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Neonatal Exposure to Sevoflurane Induces Abnormal Social Behaviors and Deficits in Fear Conditioning in Mice

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Background: **Neonatal exposure to anesthetics that block *N*-methyl-D-aspartate receptors and/or hyperactivate γ-aminobutyric acid type A receptor has been shown to cause neuronal degeneration in the developing brain, leading to functional deficits later in adulthood. The authors investigated whether exposure of neonatal mice to inhaled sevoflurane causes deficits in social behavior as well as learning disabilities.**

Methods: **Six-day-old C57BL/6 mice were exposed to 3% sevoflurane for 6 h. Activated cleaved caspase-3 immunohistochemical staining was used for detection of apoptosis. Cognitive functions were tested by pavlovian conditioned fear test. Social behavior was tested by social recognition and interaction tests.**

Results: **Neonatal exposure to sevoflurane significantly increased the number of apoptotic cells in the brain immediately after anesthesia. It caused persistent learning deficits later in adulthood as evidenced by decreased freezing response in both contextual and cued fear conditioning. The social recognition test demonstrated that mice with neonatal exposure to sevoflurane did not develop social memory. Furthermore, these mice showed decreased interactions with a social target compared with controls in the social interaction test, indicating a social interaction deficit. The authors did not attribute these abnormalities in social behavior to impairments of general interest in novelty or olfactory sensation, because they did not detect significant differences in the test for novel inanimate object interaction or for olfaction.**

Conclusions: **This study shows that exposure of neonatal mice to inhaled sevoflurane could cause not only learning deficits but also abnormal social behaviors resembling autism spectrum disorder.**

MANY pregnant women, newborns, and infants are exposed to a variety of anesthetic agents to prevent pain during childbirth or for surgical procedures. Anesthetic agents sometimes have to be administered during an important period of brain growth, the brain growth spurt period, which occurs from the last 3 months of pregnancy until approximately 2 yr after birth (in humans) or during the first 2 weeks after birth (in mice and rats).[1–4] To minimize risks to the fetus or neonates, it is necessary to study the effect of anesthetics not only in terms of teratogenicity, but also on the developing nervous system.

Recently, it has been demonstrated that neonatal administration of anesthetics induced widespread neurodegeneration and severe deficits in spatial learning tasks in rodents.[5,6] Jevtovic-Todorovic *et al.*[5] reported that neonatal exposure to a cocktail of anesthetics that are commonly used in pediatric surgery induced brain cell death 15 times more frequently than in control rat brains, and that these animals developed learning problems later in adulthood. Fredriksson *et al.*[6] reported that coadministration of an *N*-methyl-D-aspartate (NMDA) receptor antagonist with γ-aminobutyric acid type A ($GABA_A$) receptor agonists synergistically potentiated neonatal brain cell death and resulted in functional deficits in adult mice, although the underlying mechanism is not fully understood. The most thoroughly investigated drug that has NMDA antagonist and $GABA_A$ agonist property is ethanol, which induces fetal alcohol syndrome if the fetus is exposed during the brain growth spurt.[3,7] Although detrimental effects of anesthetics on cognitive function have been reported, to our knowledge, few studies have investigated the effects of anesthetics on social behavior. Therefore, we designed the current study to investigate the potential risks of neonatal exposure to anesthetics to cause social abnormalities.

Sevoflurane (2,2,2-trifluoro-1-[trifluoromethyl]ethyl fluoromethyl ether) is one of the most frequently used volatile anesthetics for induction and maintenance of general anesthesia during surgery and cesarean delivery because of its low blood gas partition coefficient and low pungency. It is especially useful for infants and children because of its properties of rapid induction and recovery together with less irritation to the airway.[8] Sevoflurane has been shown to enhance $GABA_A$ receptors[9] and block NMDA receptors, although more research is necessary to better characterize its effects on NMDA receptors.[10] In this investigation, we studied the potential risks of neonatal exposure to sevoflurane to cause social abnormalities and cognitive deficits in mice.

Materials and Methods

The experiments were approved by the Committee for Animal Research at National Defense Medical College (Tokorozawa, Saitama, Japan). Pregnant C57BL/6 mice were purchased from SLC (SLC Japan Inc., Shizuoka, Japan). The animals were illuminated with a 12-h light–dark cycle (light from 07:00 to 19:00), and room tem-

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perature was maintained at 21° ± 1°C. At the age of 3 weeks, the mice were weaned and housed in groups of 4 animals in a room. Mice had *ad libitum* access to water and food.

Previous studies reported that there is litter variability in the rate of apoptosis that occurs spontaneously in neonate mice.[11] Therefore, a balanced number of control and experimental animals were drawn from the same litters, so that each experimental condition had its own group of littermate controls. Only the male offspring were used in this study. A total of 51 litters, 101 control and 103 treated pups, were used in this study.

Anesthesia Treatment

Postnatal day 6 (P6) male mice were placed in an acrylic box and exposed to 3% sevoflurane or no anesthetics for 6 h. The total gas flow was 2 l/min, using air as a carrier. During anesthetic exposure, the mice were kept warm on a plate heated to 38°C. Control and experimental animals were under the same treatment and environment except that the control animals were exposed only to air.

Arterial Blood Gas Analysis

Arterial blood analysis was performed essentially as described previously.[5,12] Briefly, the pups underwent a quick arterial blood sampling from the left cardiac ventricle, and the samples were transferred into heparinized glass capillary tubes. A single sample (55 μl) was analyzed immediately after blood collection by blood gas analyzer (ABL800; Radiometer, Copenhagen, Denmark). Samples were obtained immediately after removal from the maternal cage (0 h) or at the end of anesthesia (6 h). At the time of blood sampling, the experiments were terminated by decapitation.

Laser Color Doppler

Cerebral blood flow (CBF) was measured by a laser-Doppler blood perfusion imager (Peri Scan PIM II; Perimed, Stockholm, Sweden). Mice were taken out of the chamber before and every hour during anesthetic treatment and were placed face down on the floor while being continuously exposed to sevoflurane *via* a tube with its opening positioned at the nose of the animals. Their head skins were peeled for scanning CBF, and data were captured using appropriate software (LDPIwin version 2.6; Lisca, Linköping, Sweden). The perfusion response is presented in arbitrary perfusion units. Because the arbitrary perfusion units values are not absolute blood flow, the magnitude of the difference in perfusion was calculated as the ratio between the area of maximum peak perfusion and areas of baseline perfusion. Arbitrary perfusion unit values were compared between anesthetized animals and those with mock anesthesia at baseline and at 1-h intervals for 6 h.

