

Anesthetic Sevoflurane Causes Neurotoxicity Differently in Neonatal Naïve and Alzheimer Disease Transgenic Mice

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ABSTRACT

Background: Recent studies have suggested that children undergoing surgery under anesthesia could be at an increased risk for the development of learning disabilities, but whether anesthetics contribute to this learning disability is unclear. Therefore, the authors set out to assess the effects of sevoflurane, the most commonly used inhalation anesthetic, on caspase activation, apoptosis, β -amyloid protein levels, and neuroinflammation in the brain tissues of neonatal naïve and Alzheimer disease (AD) transgenic mice.

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Methods: Six-day-old naïve and AD transgenic (B6.Cg-Tg[amyloid precursor protein swe, PSEN1dE9]85Dbo/J) mice were treated with sevoflurane. The mice were killed at the end of the anesthesia, and the brain tissues were harvested and then subjected to Western blot, immunocytochemistry, enzyme-linked immunosorbent assay, and real-time polymerase chain reaction.

Results: Herein, the authors show for the first time that sevoflurane anesthesia induced caspase activation and apoptosis, altered amyloid precursor protein processing, and increased β -amyloid protein levels in the brain tissues of neonatal mice. Furthermore, sevoflurane anesthesia led to a greater degree of neurotoxicity in the brain tissues of the AD transgenic mice when compared with naïve mice and increased tumor necrosis factor- α levels in the brain tissues of only the AD transgenic mice. Finally, inositol 1,4,5-trisphosphate receptor antagonist 2-aminoethoxydiphenyl borate attenuated sevoflurane-induced caspase-3 activation and β -amyloid protein accumulation *in vivo*.

Conclusion: These results suggest that sevoflurane may induce neurotoxicity in neonatal mice. AD transgenic mice could be more vulnerable to such neurotoxicity. These findings should promote more studies to determine the potential neurotoxicity of anesthesia in animals and humans, especially in children.

What We Already Know about This Topic

- ❖ Anesthetics increase programmed cell death (apoptosis) in the brains of neonatal animals, accompanied by learning deficits
- ❖ Apoptosis and neuroinflammation occur in neurodegenerative disorders including Alzheimer dementia

What This Article Tells Us That Is New

- ❖ In neonatal mice, sevoflurane increased brain cell apoptosis accompanied by neuroinflammation and increased A β expression, and these effects were enhanced in neonatal transgenic Alzheimer dementia mice
- ❖ Anesthetic neurotoxicity in neonatal animals may be enhanced in genetic conditions, which overexpress A β

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A RECENT study by Wilder *et al.*¹ investigated more than 5,000 children and found that children who had early exposure to anesthesia were at an increased risk for developing a learning disability. Furthermore, the risk for the development of a learning disability increases with longer cumulative duration of anesthesia exposure. Another pilot study by Kalkman *et al.*² has also found that children who underwent surgery and anesthesia at younger than 2 yr could be at an increased risk of developing a deviant behavior later in life. These findings suggest that anesthesia may be a significant risk factor for later development of a learning disability and deviant behavior. However, these results cannot reveal whether anesthesia itself contributes to the learning disability and deviant behavior or whether the need for anesthesia is a marker for other unidentified factors that contribute to the development of learning disabilities and deviant behavior. Moreover, recent studies of twin pairs have suggested that there is no evidence for a causal relationship between anesthesia administration and later learning-related outcomes.³ Therefore, there is a need to study the effects of anesthetics, for example, sevoflurane, on biochemical changes associated with cognitive dysfunction.

Several other studies have shown that the commonly used inhalation anesthetics, for example, isoflurane and sevoflurane, may induce apoptosis in brain tissues of neonatal mice.^{4–6} However, anesthesia-induced apoptosis is not the only cause of behavioral abnormalities.⁴ β -Amyloid protein ($A\beta$), the key component of senile plaques in patients with Alzheimer disease (AD),^{7–9} is the hallmark feature of AD-associated dementia and learning or memory dysfunction (reviewed by^{10–12}). In addition, neuroinflammation and elevation of the proinflammatory cytokine tumor necrosis factor (TNF)- α have also been shown to be associated with AD-associated dementia and learning or memory dysfunction.^{13–15} However, the effects of inhalation anesthetics on caspase activation, apoptosis, $A\beta$ levels, and neuroinflammation in neonatal mice remain largely to be determined. Furthermore, there have been no studies to compare the effects of anesthetics on apoptosis, $A\beta$ accumulation, and neuroinflammation between neonatal naïve mice and neonatal AD transgenic mice. Therefore, we set out to determine the effects of the most commonly used inhalation anesthetic, sevoflurane, on caspase activation, apoptosis, levels of $A\beta$, and proinflammatory cytokine TNF- α in the brain tissues of neonatal mice (6 days old) and to determine the underlying mechanisms. Moreover, we compared the neurotoxic effects of sevoflurane in neonatal naïve mice and neonatal AD transgenic mice (B6.Cg-Tg[APP^{swe}, PSEN1^{dE9}]85Dbo/J).

Materials and Methods

Animal Treatments

The animal protocol was approved by the Standing Committee on Animals at Massachusetts General Hospital (Boston, Massachusetts). Naïve mice (C57BL/6J mice [The Jackson Laboratory, Bar Harbor, ME]) and AD transgenic mice

(B6.Cg-Tg[APP^{swe}, PSEN1^{dE9}]85Dbo/J, [The Jackson Laboratory]) were distinguished by genotyping. All animals (3–12 mice per experiment) were 6 days old at the time of anesthesia and were randomized by weight and gender into experimental groups that received either 3 or 2.1% sevoflurane plus 60% oxygen for either 6 or 2 h, and control groups received 60% oxygen for 6 or 2 h at identical flow rates in identical anesthetizing chambers. We chose sevoflurane anesthesia because a recent study by Satomoto *et al.*⁶ indicated that anesthesia with 3% sevoflurane plus 60% oxygen for 6 h does not significantly alter blood gas and brain blood flow, which is consistent with our pilot studies. The mortality rate of the mice after the administration of anesthesia with 3% sevoflurane plus 60% oxygen for 6 h in the current studies was approximately 10–15%, which could be because of the higher than clinically relevant concentration of sevoflurane. We used this high concentration of sevoflurane anesthesia to illustrate the difference of sevoflurane-induced neurotoxicity between neonatal naïve and AD transgenic mice. Moreover, we also assessed the effects of anesthesia with 2.1% sevoflurane, a more clinically relevant concentration of sevoflurane (which did not cause the death of the mice), on the effects of caspase-3 activation and $A\beta$ levels in the brain tissues of neonatal mice. Anesthetic and oxygen concentrations were measured continuously (Datex, Tewksbury, MA), and the temperature of the anesthetizing chamber was controlled to maintain the rectal temperature of the mice at $37^{\circ} \pm 0.5^{\circ}\text{C}$. In the interaction studies, the inositol trisphosphate receptor (IP3R) antagonist 2-aminoethoxydiphenyl borate (2-APB) (5 and 10 mg/kg) was administered to the mice *via* intraperitoneal injection 10 min before the anesthetic was administered. 2-APB was first dissolved in dimethyl sulfoxide to 20 $\mu\text{g}/\mu\text{l}$ and then diluted with saline to 0.25 $\mu\text{g}/\mu\text{l}$ (1:80 dilution) and to 0.5 $\mu\text{g}/\mu\text{l}$ (1:40 dilution).

Tissue Preparation

Immediately after sevoflurane anesthesia, the mouse was decapitated, and the brain cortex was harvested. The brain tissues were homogenized in an immunoprecipitation buffer (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 2 mM ethylenediaminetetraacetic acid, and 0.5% Nonidet P-40) plus protease inhibitors ([1 $\mu\text{g}/\text{ml}$ aprotinin, 1 $\mu\text{g}/\text{ml}$ leupeptin, and 1 $\mu\text{g}/\text{ml}$ pepstatin A]; [Roche, Indianapolis, IN]). The lysates were collected, centrifuged at 13,000 rpm for 15 min, and quantified for total proteins by using the bicinchoninic acid protein assay kit (Pierce, Iselin, NJ).

