclinical animal studies that isoflurane anesthesia induced cognitive impairment only in young rats.[35]

Neuroinflammation, including proinflammatory cytokine accumulation and microglia activation, is associated with cognitive impairment in humans and animals.[11,13,17] Proinflammatory cytokines, particularly TNF-α, IL-6, and IL-1β, can be released by the microglia during its activation, fueling brain inflammation and leading to cognitive dysfunction in humans[11] and animals.[16,17] Proinflammatory cytokines can induce microglia activation in discrete brain regions, leading to the production of the same proinflammatory cytokines.[36,37] These proinflammatory cytokines inhibit long-term potentiation[38,39] and induce neurobehavioral deficits.[40–46] We found that anesthesia with 3% sevoflurane for 2 h daily for 3 days (from P6–P8) in young mice was able to increase the levels of proinflammatory cytokine IL-6 and TNF-α in brain tissues (fig. 1) and the amount of IBA1-positive cells, the marker of microglia activation, in the hippocampus (fig. 5) of the mice at P8. The anesthesia with 3% sevoflurane for 2 h daily for 1 day in P6 mice (fig. 2) and 3% sevoflurane for 2 h daily for 3 days in adult (P60) mice (fig. 4) did not increase levels of IL-6, TNF-α, or IBA1 positive cells the brain tissues of the mice. Collectively, these data suggest that the multiple exposures of sevoflurane anesthesia in young mice may cause