Histopathologic Studies

Animals from both treatment and control groups were perfused transcardially with 0.1 M phosphate buffer containing 4% paraformaldehyde immediately after 6 h of sevoflurane anesthesia, and then the brains were exposed to immersion fixation for 24 h at 4°C. The brains were histologically analyzed using paraffin-embedded sections (5 μm thick). For immunohistochemistry, anti–active caspase-3 antiserum (D175; Cell Signaling Technology, Beverly, MA) was used at dilutions of 1:400 in antibody diluent (Dako, Glostrup, Denmark). Before to use, sections were dewaxed in xylene and hydrated using a graded series of ethanol. Antigenic retrieval was performed by immersing mounted tissue sections in 0.01 mM sodium citrate (pH 6.0) and heating in an autoclave (121°C) for 5 min. Deparaffinized sections were blocked for endogenous peroxidase activity as described previously,[13] followed by blocking with a nonspecific staining blocking reagent (Dako) for 1 h to reduce background staining. The sections were then incubated overnight in a humidified chamber at 4°C. Subsequently, peroxidase-conjugated secondary antibody (DAKO En Vision + system; Dako) and 3,3-diaminobenzine-tetrachloride (DAB; Vector Laboratories, Burlingame, CA) were used according to the manufacturer's instructions. Finally, the sections were counterstained with Nissl. Activated caspase-3–positive cells were counted by the investigator who was blinded to the treatment conditions.

Terminal deoxynucleotidyl transferase–mediated deoxyuridine 5-triphosphate–biotin nick end labeling staining was performed using an *in situ* apoptosis detection kit (ApopTag fluorescein; CHEMICON, Temecula, CA) according to the manufacturer's protocol. Sections were counterstained with 4′,6-diamidino-2-phenylindole (DAPI). Fluorescein was histochemically examined with a fluorescent microscope (TE-2000E; Nikon, Tokyo, Japan) equipped with interlined charge-coupled device camera (DS-U1; Nikon).

Preparation of Protein Extracts

Mice forebrain was quickly removed and were homogenized in four volumes of 20 mM Tris-HCl, pH 7.4, 2 mM EDTA, protease inhibitor cocktail (Complete, Roche Diagnostics, Penzberg, Germany), and phosphatase inhibitors (20 mM glycerophosphate, 1 mM Na_3VO_4, 2 mM NaF). After homogenization, a portion of each sample was immediately frozen at −80°C. The rest of the homogenate was centrifuged at 15,000*g* for 30 min at 4°C. The supernatant solutions were separated and stored at −80°C until use. The amount of protein in each sample was measured using a protein assay kit (BCA; Pierce, Rockford, IL).

Western Blot Analysis

The homogenate proteins were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis. The pro-

teins were transferred onto polyvinylidene fluoride membranes (Immobilon-P; Millipore, Bedford, MA). The blots were immunoreacted with anti–cleaved poly(adenosine diphosphate–ribose) polymerase (PARP; 1:1,000, rabbit polyclonal, Asp214; Cell Signaling) or anti–β-actin (1:5,000, mouse monoclonal, AC-15; Sigma, St. Louis, MO) antibodies, and the protein bands were visualized by chemiluminescence detection system (SuperSignal West Pico; Pierce).

Behavioral Studies

As described previously for CBF and histopathologic studies, some sets of mice for behavioral studies were exposed to 3% or 0% sevoflurane for 6 h at P6. They were allowed to mature, and at the appropriate ages, sevoflurane and control mice underwent behavioral tests, namely, open-field, elevated plus-maze, Y-maze, fear conditioning, social recognition, social interaction, olfactory, and novelty tests. The movement of each mouse was monitored and analyzed using a computer-operated video tracking system (SMART, Barcelona, Spain). In the tasks using apparatus with arms, arm entry was counted when all four legs of the animal entered each arm. The apparatus was cleaned after each trial. All apparatus used in this study were made by O'Hara & Co., Ltd. (Tokyo, Japan).

Open-field Test. Emotional responses to a novel environment were measured by an open-field test using 8-week-old mice, by a previously described method.[14] Activity was measured as the total distance traveled (meters) in 10 min.

Elevated Plus-maze Test. The elevated plus-maze test was performed as previously described.[14] The elevated plus maze consisted of two open arms (25 × 5 cm) and two enclosed arms being elevated to a height of 50 cm above the floor. Normally, mice prefer a closed environment to an open area. Mouse behavior was recorded during a 10-min test period. The percentage of time spent in the open arms was used as an index of anxiety-like behavior. Mice used for the test were aged 8 weeks.

Spontaneous Alternation in the Y-maze Test. This study was performed as previously described.[14] This study allowed us to assess spatial working memory. The symmetrical Y maze made of acrylic consists of three arms (25 × 5 cm) separated by 120° with 15-cm-high transparent walls. Each mouse was placed in the center of the Y maze, and the mouse was allowed to freely explore the maze for 8 min. The sequence and the total number of arms entered were recorded. The percentage of alternation is the number of triads containing entries into all three arms divided by the maximum possible number of alternations (total number of arm entries minus 2) × 100. Mice used for the test were aged 11 weeks. The motion of the animals was manually recorded.

Fear Conditioning Test. This is a simple and sensitive test of hippocampal-dependent and hippocampal-independent learning as previously described.[14] Briefly, the conditioning trial for contextual and cued fear conditioning consisted of a 5-min exploration period followed by three conditioned stimulus–unconditioned stimulus pairings separated by 1 min each: unconditioned stimulus, 1 mA foot shock intensity, 1 s duration; conditioned stimulus, 80 db white noise, 20 s duration; unconditioned stimulus was delivered during the last seconds of conditioned stimulus presentation. A contextual test was performed in the conditioning chamber for 5 min in the absence of white noise at 24 h after conditioning. A cued test (for the same set of mice) was performed by presentation of a cue (80 db white noise, 3 min duration) in alternative context with distinct visual and tactile cues. The rate of freezing response (absence of movement in any parts of the body during 1 s) was scored automatically and used to measure fear memory. The test was performed on mice of two different age groups: 8 weeks or between 14 and 17 weeks.