Western Blots Analysis

The harvested brain tissues were subjected to Western blots as described by Xie *et al.*¹⁶ Briefly, 60 μg of each lysate was separated on sodium dodecyl sulfate polyacrylamide gel electrophoresis gels and transferred to polyvinylidene difluoride blots (Bio-Rad, Hercules, CA) using a semidry electrotransfer system (Amersham Biosciences, San Francisco, CA). The blot was incubated overnight at 4°C with primary antibodies, followed by washes and incubation with appropriate secondary antibodies,

and visualized with a chemoluminescence system. A caspase-3 antibody (1:1,000 dilution; Cell Signaling Technology, Danvers, MA) was used to recognize the caspase-3 fragment (17–20 kDa) resulting from cleavage at aspartate position 175 and full-length (FL) caspase-3 (35–40 kDa). TNF- α levels were recognized by antibody ab6671 (26 kDa; 1:1,000; Abcam, Cambridge, MA). The antibody to nontargeted protein β -actin was used to control for loading differences in total protein amounts. The figures showing blots with only the caspase-3 fragment are the same Western blots with extended exposure time during the development of the film. The signal of the Western blot band was detected using Molecular Imager VersaDoc MP 5000 System (Bio-Rad). The intensity of signals was analyzed using a Bio-Rad image program (Quantity One) and a National Institutes of Health Image Version 1.37 (National Institutes of Health, Bethesda, MD). We quantified Western blots using two steps. First, we used levels of β -actin to normalize (*e.g.*, determining the ratio of FL caspase-3 amount to β -actin amount) the levels of proteins to control for loading differences in total protein amounts. Second, we presented the changes in the levels of proteins in the mice treated with sevoflurane as the percentage or fold of those in the mice treated with control conditions. One hundred percent or onefold change in the protein levels in this article refers to the control levels for comparison with experimental conditions.

Immunoblot Detection of A β

The brain samples were homogenized (150 mM NaCl with a protease inhibitor cocktail in 50 mM Tris, pH 8.0) and centrifuged (65,000 rpm for 45 min), and then the supernatant was removed. The pellet was then resuspended by sonication and incubated for 15 min in homogenization buffer containing 1% sodium dodecyl sulfate. After pelleting of insoluble material (18,000 rpm for 15 min), the sodium dodecyl sulfate extract was electrophoresed on sodium dodecyl sulfate polyacrylamide gel electrophoresis (4–12% bis-tris polyacrylamide gel; Invitrogen, Carlsbad, CA), blotted to polyvinylidene fluoride membrane, and probed with a 1:200 dilution of A β 6E10 (Convance, Berkeley, CA).^{16,17}

Quantification of A β Using a Sandwich Enzyme-linked Immunosorbent Assay

The A β 42 and A β 40 levels in the brain tissues of AD transgenic mice were measured by using sandwich enzyme-linked immunosorbent assay (ELISA). The Human A β (1–42) ELISA kit or Human A β (1–40) ELISA kit (Wako, Richmond, VA) was used to detect the levels of A β 42 or A β 40, respectively. The monoclonal antibody BAN50, the epitope of which is human A β (1–16), was coated on 96-well plates and acted as a capture antibody for the *N*-terminal portion of human A β 42 or human A β 40. Captured human A β 42 or human A β 40 was recognized by another antibody BC05 or BA27, which specifically detected the C-terminal portion of A β 42 or A β 40, respectively. The 96-well plates were incubated overnight at 4°C with test samples and control, and then BC05 or BA27 was added. The plates were then devel-

oped with tetramethylbenzidine reagent and terminated by stop solution, and well absorbance was measured at 450 nm. A β 42 and A β 40 levels in the test samples were determined by comparing the results with signals from the controls using the standard curve. The mouse brain tissue samples were prepared by using the same method in the section of the immunoblot detection of A β .

Terminal Deoxynucleotidyl Transferase dUTP Nick End Labeling Staining Assay

Mice were perfused transcardially with 0.1 M phosphate buffer with a pH of 7.4 followed by 4% paraformaldehyde in a 0.1 M phosphate-buffered saline immediately after the administration of anesthesia with 3% sevoflurane plus 60% oxygen for 6 h. The mouse brain tissues were removed and exposed to immersion fixation for 24 h at 4°C in 4% paraformaldehyde, and then 5- μ m paraffin-embedded sections were made from the brain tissues. TMR red kit (Roche, Palo Alto, CA) was used for terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining. Briefly, the brain sections were incubated in a permeabilization solution and then incubated with a TUNEL reaction mixture. Finally, the sections were incubated with 10 μ g/ml Hoechst 33342 in a humidified dark chamber. The sections from the same brain areas between control group mice and the sevoflurane-treated mice were then analyzed in a mounting medium under a fluorescence microscope. The TUNEL-positive cells and total cells in five areas of the brain section from each of the mice in the experiments were counted under a 20 \times objective microscope lens by an investigator who was blinded to the experiments.

For the double immunocytochemistry staining to identify the cell type of the TUNEL-positive cells, the mouse brain tissues were quickly removed after sevoflurane anesthesia, put into a container with dry ice and ethanol, and then kept in a –80°C freezer. Five-micrometer frozen sections were cut using a cryostat. The sections were fixed successively with 100% methanol at –20°C for 20 min and incubated with permeabilization solution (7.8% gelatin and 1.25 ml saponin [10%] in 500 ml phosphate-buffered saline) for 30 min. The sections were incubated in 10% donkey serum in permeabilization solution for 30 min and then incubated with antibody for NeuN to identify neurons (1:500, mab 377, anti-mouse; Millipore, Billerica, MA), antibody for Glial fibrillary acidic protein to identify astrocytes (1:100, ab16997, anti-rabbit; Abcam), and antibody for Iba1 to identify microglia cells (1:100, ab5076, anti-goat; Abcam) at 4°C overnight. Then sections were exposed to secondary antibodies Alexa Fluor[®]488 goat antimouse immunoglobulin G (1:1000; Invitrogen), Alexa Fluor[®]488 goat anti-rabbit immunoglobulin G (1:1000; Invitrogen), and donkey anti-goat immunoglobulin G-Cy2 (1:100; Jackson ImmunoResearch Inc., West Grove, PA) for 1 h at 37°C in a dark chamber followed by TUNEL staining. The sections were counterstained with 10 μ g/ml Hoechst 33342 at room temperature for 10 min. Finally, the sections were mounted and immediately viewed using a fluorescence microscope.

Reverse Transcriptase Polymerase Chain Reaction

Real-time reverse transcriptase polymerase chain reaction was carried out using the QuantiTect SYBR Green real-time polymerase chain reaction kit (Qiagen, Valencia, CA). TNF- α messenger ribonucleic acid levels were determined and standardized with glyceraldehyde-3-phosphate dehydrogenase as an internal control. Primers of mouse TNF- α (ID

No., QT00104006) and mouse glyceraldehyde-3-phosphate dehydrogenase (ID No., QT01658692) were purchased from Qiagen.

Statistics

Given the potential presence of background caspase-3 activation and apoptosis in the brain tissues of neonatal mice, we did not