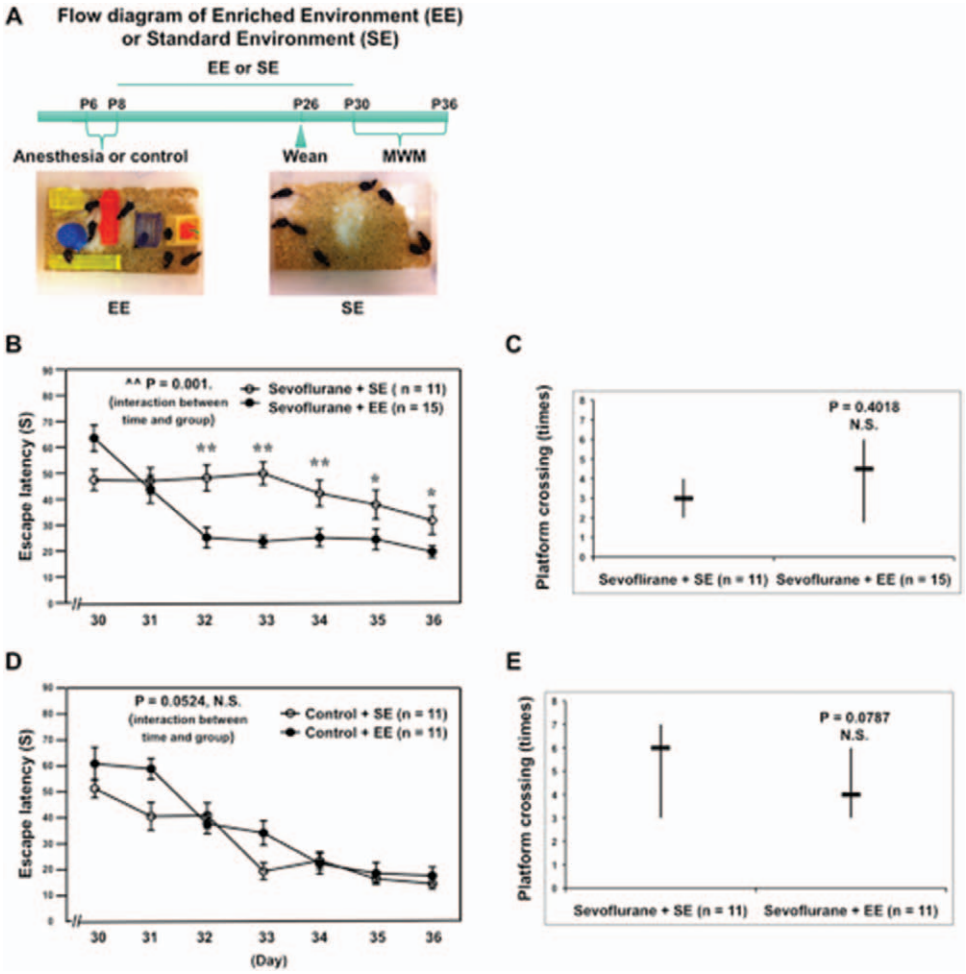

Fig. 6. EE attenuates the sevoflurane-induced cognitive impairment in mice. (*A*) The flow diagram and photographs of EE and SE. (*B*) Two-way ANOVA with repeated measurement analysis shows that there is a statistically significant interaction of time and group based on escape latency in MWM between mice following sevoflurane anesthesia plus SE and sevoflurane anesthesia plus EE (control, n = 11; sevoflurane, n = 15). (*C*) The Mann–Whitney *U* test shows that the platform crossing times of mice swimming in the MWM following the sevoflurane anesthesia plus EE is not significantly different from that of mice following the sevoflurane anesthesia plus SE (control, n = 11; sevoflurane, n = 15). (*D*) Two-way ANOVA with repeated measurement analysis shows that there is no statistically significant interaction of time and group based on escape latency of MWM between the control condition plus SE and the control condition plus EE (control, n = 11; sevoflurane, n = 11). (*E*) The Mann–Whitney *U* test shows that there is no significant difference in platform crossing times of mice swimming in the MWM between the control condition plus SE and the control condition plus EE (control, n = 11; sevoflurane, n = 11). EE, enriched environment; MWM = Morris water maze; SE = standard environment.

cognitive impairment *via* inducing neuroinflammation. The findings that the same sevoflurane anesthesia increased the amount of IBA1-positive cells in the hippocampus of young (P6) mice but not adult (P60) mice further suggest that mice at different ages may have different neuroinflammation reactions to anesthesia.

The mechanisms by which anesthesia induces neuroinflammation remain to be determined. Anesthetics have been shown to increase cytosolic calcium levels.[47–50] The elevation of cytosolic calcium is associated with increased levels of proinflammatory cytokines,[51] potentially through activation of nuclear factor-κB signaling.[52–57] Therefore, we postulate that anesthetics can increase calcium levels to trigger generation of TNF-α and IL-6 *via* nuclear factor-κB signaling, and the cytokines then induce microglia activation to generate more proinflammatory cytokines, ultimately leading to neuroinflammation.

Finally, 9% desflurane for 2 h daily for 3 days in P6 mice did not increase the levels of IL-6 and TNF-α in the brain tissues (fig. 3). These different effects between sevoflurane and desflurane are consistent with the previous findings that sevoflurane can induce apoptosis and Aβ accumulation,[21,22,29] whereas desflurane does not.[30,31] The exact mechanisms by which sevoflurane and desflurane have different effects on neurotoxicity and cognitive impairment remain to be determined. Our recent studies suggest that sevoflurane may

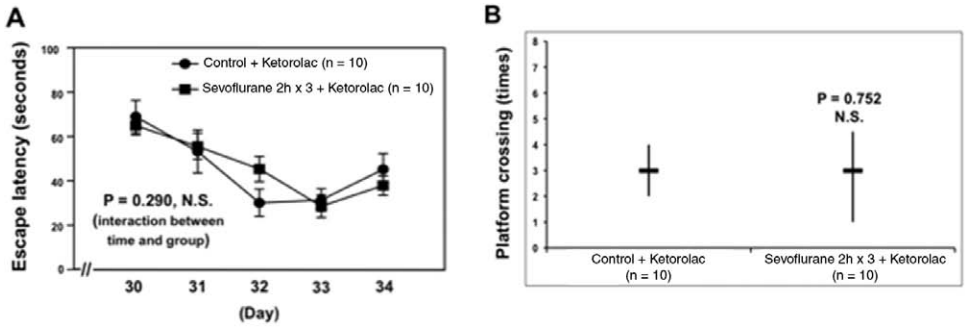

Fig. 7. Ketorolac attenuates the sevoflurane-induced cognitive impairment in mice. (*A*) Two-way ANOVA with repeated measurement analysis shows that there is no statistically significant interaction of time and group based on escape latency of MWM between the sevoflurane plus saline– and sevoflurane plus ketorolac–treated mice (control, n = 10; sevoflurane, n = 10). (*B*) The Mann–Whitney *U* test shows that there is no significant difference in platform crossing times of mice swimming in the MWM between the sevoflurane plus saline and sevoflurane plus ketorolac graoups (control, n = 10; sevoflurane, n = 10). MWM, Morris water maze.

affect the Wnt pathway (Yiying Zhang, M.D., M.S., Massachusetts General Hospital and Harvard Medical School, Charlestown, MA, unpublished data; most recent communication, January 27, 2012). Therefore, in future studies, we may also compare the effects of sevoflurane and desflurane anesthesia on the Wnt pathway to determine the underlying mechanisms of different effects of sevoflurane and desflurane on neurotoxicity and cognitive impairment.