Social Recognition Test. Social recognition test was conducted as described previously.[15] We transferred 18-week-old mice from group to individual housing for 7 days before testing to permit establishment of a home cage territory. Testing began when a stimulus female mouse was introduced into the home cage of each male mouse for 1-min confrontation. At the end of the 1-min trial, the stimulus animal was removed and returned to an individual cage. This sequence was repeated for four trials with 10-min intertrial intervals, and each stimulus was introduced to the same male resident in all four trials. In a fifth trial, another stimulus mouse was introduced to a resident male mouse.

Social Interaction Test. Caged social interaction for social *versus* inanimate targets was performed in an open field using two cylinder cages allowing olfactory and minimal tactile interaction as described previously.[16] The cylinder cages were 10 cm in height, with a bottom diameter of 9 cm and bars spaced 7 mm apart.

Olfactory Test. Fifteen-week-old mice were habituated to the flavor of a novel food (blueberry cheese) for 3 days before testing. On the fourth day, after 24 h of food deprivation, a piece of blueberry cheese was buried under 2 cm of bedding in a clean cage. The mice were placed in the cage, and the time required to find the food was measured manually.

Novelty Test. Activity was measured as the total duration of interaction with an inanimate novel object (red tube) in 10 min.

The same set of mice underwent social recognition (at 19 weeks of age), social interaction (at 14 weeks of age), olfactory (at 15 weeks of age), and novelty (at 15 weeks of age) tests. In other analyses, each test was conducted with a new set of animals.

Statistical Analysis

Statistical analysis was performed using Statview software (SAS, Cary, NC). Comparisons of the means of two

Table 1. Arterial Blood Gas Analysis

			Arterial Blood Gas			
	Time, h	n	pH	$Paco_2$, mmHg	Pao_2, mmHg	Sao_2, %
Sham operation	0	8	7.40 ± 0.09	26.0 ± 4.6	100.2 ± 5.7	95.8 ± 1.1
	6	8	7.38 ± 0.04	29.4 ± 6.2	95.6 ± 10.0	96.1 ± 1.0
3% Sevoflurane	0	8	7.32 ± 0.08	26.9 ± 4.6	98.5 ± 6.9	95.4 ± 1.1
	6	8	7.46 ± 0.06	27.5 ± 6.9	80.9 ± 4.8	95.4 ± 0.8

Neonatal exposure to sevoflurane does not induce significant cardiorespiratory dysfunction. Analysis of arterial blood gas revealed no significant differences in any of the measured parameters between mice exposed for 6 h to sevoflurane and control (sham operation) exposed to air for 6 h (*t* test, all *P* values > 0.05). $Paco_2$ = arterial carbon dioxide tension; Pao_2 = arterial oxygen tension; Sao_2 = arterial oxygen saturation.

groups were performed using the Student *t* test. In the Y-maze task, comparisons of group performance relative to random levels were performed using the one-sample *t* test. Data of the social recognition task were analyzed by repeated-measures two-way analysis of variance. Values are presented as mean ± SEM.

Results

Neonatal Exposure to Sevoflurane Did Not Induce Significant Disturbance in Ventilation, Oxygenation, or CBF

To examine the effect of neonatal exposure to sevoflurane, we exposed P6 mice to 3% sevoflurane for 6 h. Hypoxia is a known cause of neuronal cell death.[17] To assess the adequacy of ventilation and oxygenation, we examined the blood gas data in mice during the anesthesia. Control samples were obtained from pups exposed to air during the same period. We found that pH, arterial carbon dioxide tension, arterial oxygen tension, and arterial oxygen saturation did not differ significantly from sham control (table 1). These results, together with the fact that pups looked pink throughout the 6 h of gas exposure, led us to conclude that it was unlikely that apoptosis in this protocol was caused by hypoxia/hypoventilation.

Further, to assess the adequacy of cerebral perfusion, we measured CBF during anesthesia using a laser-Doppler blood perfusion imager. Control mice were exposed to air for corresponding period of the sevoflurane treated mice. Anesthesia treatment with sevoflurane did not affect CBF compared with control mice at any point during the 6 h of anesthesia (figs. 1A and B).

Neonatal Exposure to Sevoflurane Induced Extensive Apoptotic Neurodegeneration

Sevoflurane anesthesia significantly increased cleaved caspase-3 apoptosis in the mice immediately after exposure (table 2 and figs. 2–4). Figures 2B and D showed that the increased apoptosis was most robust in the caudate/putamen, retrosplenial cortex, dorsal hippocampal commissure, and neocortex in the brains of pups with sevoflurane exposure. In other sections, thal-