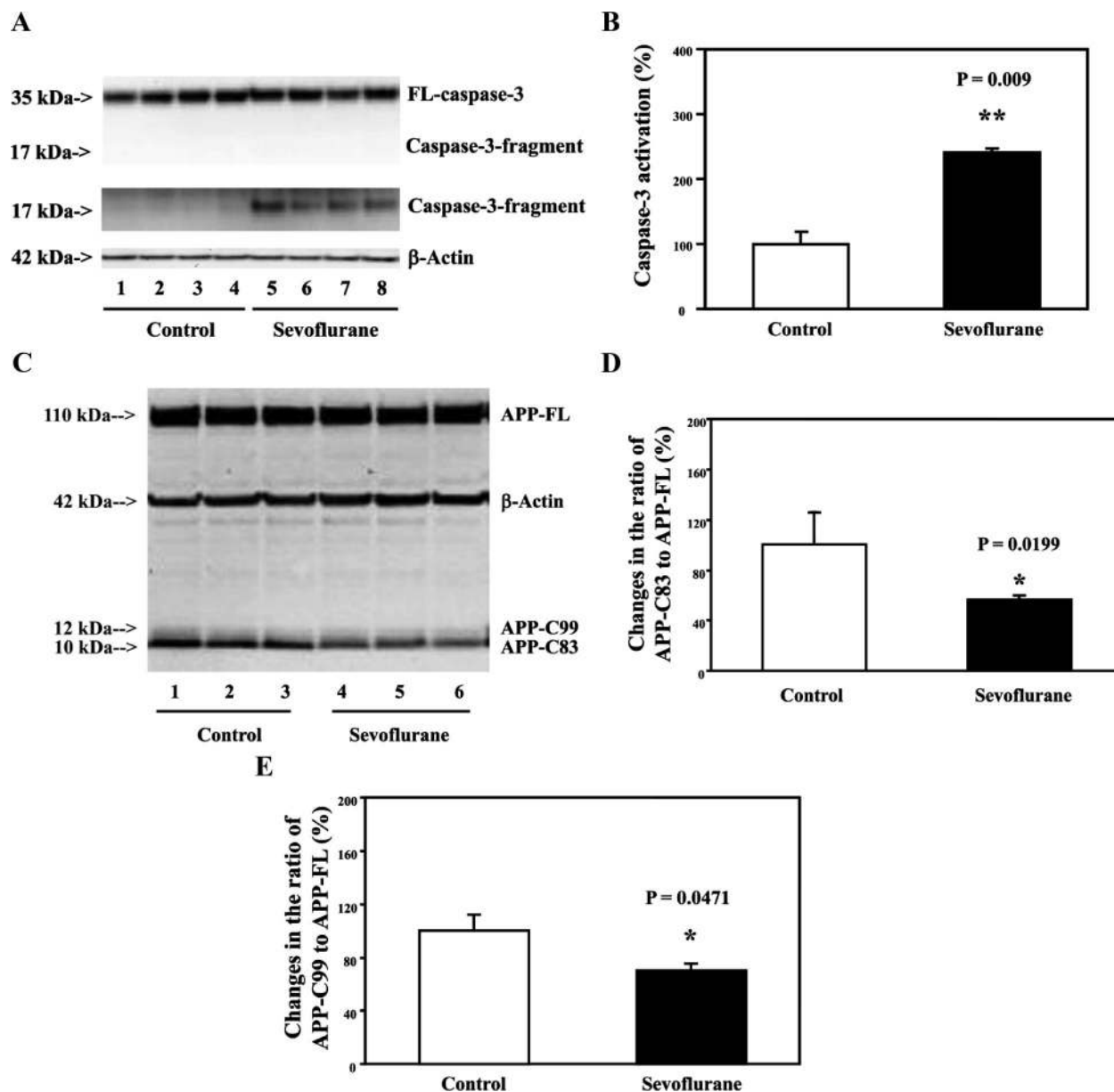


Fig. 1. Anesthesia with 3% sevoflurane for 6 h induces caspase-3 activation and amyloid precursor protein (APP) processing in the brain tissues of neonatal naïve mice. (A) Anesthesia with 3% sevoflurane for 6 h (lanes 5–8) induces caspase-3 cleavage (activation) when compared with the control condition (lanes 1–4) in the brain tissues of neonatal naïve mice. (B) Caspase-3 activation assessed by quantifying the ratio of cleaved (activated) caspase-3 fragment (17–20 kDa) to FL-caspase-3 (35–40 kDa) in the Western blot. Quantification of the Western blot shows that sevoflurane anesthesia (*black bar*) induces caspase-3 activation compared with the control condition (*white bar*). (C) Sevoflurane anesthesia (lanes 4–6) reduces the levels of APP-C83 and APP-C99 when compared with the control condition (lanes 1–3). (D) Quantification of the Western blot shows that sevoflurane anesthesia (*black bar*) decreases the ratio of APP-C83 to APP-FL when compared with the control condition (*white bar*). (E) Quantification of the Western blot shows that sevoflurane anesthesia (*black bar*) decreases the ratio of APP-C99 to APP-FL when compared with the control condition (*white bar*). We have averaged the results from six independent experiments. FL = full length. * $P < 0.05$; ** $P < 0.01$.

use absolute values to describe changes in caspase-3 activation and apoptosis. Instead, caspase-3 activation and cell apoptosis were presented as a percentage or fold of those in the control group in naïve mice or AD transgenic mice. One hundred percent or onefold caspase-3 activation or apoptosis refers to the control levels for the purposes of comparison with experimental conditions. We presented the changes in the levels of caspase-3 activation, apoptosis, levels of A β , and TNF- α in treated mice as percentages or folds of those in mice in the control condition. Data were expressed as mean \pm SD. The number of samples varied from 3 to 12, and the samples were normally distributed. A randomization table generated using a computer random number generator and stored in a Microsoft Excel spreadsheet was used to randomize animals to conditions. ANOVA or Student *t* test was used to compare the differences from the control group. Only a single measurement of each outcome value was collected from each experimental animal. As a result, no repeated measurement was involved in the analysis. *Post hoc* adjustment for multiple comparisons was conducted using the Bonferroni method. *P* values of less than 0.05 (* or #) and 0.01 (** or ##) were considered statistically significant. The significance testing was two tailed, and we have used SAS software (Cary, NC) to analyze the data.

Results

Sevoflurane Induced Caspase-3 Activation and Amyloid Precursor Protein Processing in the Brain Tissues of Neonatal Mice

We have previously reported that the commonly used inhalation anesthetic, sevoflurane, can cause neurotoxicity by inducing apoptosis and enhancing A β levels *in vitro* and in brain tissues of adult naïve mice.¹⁸ But the effects of sevoflurane on apoptosis, A β accumulation, and neuroinflammation in neonatal mice remain largely to be determined. Furthermore, the comparison of these effects between neonatal naïve mice and AD transgenic mice has not been done. Therefore, we set out to determine and compare the effects of sevoflurane on apoptosis, A β accumulation, and neuroinflammation in neonatal (6 days old) naïve (C57BL/6J) and AD transgenic (B6.Cg-Tg[APP^{swe}, PSEN1^{dE9}]85Dbo/J) mice.

Caspase-3 activation is one of the final steps of cellular apoptosis.¹⁹ Therefore, we assessed the effects of sevoflurane on caspase-3 activation in the brain tissues of neonatal naïve mice by quantitative Western blot analyses. The 6-day-old neonatal naïve mice were treated with 3% sevoflurane plus 60% oxygen for 6 h, and the brain tissues were harvested at the end of the experiment and subjected to Western blot analysis by which caspase-3 antibody was used to detect both caspase-3 fragment (17–20 kDa) and FL-caspase-3 (35–40 kDa). Caspase-3 immunoblotting showed visible increases in the protein levels of caspase-3 fragment after sevoflurane anesthesia when compared with the control condition (fig. 1A). The blot with only the caspase-3 fragment is the same Western blot with extended exposure time during the develop-

ment of the film. Quantification of the Western blot, by determining the ratio of cleaved (activated) caspase-3 fragment (17–20 kDa) to FL-caspase-3 (35–40 kDa), revealed that sevoflurane anesthesia led to a 242% increase in caspase-3 cleavage (activation) when compared with the control condition (fig. 1B; *P* = 0.009).

Given that sevoflurane-induced caspase activation and apoptosis may lead to alterations in amyloid precursor protein (APP) processing *in vitro*,¹⁸ we asked whether sevoflurane can also alter APP processing in the brain tissues of neonatal mice. APP immunoblotting showed visible decreases in the protein levels of APP-C83 and APP-C99 after the administration of anesthesia with 3% sevoflurane for 6 h when compared with control conditions (fig. 1C). The quantification of the Western blot, by determining the ratio of APP-C-terminal fragments (APP-C83 fragment [10 kDa] and APP-C99 fragment [12 kDa]) to APP-FL (110 kDa), revealed that sevoflurane anesthesia led to a 45% and 33% decrease in the ratio of APP-C83 to APP-FL (fig. 1D, *P* = 0.0199) and APP-C99 to APP-FL (fig. 1E, *P* = 0.0471), respectively, when compared with the control condition in the brain tissues of neonatal naïve mice. These results suggest that sevoflurane can alter the APP processing by decreasing the levels of APP-C-terminal fragments (APP-C83 and APP-C99).

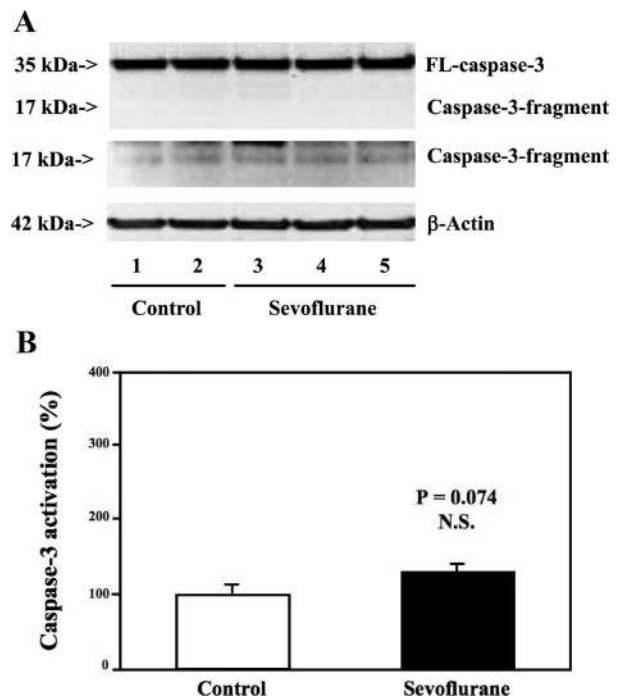


Fig. 2. Anesthesia with 3% sevoflurane for 2 h does not induce caspase-3 activation in the brain tissues of neonatal naïve mice. (A) Anesthesia with 3% sevoflurane for 2 h (lanes 3–5) does not induce caspase-3 cleavage (activation) when compared with the control condition (lanes 1 and 2) in the brain tissues of neonatal naïve mice. (B) Quantification of the Western blot shows that anesthesia with 3% sevoflurane for 2 h (black bar) does not induce caspase-3 activation compared with the control condition (white bar). We have averaged the results from four independent experiments. FL = full length; N.S. = not significant.

