

Sevoflurane exposure in 7-day-old rats affects neurogenesis, neurodegeneration and neurocognitive function

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Abstract: Objective Sevoflurane is widely used in pediatric anesthesia and former studies showed that it causes neurodegeneration in the developing brain. The present study was carried out to investigate the effects of sevoflurane on neurogenesis, neurodegeneration and behavior. **Methods** We administered 5-bromodeoxyuridine, an S-phase marker, before, during, and after 4 h of sevoflurane given to rats on postnatal day 7 to assess dentate gyrus progenitor proliferation and Fluoro-Jade staining for degeneration. Spatial reference memory was tested 2 and 6 weeks after anesthesia. **Results** Sevoflurane decreased progenitor proliferation and increased cell death until at least 4 days after anesthesia. Spatial reference memory was not affected at 2 weeks but was affected at 6 weeks after sevoflurane administration. **Conclusion** Sevoflurane reduces neurogenesis and increases the death of progenitor cells in developing brain. This might mediate the late-onset neurocognitive outcome after sevoflurane application.

Keywords: sevoflurane; neurogenesis; progenitor; proliferation; degeneration

1 Introduction

Recent studies show that anesthetic agents are toxic to the developing brain and that this injury results in long-term impairment of cognitive function. Such neurotoxicity has now been demonstrated not only for isoflurane^[1] but also for ketamine^[2], midazolam^[2], diazepam, pentobarbital^[3], thiopental^[4], N₂O^[5] and propofol^[4,6-8]. Pregnant women, newborns, and infants are often exposed to anesthetic agents during childbirth or for surgical procedures. The administration of anesthetics often occurs during an important period of brain growth, the growth-spurt, ranging from the last 3 months of pregnancy until ~2 years after birth in humans or during the first 2 weeks after birth in

rodents^[9-12]. Therefore, it is important to study the mechanisms underlying the deleterious effects of these agents on the developing nervous system during this critical period.

Although the widely-recognized neurotoxic effect of anesthesia is neonatal brain cell death, recent studies suggest that cell death after a certain anesthetic agent such as isoflurane by itself is insufficient to cause neurocognitive dysfunction^[13]. If cell death was directly responsible for cognitive impairment, one would expect compromised neurobehavioral activity immediately after neuronal loss followed by gradual improvement. However, although cell death occurs immediately after isoflurane exposure, cognitive deficits do not become apparent until 4–6 weeks later^[5,13,14]. It has therefore been suggested that anesthesia also impairs neurogenesis^[13] and this may indeed contribute to anesthesia-induced neurocognitive dysfunction in rats.

The developing brain has a high degree of plasticity.

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In rats at postnatal day 7 (P7), cell birth is a very common event. Neurogenesis occurs during development and persists in adulthood in the subventricular zone and the hippocampal dentate gyrus (DG). The peak of DG neuronal expansion occurs around P7^[15]. Appropriate neurogenesis in the DG is crucial to hippocampus-dependent episodic learning and memory. Any intervention that affects this process could result in impaired hippocampal learning^[16,17].

Sevoflurane (2,2,2-trifluoro-1-[trifluoromethyl]ethyl fluoromethylether) is one of the most frequently used volatile anesthetics 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^[14,18]. It has been recently shown that neonatal exposure to sevoflurane causes learning disabilities^[14]. In this study, we tested the hypothesis that sevoflurane induces acute brain cell death and decreases progenitor proliferation in P7 rats, and that the impaired neurogenesis in the DG causes the delayed onset of neurocognitive deficits in these animals.