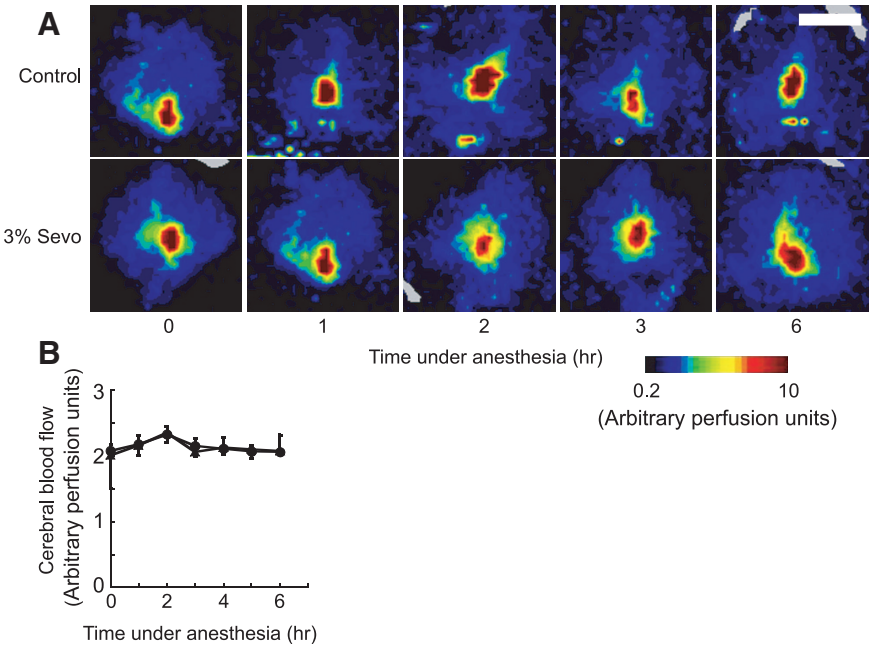

Fig. 1. Neonatal exposure to sevoflurane (Sevo) did not induce hypoperfusion of the brain. (*A*) Representative images of laser color Doppler for cerebral blood flow in a control mouse (*upper panel*) and in a sevoflurane exposed mouse (*lower panel*). The degree of perfusion is shown by the color code, with *red* representing high perfusion and *blue* representing lower perfusion. No different patterns of cerebral blood flow were observed between control and anesthetized mice throughout the 6-h exposure period. (*B*) Time course of cerebral blood flow measured by laser color Doppler during 6 h of 3% sevoflurane administration. The degree of perfusion is presented in arbitrary perfusion units. There was no difference between sevoflurane and control groups during the 6-h period (control, n = 3; 3% sevoflurane, n = 4). *Scale bar*: 5 mm.

Table 2. Brain Regions in Which Sevoflurane-induced Neurodegeneration Was Heavily Concentrated

Brain Region	Severity, Fold Increase
CA1 (hippocampus)	4.8
CA3 (hippocampus)	29.7
Dentate gyrus	32.5
Dorsal hippocampal commissure	65.2
Frontal cortex	66.7
Temporal cortex	148.0
Amygdala	51.0
Caudate/putamen	47.7
Mammillary complex	21.1
Retrosplenial cortex	166.5
Subiculum	121.5
Pontine nuclei	38.8
Inferior colliculus	32.1
Thalamus	376.7

Severity of damage is expressed as the fold increase (*i.e.*, how many times greater) in the density of degenerating neurons labeled by activated cleaved caspase-3 immunohistochemical staining in the sevoflurane-treated brain (n = 6 mice) compared with the rate of degeneration in the same region of control brain (n = 6 mice).

amus, subiculum, inferior colliculus, and pontine nuclei were also shown to be damaged severely (figs. 3B and C). Figure 4B indicated that severe neuronal damage occurred in the extrahippocampal circuit, which is believed to be important for mediating learning and memory functions,[18] including dorsal hippocampal commissure as well as thalamus and retrosplenial cortex. Apoptosis were also observed in amygdala (fig. 4D), hippocampus (fig. 4F), frontal cortex (fig. 4H), and mammillary complex (fig. 4J). These data indicated that the apoptotic response to sevoflurane was robust and followed a pattern that was characteristic of the pattern reported for other anesthetic drugs or ethanol.[18]

It was reported that caspase-3 cleavage may occur under a condition that is a nonapoptotic event,[19] raising questions about the reliability of cleaved caspase-3 immunostaining for detecting cell death. Therefore, to verify that the cleaved caspase-3 immunoreactivity represent authentic apoptosis, we also performed terminal deoxynucleotidyl transferase–mediated deoxyuridine 5-triphosphate–biotin nick end labeling as another independent measure of apoptotic cell death. We found the same pattern of staining as observed by cleaved caspase-3 staining (figs. 4L, N, P, and R).

Further, to verify that the previously described immunohistochemically detectable reactivity represented authentic apoptosis and to quantify the apoptosis response, we examined cortex extracts from control and sevoflurane-treated pups by Western blot analysis using antibody specific for cleaved PARP. PARP is one of the main cleavage targets of caspase-3 *in vivo*, and the cleavage is readily detected in many apoptosis model.[20] Western immunoblotting with anticleaved PARP antibody detected immunoreactivity in sevoflurane-exposed pup extracts, whereas the band was under detection level in control pub brain extracts (fig. 4S).

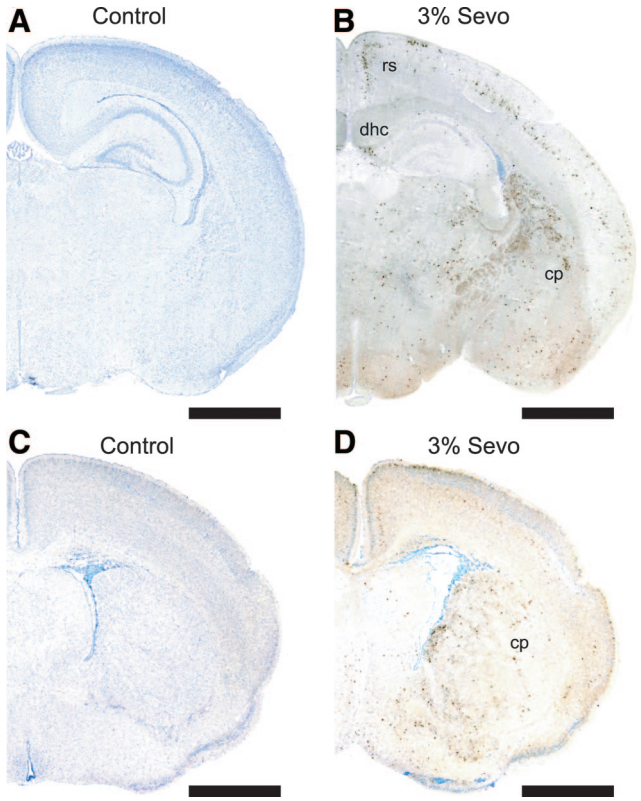

Fig. 2. The apoptotic response to sevoflurane (Sevo) was robust in pup brain. Light microscopic views of the mouse brain after exposure to room air for 6 h (*A* and *C*) and to 3% sevoflurane for 6 h (*B* and *D*). Sections were immunochemically stained to reveal caspase-3 activation (*A–D*). *Black dots* represent caspase-3–positive cells, which indicate apoptosis. A substantially higher density of cleaved caspase-3–positive profile is present in sevoflurane-treated brain. cp = caudate/putamen; dhc = dorsal hippocampal commissure; rs = retrosplenial cortex. *Scale bars*: 1 mm.