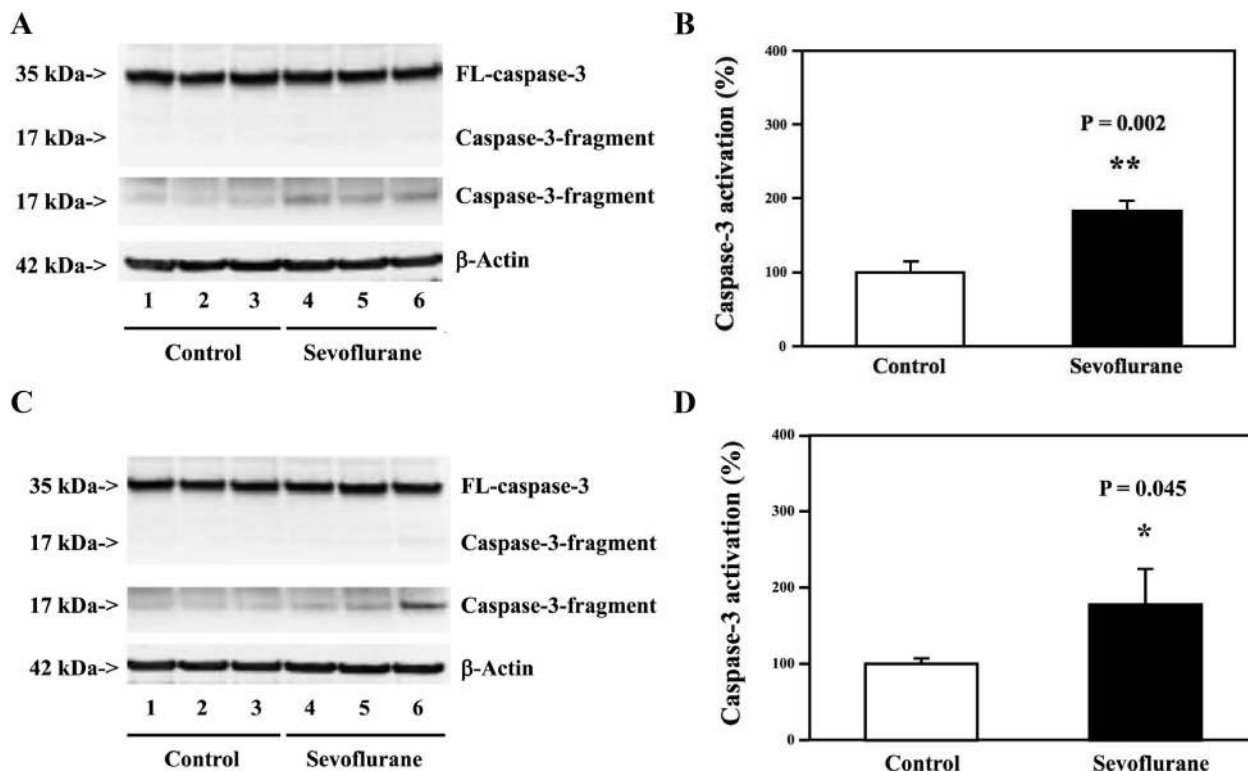


Fig. 3. Anesthesia with 2.1% sevoflurane for 6 h induces caspase-3 activation in the brain tissues of neonatal naïve and Alzheimer disease (AD) transgenic mice. (A) Anesthesia with 2.1% sevoflurane for 6 h (lanes 4–6) induces caspase-3 cleavage (activation) when compared with control condition (lanes 1–3) in the brain tissues of neonatal naïve mice. (B) Caspase-3 activation assessed by quantifying the ratio of the cleaved (activated) caspase-3 fragment (17–20 kDa) to FL-caspase-3 (35–40 kDa) in the Western blot. Quantification of the Western blot shows that sevoflurane anesthesia (*black bar*) induces caspase-3 activation compared with the control condition (*white bar*). (C) Anesthesia with 2.1% sevoflurane for 6 h (lanes 4–6) induces caspase-3 cleavage (activation) when compared with the control condition (lanes 1–3) in the brain tissues of neonatal AD transgenic mice. (D) Quantification of the Western blot shows that sevoflurane anesthesia (*black bar*) induces caspase-3 activation compared with the control condition (*white bar*), normalized to β -actin levels. We have averaged the results from 10 independent experiments. FL = full length. * $P < 0.05$; ** $P < 0.01$.

Next, we investigated whether anesthesia with the same concentration (3%) of sevoflurane but for a shorter treatment time can also induce caspase-3 activation in the brain tissues of neonatal naïve mice. We found that anesthesia with 3% sevoflurane for 2 h did not increase caspase-3 activation (figs. 2A and B): 100% *versus* 128%, $P = 0.074$. These findings suggest that the commonly used inhalation anesthetic sevoflurane can induce caspase-3 activation in the brain tissues of neonatal mice in a time-dependent manner.

Finally, we investigated whether a lower concentration of sevoflurane with the same treatment time (6 h) can induce caspase-3 activation in the brain tissues of neonatal mice. We were able to show that anesthesia with 2.1% sevoflurane for 6 h induced caspase-3 activation in the brain tissues of neonatal naïve mice (figs. 3A and B): 100% *versus* 183%, $P = 0.002$, and of neonatal AD transgenic mice (figs. 3C and D): 100% *versus* 178%, $P = 0.045$. These results suggest that a specific length of treatment time (*e.g.*, 6 h) of sevoflurane anesthesia may be needed before we can observe sevoflurane-induced caspase activation *in vivo*.

Sevoflurane Induced a Greater Degree of Caspase-3 Activation in Neonatal AD Transgenic Mice

We next investigated whether the same sevoflurane anesthesia can also induce caspase-3 activation in the brain tissues of neonatal AD transgenic mice. The APP^{swe}/PSEN1^{dE9} mouse is a particularly aggressive AD transgenic mouse model generated with mutant transgenes for APP (APP^{swe}: KM594/5NL) and presenilin 1 (deletion of exon 9 [dE9]).²⁰ Therefore, we assessed and compared the effects of anesthesia with 3% sevoflurane for 6 h on caspase-3 activation in the brain tissues of 6-day-old naïve and AD transgenic mice. Caspase-3 immunoblotting showed visible increases in the protein levels of caspase-3 fragment after sevoflurane anesthesia when compared with the control condition (fig. 4A) in both neonatal naïve (lane 1 *vs.* lane 2) and AD transgenic mice (lane 3 *vs.* lane 4). The blot with only the caspase-3 fragment is the same Western blot with extended exposure time during the development of the film. Moreover, caspase-3 immunoblotting showed that sevoflurane anesthesia induced a more visible increase in the band of caspase-3 fragment in neonatal AD transgenic mice than that in neonatal naïve mice. We quantified the Western blot using fold change.

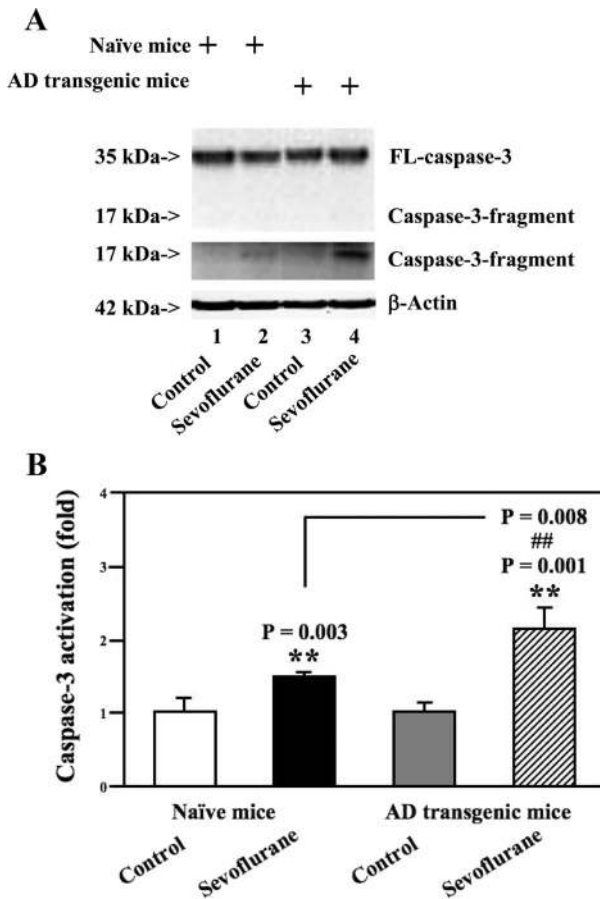


Fig. 4. Anesthesia with 3% sevoflurane for 6 h induces a greater degree of caspase-3 activation in the brain tissues of neonatal Alzheimer disease (AD) transgenic mice than that in neonatal naïve mice. (A) Anesthesia with sevoflurane anesthesia (lanes 2 and 4) induces caspase-3 activation when compared with the control condition (lanes 1 and 3) in naïve mice and AD transgenic mice, respectively. Sevoflurane anesthesia induces a greater degree of caspase-3 activation in AD transgenic mice (lane 4) than in naïve mice (lane 2). (B) Quantification of the Western blot shows that sevoflurane anesthesia (black bar and hatched bar) induces caspase-3 activation compared with the control condition (white bar and gray bar) in both neonatal naïve and AD transgenic mice, respectively. Sevoflurane anesthesia induces a greater degree of caspase activation in AD transgenic mice (hatched bar) than in naïve mice (black bar). We have averaged the results from four independent experiments. FL = full length. ** $P < 0.01$; ## $P < 0.01$.