2 Material and methods

2.1 Surgeries Male Sprague Dawley rats at P7 were placed in an acrylic box. The room temperature was maintained at 25°C by an air-conditioner^[14]. The rats were anesthetized in groups of six at 1 minimum alveolar concentration for 4 h and confirmed by tail clamping. Movement was defined as any movement other than breathing. Tail clamping with an alligator clamp was repeated every 30 min from the induction of general anesthesia. In the pilot study, we established that sevoflurane concentrations between 3% and 5% maintained a sufficient depth of anesthesia, as determined by 50% of mice lacking a reaction to a painful stimulus (tail clamping). The concentrations of sevoflurane are similar to those used by others^[19]. The anesthetic concentration was initially set to 2% sevoflurane with 2L/min O₂. Then it was adjusted according to the responses to tail clamping. Animals were closely monitored during the experiment and the concentration of sevoflurane was adjusted between 3% and 5% to maintain normal skin

color and adequate respiratory effort. Control rats were placed in the anesthesia box for 4 h without exposure to anesthetic but in conditions otherwise identical to the animals in the sevoflurane group. All experiments were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and approved by Animal Care and Use Committee of Fudan University.

2.2 Blood gas analysis To exclude severe hypoxia, hypercapnia or lactic acidosis, blood gas analysis was performed in two animals per group. The pups were decapitated immediately after anesthesia and mixed arterial and venous blood was collected from the neck vessels. Blood pH, *p*CO₂, and *p*O₂ were analyzed immediately in a blood analyzer (GEM Premier 3000, Instrumentation laboratory, Bedford, MA).

2.3 Bromodeoxyuridine (BrdU) injections BrdU (Sigma-Aldrich, St. Louis, MO) was injected intraperitoneally (i.p.) to target the processes of progenitor proliferation. The rats were divided into three groups according to the time of injection. In group I, 300 mg/kg BrdU was injected 4 h before anesthesia. In group II, 300 mg/kg BrdU was injected immediately before anesthesia. In group III, 50 mg/kg BrdU was injected 4 days after anesthesia twice at a 12-h interval. All the rats were perfused 12 h after the last injection. Group I was used to determine the effect of sevoflurane on the proliferation of progenitor cells labeled before anesthesia. Group II targeted the proliferation of progenitor cells that were in the S-phase of the cell cycle during the time of anesthesia. Group III was used to determine its effect after 4 days.

2.4 Immunohistochemistry Animals were deeply anesthetized with 10% chloral hydrate (360 mg/kg, i.p.) followed by transcardial perfusion with 4% paraformaldehyde (PFA) in 0.1 mol/L phosphate buffered saline (PBS), pH 7.4. Each brain was removed, post-fixed overnight in ice-cold 4% PFA/PBS, and placed sequentially in 20% and 30% sucrose until completely submerged. Coronal sections (40 μm) were cut on a microtome and stored in preservation solution. For immunohistochemical detection of BrdU-labeled nuclei, DNA was denatured by incubation with

2N hydrochloric acid for 30 min at 37°C followed by neutralization with 0.1 mol/L boric acid (pH 8.5) for 10 min at room temperature (RT). Sections were washed 3 times with PBS for 5 min each between each of these steps. Nonspecific epitopes were blocked by incubation with 3% donkey serum and 0.1% Triton X-100 in PBS for 30 min at RT. Primary antibody (rat anti-BrdU, 1:200; AbD Serotec, Raleigh, NC) was then applied in PBS and 0.2% bovine serum albumin, and slides were incubated overnight at 4 °C. On day 2, slides were washed 3 times with PBS, followed by incubation with the secondary fluorescent antibody Alexafluor 594 (1:500; Invitrogen, Carlsbad, CA). After 2 h of incubation at RT, excess antibody was washed out with PBS. DAPI (Sigma-Aldrich) was then used to counterstain the nuclei. Fluorescence was visualized using a conventional microscope (DM IRG; Leica, Bannockburn, IL) with a 10× objective lens.