General Behavior Was Normal in Mice with Neonatal Exposure to Sevoflurane

To examine responses to a novel environment, mice with neonatal exposure to sevoflurane were assayed in an open-field test. These mice did not differ from control animals in their exploratory behavior (fig. 5A; *t* test, $t = 1.24$, $P > 0.05$). To study whether anxiety-related behavior of mice with neonatal exposure to sevoflurane was affected, mice underwent an elevated plus-maze test. Anxiety-related behavior was assessed by the percentage of time spent in the open arms of the test equipment. Anesthetized mice did not differ significantly in the percentage of time spent in the open arms (fig. 5B; *t* test, $t = 0.76$, $P > 0.05$). These results indicate that the emotional state of mice with neonatal exposure to sevoflurane did not differ grossly from controls under the conditions of this study.

Further, to examine whether exposure of the developing brain to sevoflurane was associated with changes in

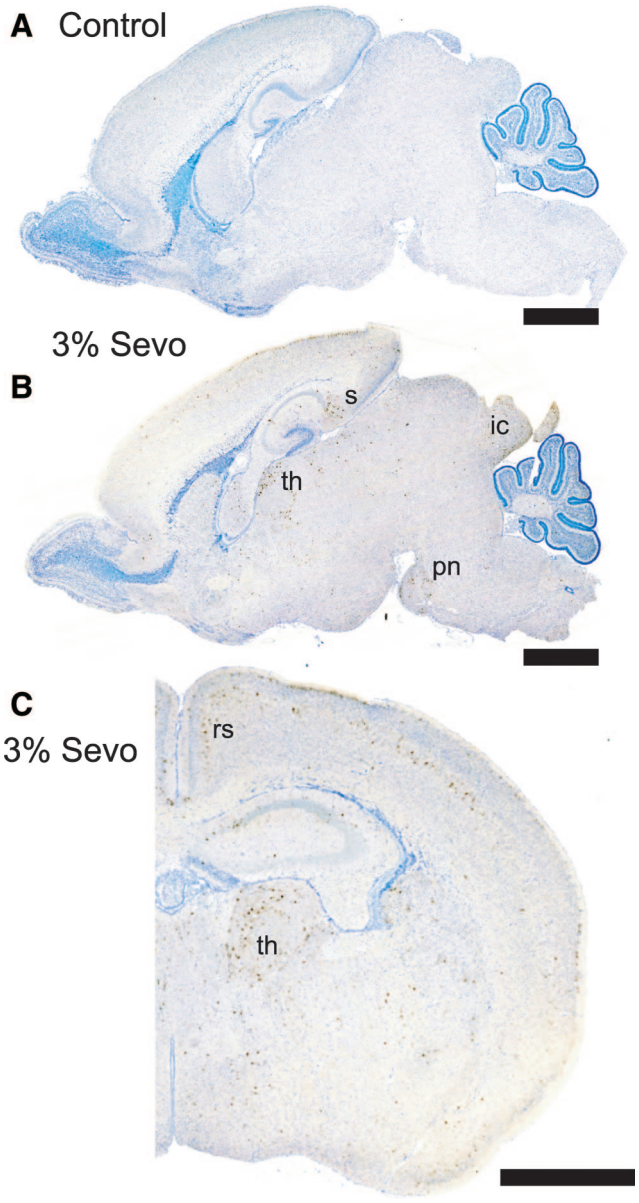

Fig. 3. Thalamus and other regions were also severely damaged after neonatal exposure to sevoflurane (Sevo). Sagittal (*A* and *B*) and coronal (*C*) views of the mouse brain after exposure to room air for 6 h (*A*) and to 3% sevoflurane for 6 h (*B* and *C*) as described in figure 2. A substantially higher density of cleaved caspase-3–positive profile is present in thalamus (th) as well as subiculum (s), inferior colliculus (ic), and pontine nuclei (pn). rs = retrosplenial cortex. *Scale bars*: 1 mm.

spatial working memory, mice underwent a Y-maze spontaneous alternation task. Working memory refers to a cognitive function that provides concurrent temporary storage and manipulation of the information necessary for complex cognitive tasks. This test examines whether mice remember the position of the arm selected in the preceding choice. Mice with and without sevoflurane exposure performed this task with 64.3 ± 7.3% and 60.0 ± 8.3% correct choices, respectively, which were well above the expected results of random choices (random choice = 50%; one-sample *t* test, $P < 0.05$), there being no difference compared with control (fig. 5C; *t* test, $t = 1.20$, $P > 0.05$). This result suggests that spatial working memory was not affected by exposure of the developing brain to sevoflurane.

Neonatal Exposure to Sevoflurane Induced Deficits in Contextual and Cued Fear Conditioning

To assess the influence of neonatal exposure to sevoflurane on long-term memory, mice underwent contextual/cued fear conditioning. In this paradigm, mice learn to associate previous neutral auditory cues and the apparatus (context) with electric foot shock in a single training session, such that robust long-term memory was established for an experimental context (hippocampus dependent) and an auditory cue (hippocampus independent).[21,22] Long-term memory was assessed based on the freezing reaction of the mice in response to the context or the conditioned cue. The freezing response to the same context in mice with neonatal exposure to sevoflurane was reduced significantly compared with controls after a 24-h retention delay at 8 weeks of age (fig. 6A; *t* test, $t = 3.10$, $P < 0.01$). The response of mice with sevoflurane to the cued fear conditioning was also reduced significantly after a 48-h retention delay compared with control mice (fig. 6B; *t* test, $t = 3.16$, $P < 0.01$).

It was reported that exposure of infant mice to ethanol induced neuroapoptosis and subsequent memory impairments that were very severe at P30 and less severe at P75.[18] This result provided evidence favoring the interpretation that recovery of some type of learning functions might occur in later adulthood in ethanol-treated mice. Therefore, further to examine whether the neonatal exposure to sevoflurane causes permanent neurocognitive deficits in mice and how it evolves over time, we undertook an assessment of hippocampal function at later time point using another set of mice.