Onefold in the article referred to the ratio of the activated (cleaved) caspase-3 fragment to FL-caspase-3 in the control group of either naïve mice or AD transgenic mice. The quantification of the Western blot revealed that sevoflurane anesthesia induced caspase-3 activation in the brain tissues of both neonatal naïve mice (1-fold vs. 1.48-fold, $P = 0.003$) and AD transgenic mice (1-fold vs. 2.45-fold, $P = 0.001$) (fig. 4B). Moreover, sevoflurane anesthesia induced a greater degree of caspase-3 activation in the brain tissues of neonatal AD transgenic mice than that in neonatal naïve mice: 2.13-fold vs. 1.48-fold, $P = 0.008$ (fig. 4B). These findings suggest that sevoflurane may

cause a greater degree of neurotoxicity in neonatal AD transgenic mice than that in neonatal naïve mice.

Sevoflurane Induced More TUNEL-positive Cells in Neonatal AD Transgenic Mice

Given that caspase-3 activation alone may not represent apoptotic cell damage,²¹ we also assessed the effects of 3% sevoflurane plus 60% oxygen for 6 h on cellular apoptosis using the TUNEL study. We quantified the TUNEL-positive cells using fold change. Onefold in the article referred to the ratio of TUNEL-positive cells to the total cells in the control group of either naïve mice or AD transgenic mice. We found that sevoflurane anesthesia increased TUNEL-positive cells (apoptosis) when compared with the control condition in the brain tissues of neonatal naïve mice (figs. 5A and B; 1-fold vs. 2.08-fold; $P = 0.001$) and neonatal AD transgenic mice (figs. 5A and B; 1-fold vs. 2.13-fold; $P = 0.0004$). Consistent with findings in studies on caspase-3 activation (fig. 4), sevoflurane anesthesia induced more TUNEL-positive cells (apoptosis) in the brain tissues of neonatal AD transgenic mice than those in neonatal naïve mice: 2.45-fold vs. 2.08-fold, $P = 0.012$ (figs. 5A and B). Furthermore, double immunocytochemistry staining indicated that the majority of the TUNEL-positive cells in the brain tissues of neonatal AD transgenic mice after sevoflurane anesthesia were neurons (NeuN staining) but not microglia cells (Iba1 staining) or astrocytes (Glial fibrillary acidic protein staining) (fig. 5C). Taken together, these findings suggest that sevoflurane can induce apoptosis in the neurons of the brain of neonatal mice. These findings also suggest that neonatal AD transgenic mice are more vulnerable to such sevoflurane-induced neurotoxicity.

Sevoflurane Enhanced A β Levels in Neonatal Naïve and AD Transgenic Mice

Sevoflurane has been shown to induce apoptosis, which then leads to A β accumulation *in vitro* and in the brain tissues of adult mice.¹⁸ Given that sevoflurane can induce apoptosis and alter APP processing in the brain tissues of neonatal mice, we next asked whether sevoflurane can also enhance A β levels in the brain tissues of these neonatal mice. The harvested brain tissues were subjected to Western blot analysis, by which antibody 6E10 was used to detect A β levels as described in our previous studies.¹⁶ A β immunoblotting revealed that an anesthesia administration of 3% sevoflurane for 6 h caused visible increases in A β levels in the Western blot when compared with control conditions (fig. 6A). Quantification of the Western blot revealed that sevoflurane anesthesia increased A β levels in both neonatal naïve mice: 100% vs. 401%, $P = 0.023$, and AD transgenic mice: 287% vs. 491%, $P = 0.042$, when compared with the control condition (fig. 6B). Note that the baseline A β level in the brain tissues of neonatal AD transgenic mice was higher than those in neonatal naïve mice: 100% vs. 287%, $P = 0.009$. Furthermore, sandwich ELISA identified that sevoflurane anesthesia increased A β 42 levels: 100% vs. 233%, $P = 0.007$ (fig. 6C), but not A β 40 levels (fig. 6D), in the

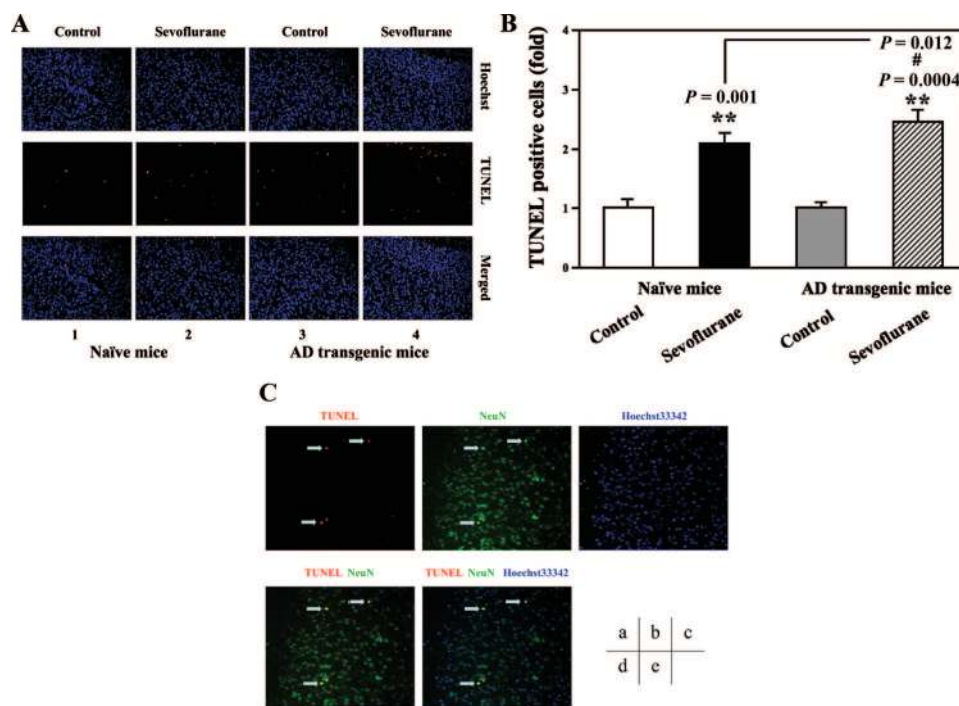


Fig. 5. Anesthesia with 3% sevoflurane for 6 h induces more TUNEL-positive cells in the brain tissues of neonatal Alzheimer disease (AD) transgenic mice than in neonatal naïve mice. (A) Sevoflurane anesthesia (columns 2 and 4) increases TUNEL-positive cells (apoptosis) when compared with the control condition (columns 1 and 3) in the brain tissues of neonatal naïve and AD transgenic mice, respectively. Note that sevoflurane anesthesia causes more TUNEL-positive cells (apoptosis) in neonatal AD transgenic mice (column 4) when compared with neonatal naïve mice (column 2). (B) Quantification of the TUNEL image shows that sevoflurane anesthesia (black bar and hatched bar) increases TUNEL-positive cells (apoptosis) compared with the control condition (white bar and gray bar) in neonatal naïve mice and AD transgenic mice, respectively. Sevoflurane anesthesia induces more TUNEL-positive cells (apoptosis) in neonatal AD transgenic mice (hatched bar) when compared with neonatal naïve mice (black bar). (C) Immunocytochemistry imaging studies show that the majority of TUNEL-positive cells (e.g., indicated by the arrows) are neurons detected by positive NeuN staining. (a) TUNEL (apoptosis) staining; (b) NeuN (neuron) staining; (c) Hoechst33342 (nuclei) staining; (d) merged picture of TUNEL (apoptosis) staining and NeuN (neuron) staining; (e) merged picture of TUNEL (apoptosis) staining, NeuN (neuron) staining, and Hoechst 33342 (nuclei) staining (original magnification $\times 200$). We have averaged the results from five independent experiments. TUNEL = terminal deoxynucleotidyl transferase deoxyuridine triphosphate nick end labeling. ** $P < 0.01$ or # $P < 0.05$.

brain tissues of neonatal AD transgenic mice, but not in the brain tissues of neonatal naïve mice (data not shown). In addition, we were able to show that anesthesia with 2.1% sevoflurane for 6 h also increased the levels of A β 42 in the brain tissues of neonatal AD transgenic mice (data not shown). Taken together, these results suggest that sevoflurane may specifically increase A β 42 levels in the brain tissues of neonatal mice.