2.5 Fluoro-Jade (FJ) staining Sections of P7 rat brain were mounted on glass slides and covered in 100% ethanol for 3 min followed by 70% ethanol and deionized water (dH₂O) for 1 min each. Slides were incubated with 0.06% potassium permanganate for 20 min on a shaker at RT before a 1-min wash with dH₂O. Slides were then incubated with FJ staining solution [4 mL 0.01% FJ (in dH₂O) stock solution, 36 mL 0.1% acetic acid in dH₂O, and 40 µL DAPI] for 30 min at RT before 3 washes with dH₂O for 1 min each. After air-drying, slides were briefly rinsed with xylene. The DG areas of three slices per brain were visualized using a microscope (DM IRB; Leica) by an observer blinded to group assignment.

2.6 Real-time quantitative polymerase chain reaction (qPCR) Twelve hours after sevoflurane anesthesia, the rat pups were decapitated and the brains harvested. The DG area was selected. Total RNA was extracted using TRIzol reagent (Invitrogen). RNA was reverse transcribed to cDNA using the Qiagen Omniscript RT kit (Qiagen, Valencia, CA) following the manufacturer's instructions. qPCR was performed for quantification of ki-67 and β-actin mRNA on a Rotor-Gene 3000 (Corbett Research, Sydney, Australia) based on the published methods^[20,21]. Changes in gene expression were deter-

mined using the $\Delta\Delta C_t$ method^[22]. β-actin was used as a reference gene for loading control. Expression of the gene of interest was defined relative to control animals, in which the expression was defined as 100%. The primer sequences for the target genes were as follows: Ki-67 forward 5'-GTGACAGGCACACCGGCACC-3'; reverse 5'-GGGTCACTGGCAGCAGCTGG-3'; β-actin forward 5'-ATGTGGATCAGCAAGCAGGAGTA-3'; reverse 5'-TTGTCAAAGAAAGGGTGTAAAACG-3'.

2.7 Western blot The harvested DG samples were subjected to Western blot as described by Xie *et al.*^[23]. Briefly, 30 µg tissue lysate was separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride blots (Bio-Rad, Hercules, CA) using a semidry electrotransfer system (Amersham Biosciences, San Francisco, CA). After blocking with 5% non-fat milk, the blot was incubated overnight at 4°C with rabbit anti-cleaved caspase-3 (1:500; Cell Signaling Technology, Danvers, MA), followed by TBST washes and incubation with goat anti-rabbit IgG (1:20 000; Jakson ImmunoResearch, West Grove, PA) and visualized with a chemoluminescence system (Amersham ECL Plus™; GE Healthcare, Piscataway, NJ). β-actin was used as a loading control. The signal of the Western blot band was detected using the Molecular Imager VersaDoc MP 5000 System (Bio-Rad). The signal intensity was analyzed using a Bio-Rad image program (Quantity One) and ImageJ version 1.37 (National Institutes of Health, Bethesda, MD).

2.8 Morris water maze (MWM) test A black circular tank (160 cm in diameter, 60 cm deep) was filled with water (21 ± 1°C) to a depth of 32 cm. The tank was surrounded by several visual cues. The animals were subjected to the MWM test twice: once at 2 weeks and once at 6 weeks after anesthesia. Each animal was tested for 6 consecutive days during each of the MWM tests. Briefly, a platform (9 × 9 cm²) was submerged 0.5 cm below the water surface in the middle of one of the four virtual quadrants. Each day the animals were lowered into the water facing the wall from four different starting points. For example, if the goal was SE, then the start locations were N, W, NE or SW. Although not equidistant from the goal, these start positions

are closer to being equal in length than using start positions adjacent to the goal. Animals that did not find the escape platform within 60 s were placed onto it by the researcher. All rats were allowed to remain on the platform for 10s for orientation and were then removed to rest for 15 s in a cage until the next trial. In each trial, the escape latency to reach the platform was measured by a computerized tracking system (Shanghai Jiliang Software Technology Co., Ltd., China). The four daily trials were averaged for each animal. On day 7, the escape platform was removed and the time spent in each quadrant during a single 60-s trial and the number of platform-site crossings were recorded.