We found that neonatal exposure to sevoflurane caused deficits in the fear conditioning test at 14–17 weeks of age similar to the results at 8 weeks. The freezing response of mice with sevoflurane was reduced significantly in contextual tests compared with that of controls after a 24-h retention delay (fig. 6C; *t* test, $t = 3.48$, $P < 0.01$). The freezing response of sevoflurane-exposed mice to cued fear was also reduced significantly compared with that of controls after a 48-h retention delay at 14–17 weeks of age (fig. 6D; *t* test, $t = 2.11$, $P < 0.05$). These results strongly suggested that exposure of P6 mice to sevoflurane caused hippocampal-dependent and -independent neurocognitive deficits that persisted for relatively long time periods (at least from 8 to 14–17 weeks of age) of the mice's lifespan.

Neonatal Exposure to Sevoflurane Induced Abnormal Social Interaction

Mice are a social species and exhibit social interaction behavior.[23] Therefore, we investigated whether mice

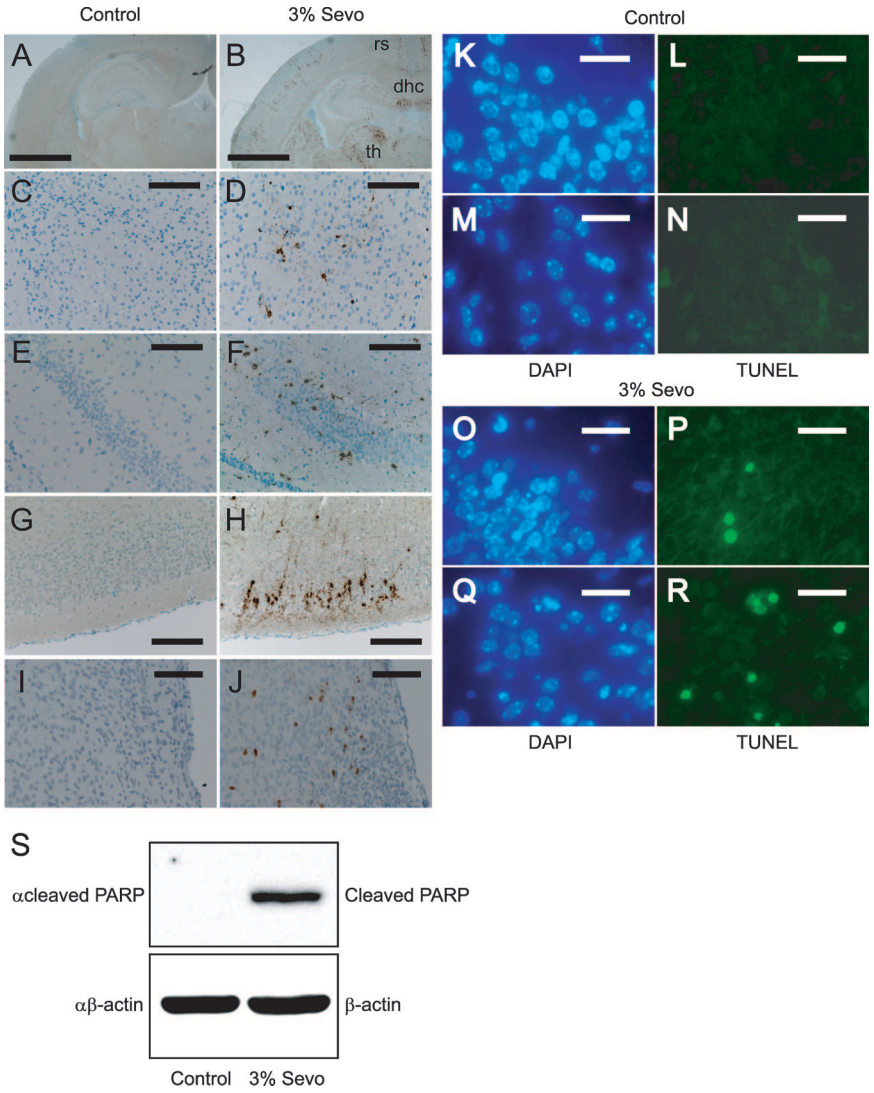

Fig. 4. Inhaled sevoflurane increased apoptosis in several regions of the brain. Light microscopic views of the mouse brain after exposure to room air for 6 h (*A*, *C*, *E*, *G*, and *I*) and to 3% sevoflurane (Sevo) for 6 h (*B*, *D*, *F*, *H*, and *J*). Higher density of cleaved caspase-3–positive profile was present in extrahippocampal circuit (*A* and *B*), amygdala (*C* and *D*), hippocampus (*E* and *F*), frontal cortex (*G* and *H*), and mammillary complex (*I* and *J*). *Scale bars*: 1 mm in *A* and *B*, 50 μm in *C–J*, and 100 μm in *I* and *J*. (*K–R*) Terminal deoxynucleotidyl transferase–mediated deoxyuridine 5-triphosphate–biotin nick end labeling (TUNEL; *L*, *N*, *P*, and *R*) showed similar pattern of neuroapoptosis to cleaved caspase-3 staining. Sections were counter stained with 4′,6-diamidino-2-phenylindole (DAPI; *K*, *M*, *O*, and *Q*). Representative images of cortex (*K*, *L*, *O*, and *P*) and caudate (*M*, *N*, *Q*, and *R*) are shown. *Scale bars*: 500 μm. (*S*) Poly(adenosine diphosphate–ribose) polymerase (PARP) was cleaved after neonatal exposure to sevoflurane. Protein extracts of control (exposure to room air for 6 h) and sevoflurane-exposed cortex were prepared and analyzed for cleaved PARP immunoreactivity on Western blot. Representative blot from three independent results (from three pairs of pups) was shown. β-Actin reactivity was used as a protein loading control. dhc = dorsal hippocampal commissure; rs = retrosplenial cortex; th = thalamus.

with neonatal exposure to sevoflurane display abnormal social behaviors. First, we investigated social memory, which depends predominantly on olfactory cues. This ability is needed for social familiarity and can be identified as a consistent decrease in olfactory investigation during repeated encounters with a female in the social recognition test. Control mice showed a significant decline in the time spent in investigating a female with subsequent presentation of the same female in trials 3 and 4, as compared with trial 1 (fig. 7A). This decrease was not due to a general decline in olfactory investigation, because presentation of a novel female during trial 5 resulted in a similar amount of investigation as trial 1 with the original female. In contrast, mice with neonatal exposure to sevoflurane showed high levels of sustained investigation at each encounter with the same female and the same level of investigation when presented with a new female at trial 5, significantly different from the response of controls (analysis of variance, $F = 14.51$, $P < 0.001$ [between control and sevoflurane administration]; $F = 28.34$, $P < 0.0001$ [between trials]; $F = 16.64$, $P < 0.0001$ [interaction between trials and sevoflurane