IP3R Antagonist 2-Aminoethoxydiphenyl Borate Attenuated Sevoflurane-induced Caspase-3 Activation and Increases in A β Levels

The underlying mechanism by which inhalation anesthetics induce apoptosis and enhance A β accumulation is largely unknown. Several studies have shown that the inhalation anesthetic isoflurane may increase cytosolic calcium levels, leading to apoptosis.^{22,23} Therefore, we investigated whether sevoflurane-induced apoptosis and A β accumulation in neonatal mice are also associated with IP3R. For this purpose, we assessed the effects of the IP3R antagonist 2-APB on sevoflu-

rane-induced caspase-3 activation and A β accumulation in the brain tissues of neonatal naïve mice. As can be seen in figure 7A, anesthesia with 3% sevoflurane for 6 h led to caspase-3 activation when compared with the control condition. Five milligram per kilogram (lanes 6–8) and 10 mg/kg (lanes 4 and 5) of 2-APB attenuated sevoflurane-induced caspase-3 activation in a dose-dependent manner. Quantification of the Western blot showed that sevoflurane anesthesia induced caspase-3 activation: 100% versus 356%, $P = 0.002$ (fig. 7B). The IP3R antagonist 2-APB attenuated sevoflurane-induced caspase-3 activation in a dose-dependent manner, 5 mg/kg 2-APB (gray bar): 356% versus 149%, $P = 0.001$; 10 mg/kg 2-APB (hatched bar): 356% versus 115%, $P = 0.005$. Moreover, we were able to show that 2-APB also attenuated sevoflurane-induced increases in A β levels (figs. 7C and D), 304% versus 157%, $P = 0.042$. These findings suggest that IP3R may be involved in sevoflurane-induced caspase activation, apoptosis, and A β accumulation.

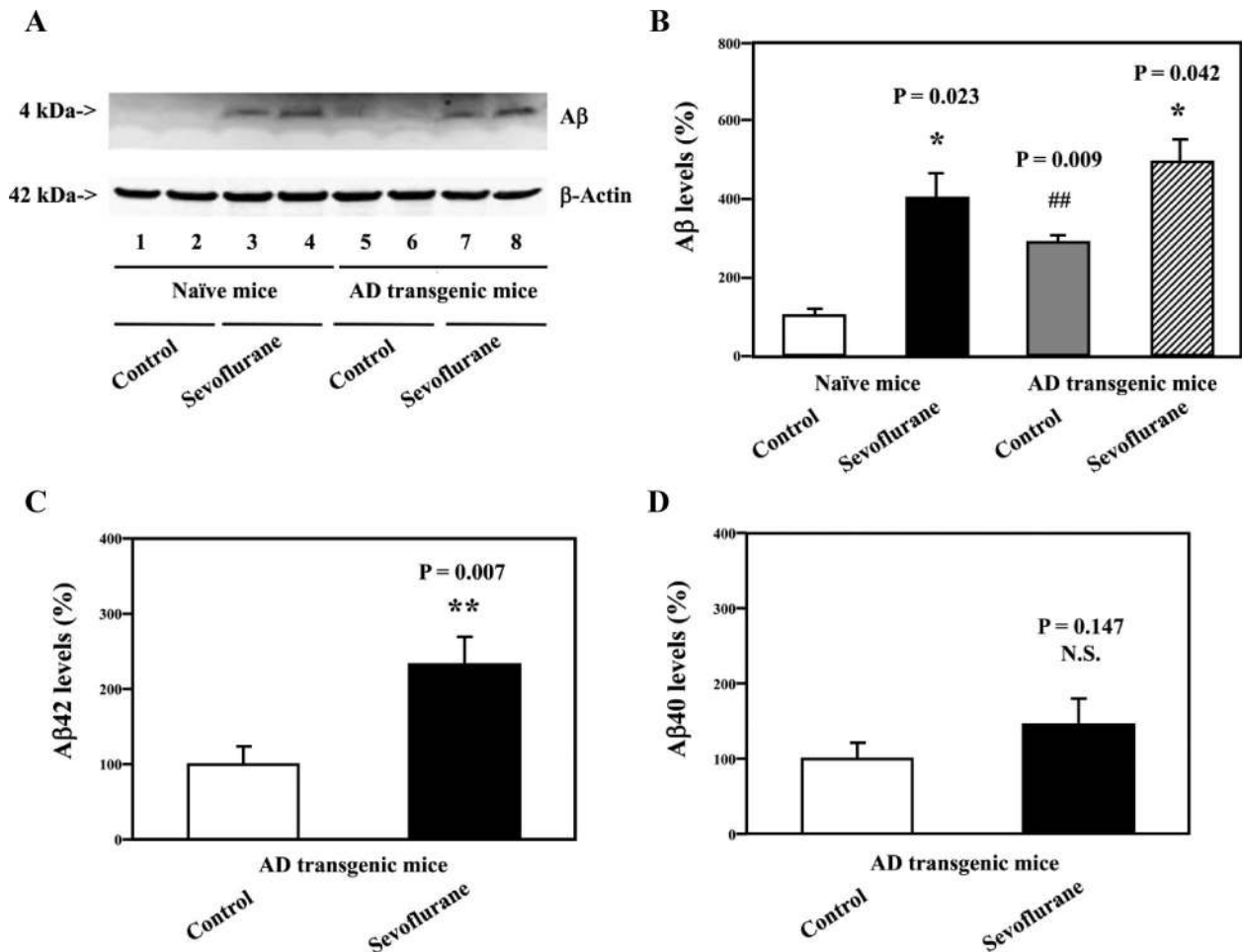


Fig. 6. Anesthesia with 3% sevoflurane for 6 h increases A β levels in the brain tissues of neonatal naïve and Alzheimer disease (AD) transgenic mice. (A) Sevoflurane anesthesia (lanes 3 and 4 and lanes 7 and 8) increases A β levels when compared with the control condition (lanes 1 and 2 and lanes 5 and 6) in the brain tissues of neonatal naïve and AD transgenic mice, respectively. (B) Quantification of the Western blot shows that sevoflurane anesthesia increases A β levels (black bar and hatched bar) when compared with the control condition (white bar and gray bar) in the brain tissues of neonatal naïve mice and AD transgenic mice, respectively. (C) Sandwich enzyme-linked immunosorbent assay (ELISA) shows that sevoflurane anesthesia increases A β 42 levels in the brain tissues of AD transgenic mice. (D) ELISA sandwich shows that sevoflurane anesthesia does not increase A β 40 levels in the brain tissues of AD transgenic mice. We have averaged the results from six independent experiments. A β = β -amyloid protein; N.S. = not significant. * $P < 0.05$; ** $P < 0.01$; ## $P < 0.01$.

Sevoflurane Increased TNF- α Levels in Neonatal AD Transgenic Mice

Increasing evidence suggests that neurons and microglia cells can produce inflammatory mediators including proinflammatory cytokine TNF- α .²⁴ TNF- α is a death-inducing cytokine, which can induce both apoptosis and necrosis through receptor-interacting protein 3, a protein kinase.²⁵ Therefore, we assessed the effects of sevoflurane on neuroinflammation by determining TNF- α levels in the brain tissues of neonatal naïve and AD transgenic mice after administration of 3% sevoflurane anesthesia for 6 h. We were able to show that sevoflurane anesthesia increased protein levels (figs. 8A and B, 100% vs. 219%, $P = 0.001$) and messenger RNA levels (fig. 8C, $P = 0.002$) of TNF- α levels in the brain tissues of neonatal AD transgenic mice but not in the brain tissues of neonatal naïve mice (figs. 8D–F) in the current experiments. These results suggest that sevoflurane may increase TNF- α levels by enhancing its gener-

ation in the brain tissues of neonatal AD transgenic mice, leading to neuroinflammation.

Discussion

Several studies have suggested that anesthesia may be a significant risk factor in children for the later development of learning disabilities and/or deviant behavior.^{1,2} However, it is still possible that the need for anesthesia is a marker for other unidentified factors, rather than anesthesia itself, that contributes to the development of the learning disability and/or deviant behavior. Thus, it is important to assess the effects of sevoflurane, the most commonly used inhalation anesthetic (especially in pediatric patients), on the biochemical changes that are associated with cognitive dysfunction in neonatal mice, which include apoptosis, A β accumulation, and neuroinflammation.