2.9 Data analysis Data are expressed as mean \pm SEM. Two-group comparisons were evaluated using Student's *t*-test. Multiple comparisons were evaluated by ANOVA followed by Dunnett's or Tukey's test. Differences were considered statistically significant for $P < 0.05$.

3 Results

3.1 Blood gas analysis There was no significant difference between the two groups in PH (sevoflurane 7.403 ± 0.004 versus control 7.403 ± 0.011 ; $P > 0.05$, $n = 6$), pO_2 (37.83 ± 2.845 versus 41.50 ± 3.998 mmHg; $P > 0.05$, $n = 6$) or pCO_2 (41.00 ± 1.487 versus 42.67 ± 2.115 mmHg; $P > 0.05$, $n = 6$).

3.2 Sevoflurane induces neuronal degeneration in DG FJ staining was performed to detect neuronal cell death in the DG area^[24,25]. FJ is an anionic X fluorescein derivative which is used to stain degenerating neurons. This technique was originally developed by Schmued *et al.*^[26] to replace hematoxylin and eosin or Nissl stains with a more sensitive and specific marker in tissue slices. Sevoflurane enhanced FJ staining in P7 rat pups immediately after anesthesia [number of FJ-positive cells per $50 \times 50 \mu m^2$: 75.4 ± 2.0 (sevoflurane) versus 67.3 ± 1.8 (control); $P < 0.05$, $n = 6$], and this effect remained 4 days later (74.9 ± 1.0 versus 61.6 ± 1.5 ; $P < 0.05$, $n = 6$) (Fig. 1A–C).

Caspase-3 activation is one of the final steps in apoptosis^[27]. Therefore, we next assessed the effects of sevoflurane on caspase-3 activation in the DG area of P7 rats by Western blot analysis after animal recovery from anesthe-