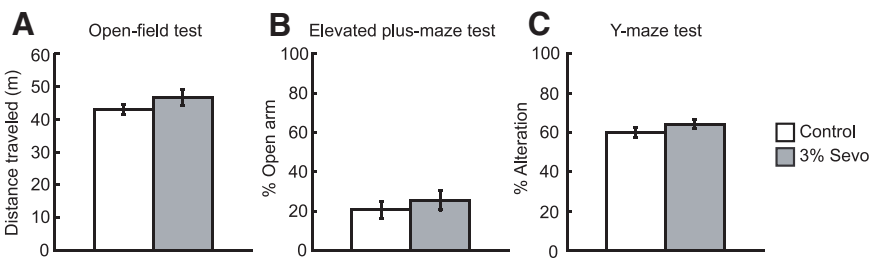

Fig. 5. Behavioral effects of neonatal sevoflurane exposure were assessed by the open-field test (total distance traveled in 10 min; control, n = 10; sevoflurane, n = 11; *A*), elevated plus-maze test (percentage of time spent in open arms; control, n = 18; sevoflurane, n = 20; *B*), and Y-maze test (percentage of correct alternation response; control, n = 10; sevoflurane, n = 9; *C*). No significant differences were observed between mice with neonatal sevoflurane exposure and controls in these tests.

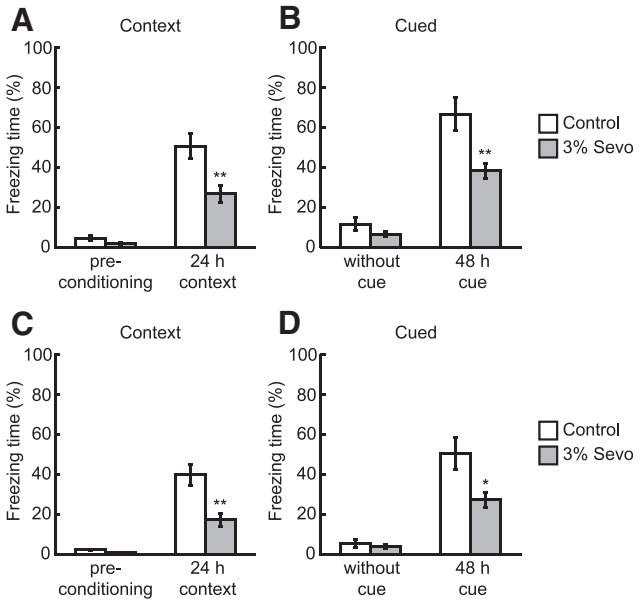

Fig. 6. Neonatal exposure of mice to sevoflurane (Sevo) induced impaired memory performance in both contextual and cued tests. (*A* and *B*) Contextual and cued tests at 8 weeks of age. (*A*) Freezing response was measured in the context before shock (basal freezing) and in the conditioning chamber (contextual fear response) 24 h after conditioning (control, n = 8; sevoflurane, n = 8). (*B*) Freezing response (for the same set of mice as in *A*) was measured in an alternative context without auditory cue (basal freezing after conditioning) or with cue 2 days after conditioning. (*C* and *D*) Contextual and cued tests for another set of mice at a different age (14–17 weeks of age) as in *A* and *B* (control, n = 9; sevoflurane, n = 9). For all figures, *asterisks* represent statistical difference (* $P < 0.05$, ** $P < 0.01$).

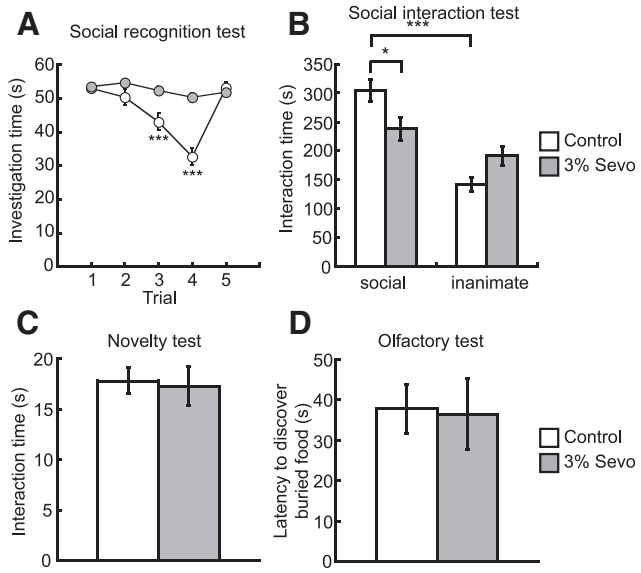

Fig. 7. Neonatal exposure to sevoflurane (Sevo) induced abnormal social behavior in adulthood. (*A*) Olfactory investigations in mice with neonatal exposure to sevoflurane were used for social recognition test. Social memory by male mice was measured as the difference in anogenital investigation. Data depict the amount of time allocated to investigating the same female during each of four successive 1-min trials. A fifth trial depicts the response to a new female. *Asterisks* represent statistical differences (* $P < 0.001$) between each trial compared with the first trial (control, n = 17; sevoflurane, n = 18). (*B*) When exposed to caged social and inanimate targets (social interaction test) in an open field, control mice showed a normal preference for the social target over an inanimate target, whereas the difference of interaction time between both targets was not significant in mice with neonatal exposure to sevoflurane. Furthermore, mice with neonatal exposure to sevoflurane spent significantly less time interacting with the social target compared with controls (control, n = 17; sevoflurane, n = 18). *Asterisks* represent statistical differences (* $P < 0.05$, *** $P < 0.001$). (*C*) Time spent interacting with a novel inanimate object was not significantly affected by neonatal exposure to sevoflurane (control, n = 17; sevoflurane, n = 18). (*D*) Mice with neonatal exposure to sevoflurane did not show significant differences from controls in latency to find a buried treat after overnight food deprivation (control, n = 17; sevoflurane, n = 18).**

administration]; fig. 7A). These data suggest that mice with neonatal exposure to sevoflurane do not develop social memory.