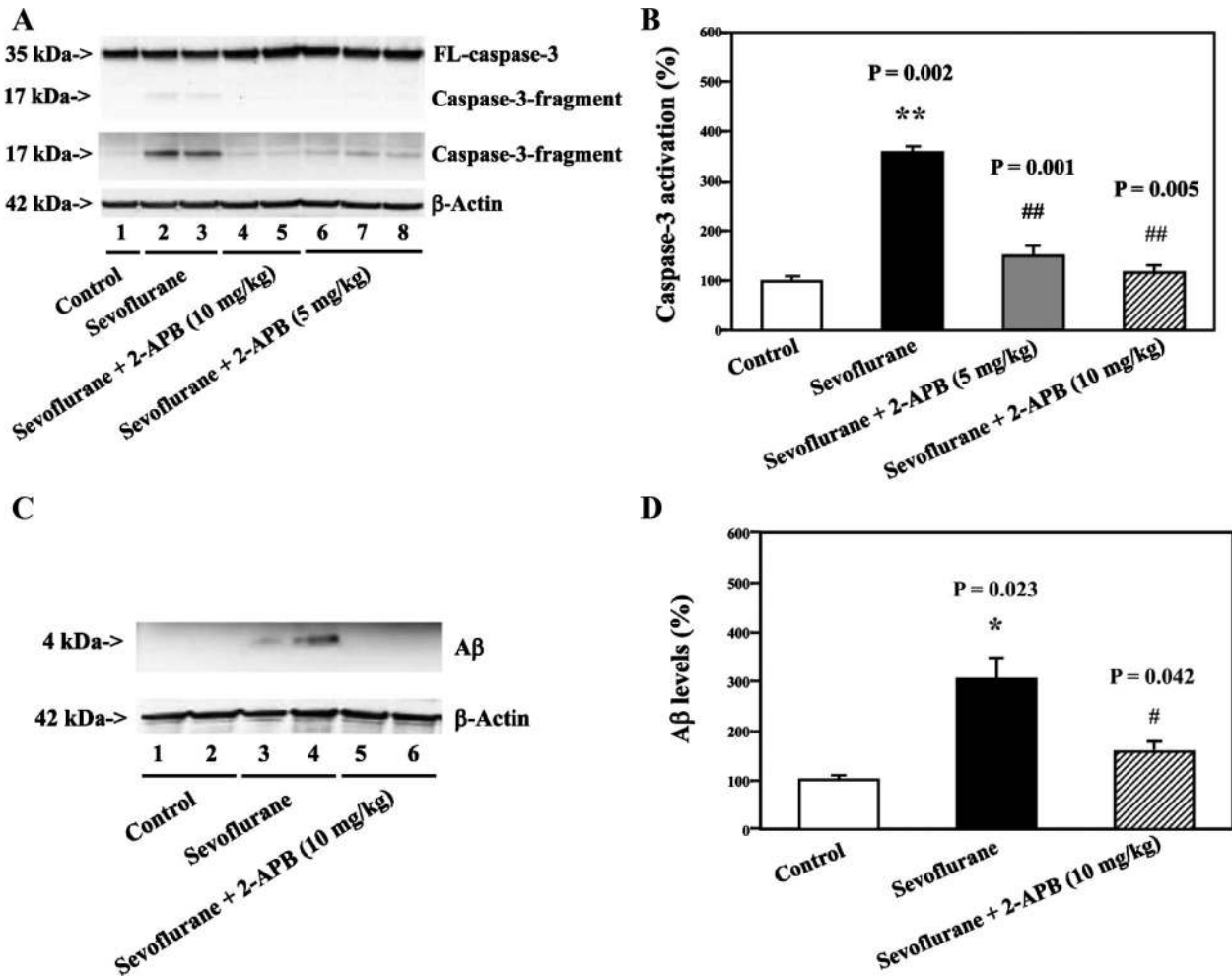


Fig. 7. 2-Aminoethoxydiphenyl borate (2-APB) attenuates sevoflurane-induced caspase-3 activation and A β accumulation in the brain tissues of neonatal naïve mice. (A) Anesthesia with 3% sevoflurane for 6 h (lanes 2 and 3) induces caspase-3 cleavage (activation) when compared with the control condition (lane 1). 2-APB (5 mg/kg: lanes 6 to 8; and 10 mg/kg: lanes 4 and 5) attenuates sevoflurane-induced caspase-3 activation. (B) Quantification of the Western blot shows that sevoflurane anesthesia (black bar) induces caspase-3 activation compared with the control condition (white bar). 2-APB (5 mg/kg: gray bar; and 10 mg/kg: hatched bar) attenuates sevoflurane-induced caspase-3 activation (black bar). (C) Sevoflurane anesthesia (lanes 3 and 4) increases A β levels when compared with the control condition (lanes 1 and 2). 2-APB treatment (lanes 5 and 6) attenuates sevoflurane-induced increases of A β levels (lanes 3 and 4). (D) Quantification of the Western blot shows that sevoflurane anesthesia (black bar) increases A β levels compared with the control condition (white bar). 2-APB treatment (10 mg/kg; hatched bar) attenuates sevoflurane-induced increase of A β (black bar). We have averaged the results from four independent experiments. A β = β -amyloid protein; FL = full length. * $P < 0.05$ or # $P < 0.05$; ** $P < 0.01$ or ## $P < 0.01$.

We have shown in the current studies that anesthesia with 3% or 2.1% sevoflurane for 6 h, but not 3% sevoflurane for 2 h, can induce caspase activation and apoptosis, alter APP processing, and increase A β levels in the brain tissues of neonatal naïve and AD transgenic mice. These findings suggest that sevoflurane can induce caspase activation and apoptosis in a time-dependent manner. Sevoflurane anesthesia may specifically induce apoptosis in neurons and increase A β 42 levels. Moreover, sevoflurane anesthesia may induce a greater degree of caspase activation and apoptosis in the brain tissues of neonatal AD transgenic mice (B6.Cg-Tg[APP^{swe}, PSEN1^{dE9}]85Dbo/J) than that in neonatal naïve mice. Finally, sevoflurane anesthesia can induce neuroinflammation by increasing proinflammatory cytokine TNF- α in the brain

tissues of AD transgenic mice, but not in the brain tissues of naïve mice. Collectively, these findings suggest that sevoflurane anesthesia may lead to neurotoxicity by inducing apoptosis and neuroinflammation and by increasing A β levels in the brain tissues of neonatal mice, and the overexpression of AD genes and/or increased A β levels in AD transgenic mice could potentiate such neurotoxicity. These findings, the extension of which is pending in studies on humans, raise novel concerns regarding the use of sevoflurane, the mostly commonly used inhalation anesthetic, in individuals with increased A β burden, including patients with Down syndrome, the unaffected carriers of APP or presenilin gene mutations, and the late onset AD risk factor, apolipoprotein e- ϵ 4, that increase A β accumulation in the brain. Future studies should include further