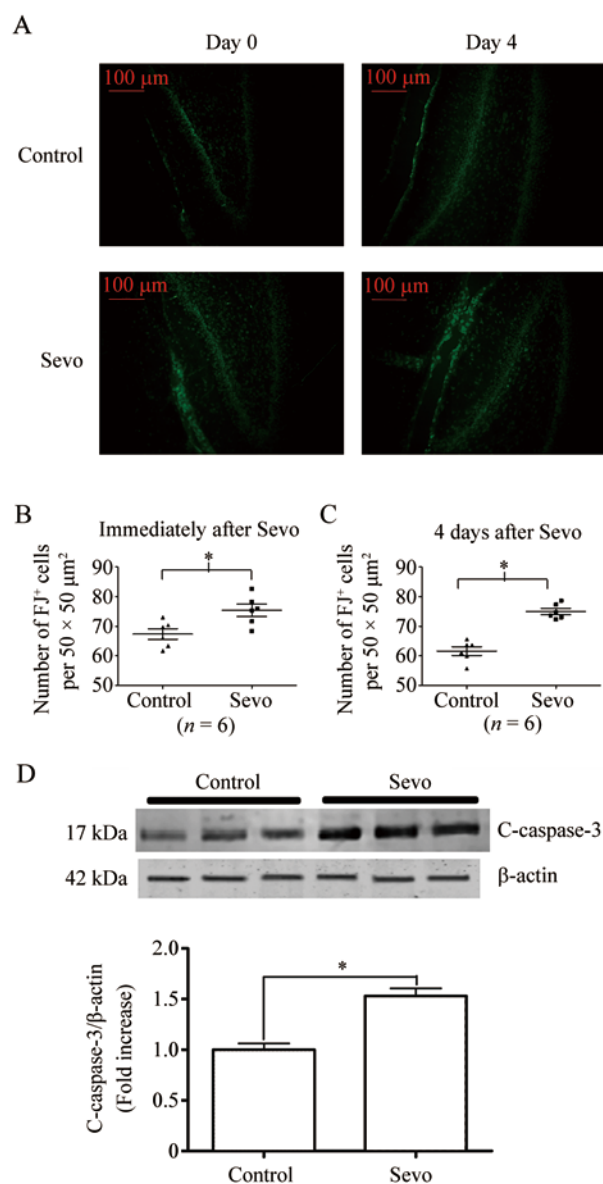


Fig. 1. Sevoflurane (Sevo) enhances Fluoro-Jade (FJ) staining in the dentate gyrus (DG) and induces caspase-3 activation indicative of neuronal cell death. **A:** Representative images showing increased numbers of FJ-positive cells in the DG area both on the day of sevoflurane anesthesia (Day 0) (left panel) and at 4 days after anesthesia (Day 4) (right panel) compared to control. Scale bars, 100 μm . **B:** Summary of the effect of sevoflurane on FJ staining in the DG at day 0. $*P < 0.05$; $n = 6$. **C:** Summary of the effect of sevoflurane on FJ staining in the DG at day 4. $*P < 0.05$; $n = 6$. **D:** Western blots showing that the expression of cleaved caspase-3 (C-caspase-3) was increased by 4-h sevoflurane exposure. Each group contained three independent animal and tissue preparations. Bar-graph summarizes the effect of sevoflurane on C-caspase-3 expression. $*P < 0.05$; $n = 3$.

sia. Caspase-3 immunoblotting showed visible increases in the protein levels of cleaved caspase-3 after sevoflurane compared with the control condition (Fig. 1D). Quantification of the Western blots, by determining the ratio of the cleaved (activated) caspase-3 fragment (17–20 kDa) to β -actin (42 kDa), revealed that anesthesia led to a >50% increase in caspase-3 activation after 4 h of anesthesia compared with the control condition. Taken together, these results suggest that sevoflurane causes significant neuronal

degeneration in the DG of P7 rats and this effect lasts for at least 4 days.

3.3 Sevoflurane interferes with neurogenesis in DG

Since abnormal neurogenesis in the DG region has been suggested as a candidate mechanism for anesthesia-induced long-term neurocognitive dysfunction^[28], we next sought to determine the effect of sevoflurane on neurogenesis in P7 rat pups. First, we measured the mRNA level of Ki-67. The Ki-67 protein (also known as MKI67) is a cellular