In a test for social *versus* inanimate preference, control mice spent significantly more time interacting with the social target than with the inanimate target (fig. 7B; *t* test, $t = 7.30$, $P < 0.0001$). In contrast, mice with neonatal exposure to sevoflurane spent a similar amount of time interacting with both targets (fig. 7B; *t* test, $t = 1.77$, $P > 0.05$). Furthermore, mice with neonatal exposure to sevoflurane exhibited decreased interaction with a social target compared with controls (fig. 7B; *t* test, $t = 2.38$, $P < 0.05$), indicating a social interaction deficit. We did not attribute the abnormalities in social recognition and interaction to impairment in general interest in novelty or olfactory sensation, because we did not detect significant differences between groups in tests for novel inanimate object interaction (fig. 7C; *t* test, $t = 0.21$, $P > 0.05$) or for olfaction (fig. 7D; *t* test, $t = 0.12$, $P > 0.05$). Therefore, it can be concluded that mice with neonatal exposure to sevoflurane demonstrated deficits in social behavior.

Discussion

This study showed that single administration of sevoflurane to neonatal mice caused a significant increase in neuroapoptosis in the brain compared with littermate controls exposed only to air. Consistent with other recent evidence that apoptotic neurodegeneration can be induced by exposure during the brain growth spurt to drugs that block NMDA receptors and/or hyperactivate $GABA_A$ receptors,[5,6,18] neonatal exposure to sevoflurane was shown to induce widespread apoptosis in several major brain regions, leading to impaired learning later in adulthood. This finding for sevoflurane is consistent with recent finding by Johnson *et al.*,[24] who found that a neonatal exposure to isoflurane triggers a significant neuroapoptosis response in the mouse brain. Our results of fear conditioning strongly suggested that exposure of P6 mice to sevoflurane caused learning deficits, although we could not rule out the possibility of the effects of sevoflurane on sensitization despite true conditioning to the auditory cue. Furthermore, this

study also showed that exposure of neonatal mice to inhaled sevoflurane caused deficits in social behavior. To our knowledge, this is the first study to show that single administration of sevoflurane, which is a commonly used anesthetic in pediatric surgery throughout the world, causes a robust neuroapoptosis response in infant mouse brain and behavioral deficits in both cognitive and social spheres. The minimum alveolar anesthetic concentration that prevents purposeful movement in response to supramaximal noxious stimulation in 50% of animals (minimum alveolar concentration) of sevoflurane in human neonates is 3.3 ± 0.2%.[8] Therefore, the concentration of sevoflurane (3%) used in this mice study would be comparable to clinically used ranges.

Most anesthetics act *via* NMDA and/or $GABA_A$ receptors. It was demonstrated that neonatal coadministration of an NMDA antagonist and a $GABA_A$ agonist was much more detrimental than either of these used alone.[5,6] Although the precise mechanism for this is yet to be understood, this evidence suggested that more severe neurodegeneration was induced when both NMDA and $GABA_A$ receptors were simultaneously altered in the developing brain. Therefore, an anesthetic that has NMDA antagonist and $GABA_A$ agonist properties would be of concern when administered to the developing brain.

Autism spectrum disorders (ASDs) are a group of common neuropsychiatric disorders characterized primarily by impairments in social, communicative, and behavioral functioning,[25] of unknown mechanism. Epidemiologic studies have shown that the prevalence of ASDs is 3–6 per 1,000 children. If neonatal exposure to anesthetics induces deficits in social behavior, a causal link between ASDs and neonatal exposure to anesthetics could be suggested, because deficits in social behaviors are a core feature of ASDs. Our findings revealed that neonatal exposure to sevoflurane induced deficits in social memory and social interaction in mice. These tests were thought to be core paradigms to test autistic behavior in mice and have been used to measure autistic behavior in other ASD models as well.[16,17,26,27] This study is the first to indicate the potential risk of general anesthetics to induce disturbances in social behaviors that resembles those seen in ASDs.

However, there is a caveat that the relevance of these mouse findings to the human situation is unknown and requires clarification. It is too early to say whether anesthetics have the same effect in humans. There may be species differences in the detrimental effects of anesthetic agents on the developing brain. Physicians could reduce any potential risks by limiting the duration of anesthetic administration in neonates.

In any case, the current results suggest the potential hazards of neonatal sevoflurane exposure in causing social behavioral alterations. We would like to insist on the need for further research to determine whether a correlation exists between anesthesia exposure during development and ASDs in human populations. What would be the potential association between the sevoflurane-induced apoptosis and sevoflurane-induced changes in social behaviors? Several authors have proposed that glutamate and GABAergic system disturbance in cortical network in ASDs may be characterized by an imbalance between excitation and inhibition in neuronal networks.[28,29] Such a change may lead to hyperexcitability or unstable neuronal networks, which may alter oscillatory rhythms in brain.[30,31] The excitatory/inhibitory balance in cortical networks may be controlled by the relative numbers and activities of glutamatergic and GABAergic neurons.[29] Indeed, reduced GABAergic inhibition by mutations of genes encoding subunits of the $GABA_A$ receptors is associated with ASDs.[28,29,32] Neural loss by a drug that may violate NMDA and $GABA_A$ receptors in the critical period, seen in the current study, might interfere with the developmental mechanisms patterning the balance between excitation and inhibition system and cause ASD-like behaviors.

Our results showed that neuronal degeneration was particularly severe in several of the specific brain regions that comprise the extrahippocampal circuit, which is believed to be important for mediating learning and memory functions. This pattern was similar to the pattern for neonatal exposure to ethanol, which has NMDA antagonist and $GABA_A$ agonist properties.[18] These pattern of brain damage and subsequent learning deficits are described in ethanol-treated mice.[18] In this regard, it is noteworthy that there is evidence that prenatal exposure to ethanol may be a factor in social difficulties in humans.[33] Further research, including electrophysiologic study, will be needed to address the molecular mechanisms that explain the relevance between neonatal exposure to sevoflurane and deficits in social behavior. It would also be necessary to study whether other drugs that cause neuroapoptosis in the critical period would induce deficits in social behavior.

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