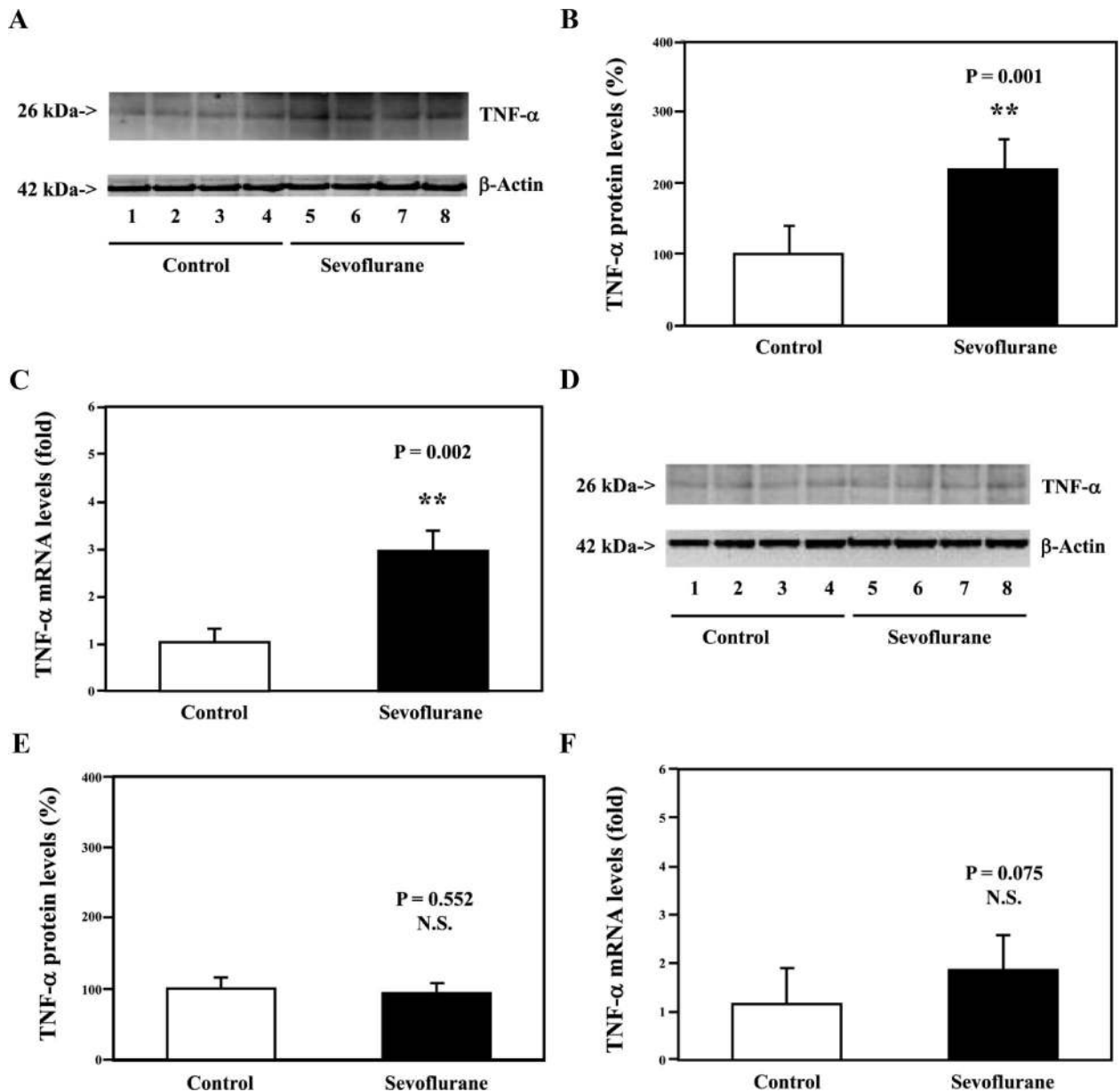


Fig. 8. Anesthesia with 3% sevoflurane for 6 h increases tumor necrosis factor (TNF)- α levels in the brain tissues of neonatal Alzheimer disease (AD) transgenic mice. (A) Sevoflurane anesthesia (lanes 5–8) increases TNF- α levels when compared with the control condition (lanes 1–4) in the brain tissues of neonatal AD transgenic mice. (B) Quantification of the Western blot shows that sevoflurane anesthesia (black bar) increases TNF- α levels compared with the control condition (white bar). (C) Sevoflurane anesthesia (black bar) increases messenger ribonucleic acid (mRNA) levels of TNF- α when compared with the control condition (white bar). (D) Sevoflurane anesthesia (lanes 5–8) does not increase TNF- α levels when compared with the control condition (lanes 1–4) in the brain tissues of neonatal naïve mice. (E) Quantification of the Western blot shows that sevoflurane anesthesia (black bar) does not increase TNF- α levels compared with the control condition (white bar). (F) Sevoflurane anesthesia (black bar) does not increase the mRNA levels of TNF- α when compared with the control condition (white bar) in the brain tissues of neonatal naïve mice. We have averaged the results from four independent experiments. N.S. = not significant. ** $P < 0.01$.

characterization of sevoflurane-induced neurotoxicity in animals and humans and determination of the causative link between sevoflurane anesthesia, apoptosis, and A β levels.

Consistent with our *in vitro* studies,¹⁸ sevoflurane anesthesia can reduce the levels of APP-C-terminal fragments including APP-C83 and APP-C99. Given that APP-C83 and APP-C99 are metabolized by γ -secretase (reviewed in^{10,12}), these findings

suggest that sevoflurane may increase the activity of γ -secretase, leading to reductions in the levels of APP-C83 and APP-C99. Future studies will include the systematic investigation of the effects of sevoflurane and other anesthetics on the levels of γ -secretase components, for example, presenilin 1, nicastrin, presenilin enhancer 2, and anterior pituitary hormones,^{26–30} and the γ -secretase activity.³¹

The mechanisms by which sevoflurane anesthesia can induce neuroinflammation only in the brain tissues of AD transgenic mice, but not in the brain tissues of neonatal naïve mice, in the current experiment are not well understood. Both A β accumulation and neuroinflammation are important parts of AD neuropathogenesis, and they can potentiate each other's neurotoxicity^(32,33; reviewed in¹¹). It is, therefore, conceivable that the higher baseline levels of A β in AD transgenic mice can facilitate the effects of sevoflurane on increasing TNF- α levels, leading to apparent neuroinflammation in the current experiment. Future studies should include systematic assessment of dose- and time-dependent effects of sevoflurane on the levels of TNF- α and other proinflammation cytokines (e.g., interleukin-6) in both naïve and AD transgenic mice to further test this hypothesis.

Even though baseline A β levels in the brain of B6.Cg-Tg(APPswe, PSEN1dE9)85Dbo/J mice were higher than those in the brain of the naïve mice, sevoflurane anesthesia did not lead to significantly greater increases of A β levels in the brain tissues of AD transgenic mice than in naïve mice. This could be because of the ceiling effects of sevoflurane-induced increases in A β levels. It is also possible that sevoflurane may enhance A β levels through a nonapoptosis pathway. A recent study³⁴ has shown that cellular stress induced by glucose deprivation can lead to increases in the levels of β -secretase (the enzyme to generate A β) and A β through phosphorylation of the translation initiation factor eIF2 α independent of caspase activation and apoptosis. Future studies should include the determination of whether anesthetics can also increase A β generation through this translation mechanism.

Nevertheless, ELISA studies showed that sevoflurane anesthesia enhanced the levels of A β 42, but not A β 40, in the brain tissues of neonatal AD transgenic mice, but not in the brain tissues of neonatal naïve mice. These findings suggest that sevoflurane anesthesia may lead to a greater degree of A β accumulation in the brain tissues of AD transgenic mice than that in naïve mice. However, it is still possible that the ELISA kit used in the current experiment was not sensitive enough to detect the non-human A β levels in the brain tissues of neonatal naïve mice.

The IP3 receptor, located in the endoplasmic reticulum membrane, regulates the release of calcium from the endoplasmic reticulum to the cytoplasm^(35 reviewed in³⁶). We have found that the IP3R antagonist 2-APB³⁷ can attenuate sevoflurane-induced caspase-3 activation and A β accumulation in neonatal naïve mice. These results have suggested that sevoflurane may act on IP3R to affect calcium homeostasis, leading to apoptosis and A β accumulation. Moreover, these findings imply that 2-APB may be able to prevent or reduce sevoflurane-induced neurotoxicity. However, it is expected that IP3 antagonism may lead to many other effects. Therefore, a specific effect of IP3 antagonism on caspase-3 activation and A β accumulation cannot be made from the results in current studies. Future studies should include assessing the effects of other IP3 antagonists, for example, xestospongion C, on sevoflurane-induced caspase activation and A β accumulation to further test this hypothesis.

One caveat of the current study is that we did not measure blood gas in each of the mice after the administration of anesthesia with 3% sevoflurane plus 60% oxygen for 6 h. This is because the same sevoflurane anesthesia has been shown not to significantly alter blood gas and brain blood flow,⁶ which is consistent with our pilot studies (see table, Supplemental Digital Content 1, which lists the values of mice blood gas measured in this study, <http://links.lww.com/ALN/A581>). In addition, the finding that anesthesia with 3% sevoflurane plus 60% oxygen for 2 h does not induce caspase-3 activation further suggests that it is sevoflurane, but not physiologic changes (e.g., alterations in oxygen, carbon dioxide or pH in blood), that causes neurotoxicity. However, it is still possible that the combination of sevoflurane and anesthesia-induced hypoxia and/or acidosis induces neurotoxicity in some mice in the current studies.

Currently, there is no satisfactory way to extrapolate the findings of apoptosis, A β accumulation, and neuroinflammation in the mouse brain to the human brain. Thus, the findings from the current studies do not present any direct evidence that inhalation of anesthetic sevoflurane can cause harm to the human brain. Determination of the *in vivo* relevance of sevoflurane on neurotoxicity in the human brain and the longitudinal learning or memory studies will be necessary before we can conclude that anesthetic sevoflurane can cause neurotoxicity in humans.

In conclusion, we have shown that sevoflurane, the most commonly used inhalation anesthetic, can induce caspase activation and apoptosis, alter APP processing, and increase A β levels in the brain tissues of neonatal naïve and AD transgenic mice. Importantly, more severe apoptosis, A β accumulation, and neuroinflammation may occur in the brain tissues of neonatal AD transgenic mice when compared with neonatal naïve mice. These findings suggest that sevoflurane may cause neurotoxicity in neonatal mice and that overexpression of mutated AD genes, that is, presenilin 1 and APP, and/or increased A β levels in AD transgenic mice may potentiate such neurotoxicity. We further found that sevoflurane-induced neurotoxicity may be associated with IP3R, and 2-APB, one of the IP3R antagonists, may attenuate sevoflurane-induced neurotoxicity. Given the findings that anesthesia could be a risk factor for the development of a learning disability in children and that sevoflurane is used extensively in pediatric patients, these current findings will hopefully lead to further studies to determine the potential neurotoxicity of sevoflurane, including confirmation studies in humans.

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