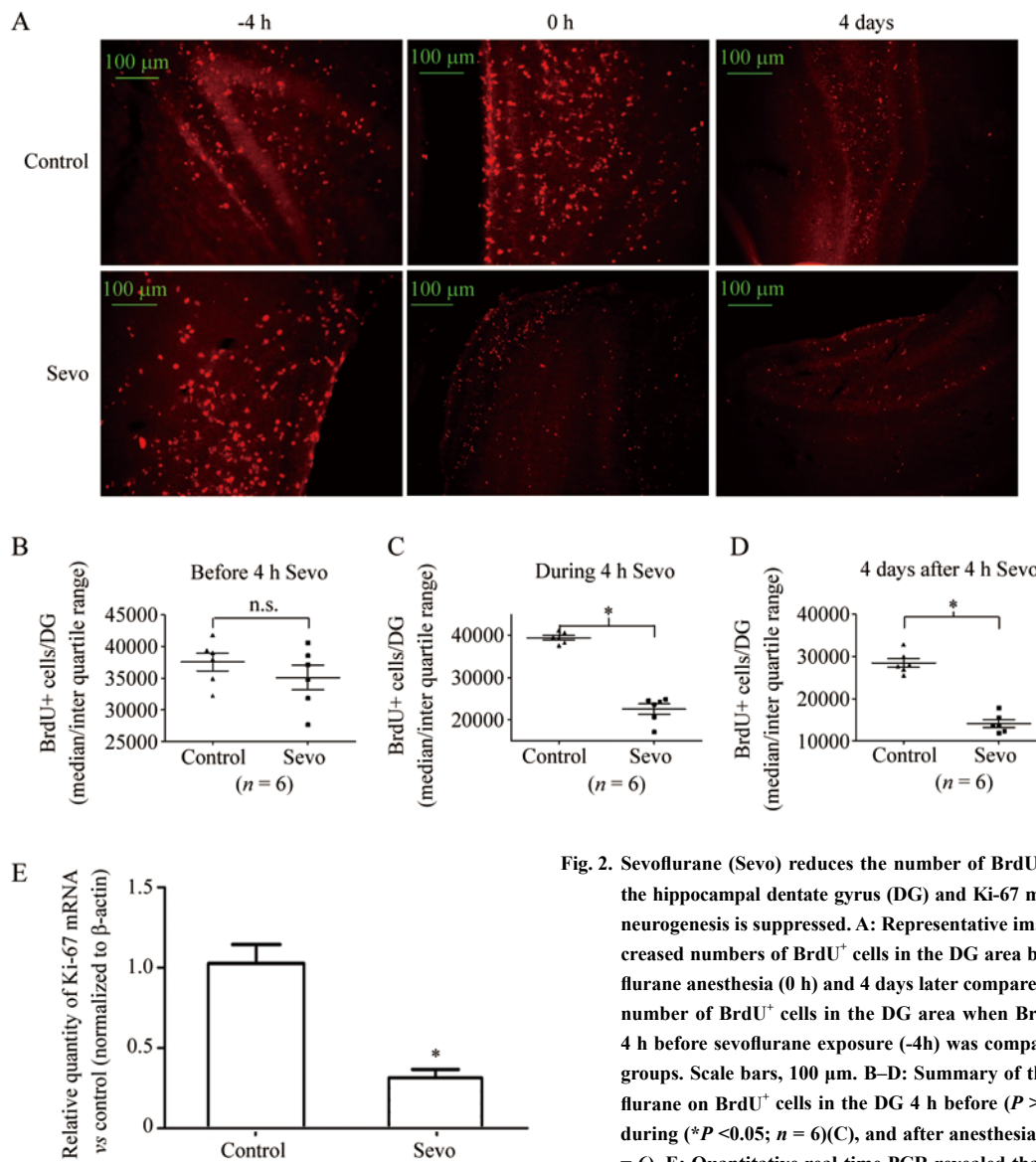


Fig. 2. Sevoflurane (Sevo) reduces the number of BrdU-positive cells in the hippocampal dentate gyrus (DG) and Ki-67 mRNA, indicating neurogenesis is suppressed. **A:** Representative images showing decreased numbers of BrdU⁺ cells in the DG area both during sevoflurane anesthesia (0 h) and 4 days later compared to control. The number of BrdU⁺ cells in the DG area when BrdU was injected 4 h before sevoflurane exposure (-4h) was comparable in the two groups. Scale bars, 100 μ m. **B–D:** Summary of the effect of sevoflurane on BrdU⁺ cells in the DG 4 h before ($P > 0.05$; $n = 6$) (B), during ($*P < 0.05$; $n = 6$) (C), and after anesthesia (D) ($*P < 0.05$, $n = 6$). **E:** Quantitative real-time PCR revealed that the level of Ki-67 in the DG was reduced by ~70% by 4-h sevoflurane exposure; $*P < 0.05$; $n = 5$.