Interestingly, there was no significant increase in the amount of IBA1-positive cells in the mouse hippocampus at P30 (data not shown), the time when the mice developed cognitive impairment (P30–P36). These results suggest that the acute sevoflurane-induced neuroinflammation may not last long enough to directly cause the recent cognitive impairment. Moreover, the findings suggest that there should be other pathologic event(s) (*e.g.*, synaptic dysfunction) between the sevoflurane-induced neuroinflammation and the sevoflurane-induced cognitive impairment. Future studies to test this hypothesis are warranted.

In the current studies, we have not found that EE can affect the amount of IBA1-positive cells in the mouse hippocampus. Thus, it is unlikely that EE attenuates the sevoflurane-induced cognitive impairment through directly mitigating the sevoflurane-induced neuroinflammation. EE may improve brain function through various mechanisms (*e.g.*, promotion of neurogenesis and synapse function).[26–28,58,59] Our current findings that EE can ameliorate the sevoflurane-induced cognitive impairment suggest that the sevoflurane anesthesia could affect neurogenesis, synapse, and other functions, leading to cognitive impairment. These findings have established a system for us to perform additional studies to determine the underlying mechanisms (*e.g.*, neurogenesis and synapse function) of the sevoflurane-induced cognitive impairment. Moreover, pending further studies, these findings may imply that susceptible children should have more exposures to EE after anesthesia and surgery.

A recent study shows that nociceptive stimulation can potentiate the anesthesia-induced neurotoxicity in the rat developing brain.[60] It is therefore possible that surgery and other perioperative factors may worsen the anesthesia-induced neuroinflammation in mouse developing brain and cognitive impairment. Other studies suggest that there is no significant difference in the incidence of postoperative cognitive dysfunction between surgery with general anesthesia and surgery without it (with epidural, spinal, or local anesthesia)[61–66] (reviewed in Mason *et al.* and Newman *et al.*[67,68]). Considered together, it is important to perform both human and animal studies to determine the interaction of anesthesia and surgery on potential neurotoxicity and postoperative cognitive dysfunction.

This study has several limitations. First, we did not investigate the effects of anesthesia on other domains of cognitive impairment (*e.g.*, executive function) and instead focused on learning and memory function because it is the major domain of cognitive impairment. However, the current data have suggested that multiple exposures to sevoflurane anesthesia in young mice may cause neuroinflammation and cognitive impairment, which will allow us to further study the upstream mechanisms of the anesthesia-induced cognitive impairment and downstream consequences of the anesthesia-induced neuroinflammation. Second, we assessed the effects of anesthesia only in P6 mice. It is unknown whether anesthesia-induced neuroinflammation and cognitive impairment can also occur in young mice and the developing brain at other ages. Therefore, future studies will include an assessment of the effects of different anesthetic(s) and anesthesia regimens on varied ages of young mice (*e.g.*, P2, P14, and P21) to further test the hypothesis that the developing brain is more susceptible to anesthesia neurotoxicity. Third, it is unknown whether the potency of 3% sevoflurane is equal to that of 9% desflurane in mice. We chose 3% sevoflurane and 9% desflurane in the current study because the minimum alveolar concentration of sevoflurane and desflurane in children is 2.5 and 8.72, respectively.[69] Finally, brain and blood cytokines could not be easily separated at the time the animals were killed. However, the desflurane anesthesia and

anesthesia with sevoflurane for 1 day neither increased brain levels of cytokines nor induced cognitive impairment; the anesthesia with sevoflurane for 3 days increased the number of IBA1-positive cells in mouse hippocampus. Finally, anti-inflammatory treatment with ketorolac (fig. 7) ameliorated the sevoflurane-induced cognitive impairment in the mice. Considered together, these findings support the conclusion that neuroinflammation may, at least partially, contribute to the sevoflurane-induced cognitive impairment.

In conclusion, we have found that the selectivity of anesthetics and anesthesia regimens are associated with neurotoxicity and age-dependent vulnerability. The findings suggest the combination of an environmental insult (*i.e.*, precipitating factors such as multiple exposures to a specific anesthetic) with age vulnerability (*i.e.*, predisposing factors such as young age) plays a role in cognitive impairment. Finally, EE and antiinflammatory treatment could be strategies used to prevent and treat anesthesia-induced cognitive impairment. These findings would promote more studies to investigate anesthesia neurotoxicity in the developing brain, ultimately leading to safer anesthesia care and better postoperative outcomes for children who could be vulnerable to brain damage.

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