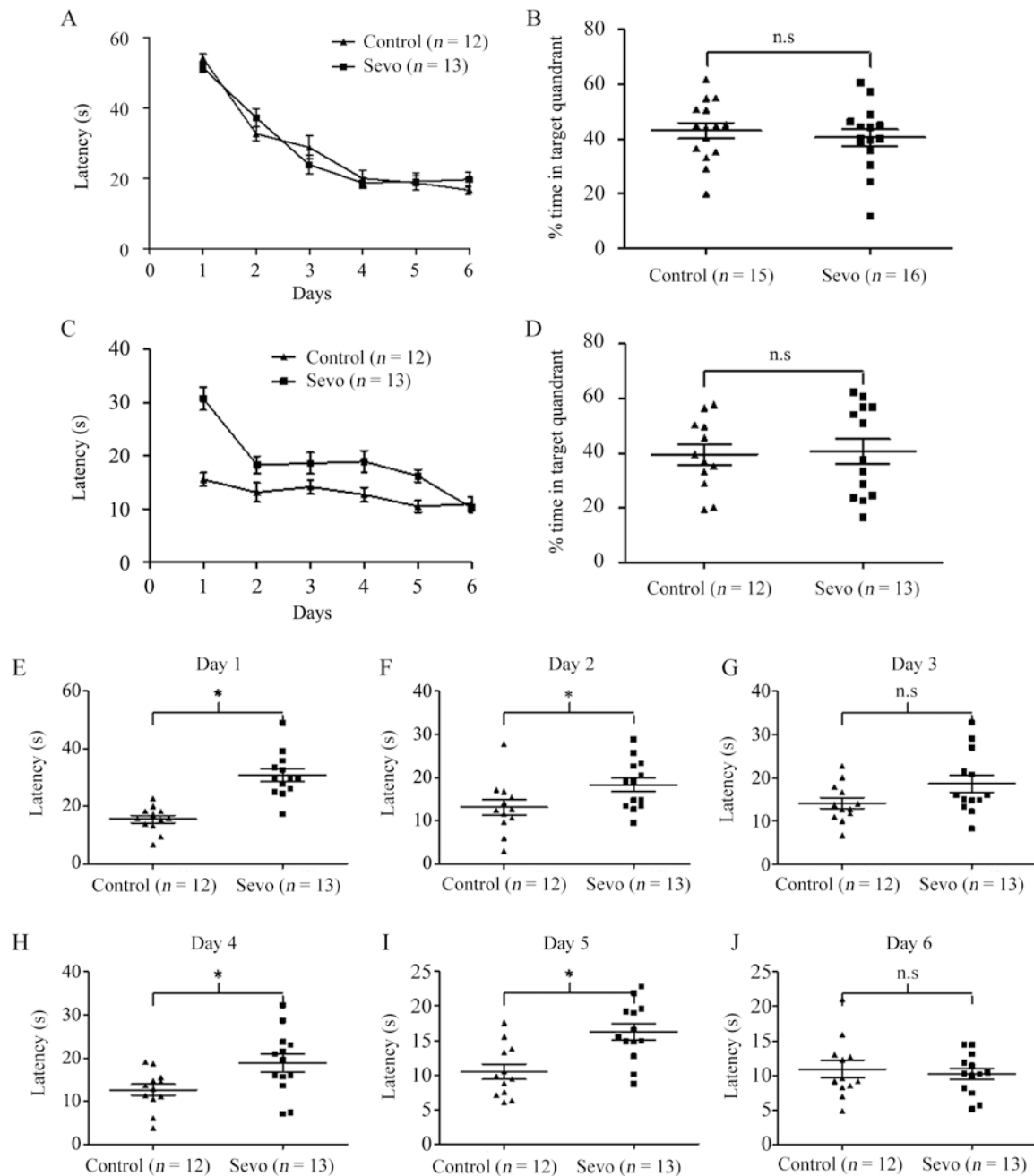


Fig. 3. Spatial learning and memory assessed by Morris water maze 2 and 6 weeks after 4 h of sevoflurane (Sevo) on postnatal day 7 (P7). Memory acquisition was assessed in cued trials by measuring the time to reach the escape platform (latency). Memory retention was assessed 24 h later in a probe trial during which the platform was removed from the pool by measuring the percentage of time spent in the target quadrant. When sevoflurane was administered at P7, neither memory acquisition from P22 to P27 (A) nor memory retention on P28 was affected (B) ($P > 0.05$). Six weeks after anesthesia, memory acquisition was affected. During the cued trials (C), the latencies on days 1, 2, 4 and 5 were significantly longer in sevoflurane-treated rats ($*P < 0.05$; $n = 12-13$; E-I); however, on the last trial (day 6), the latency of the sevoflurane group returned to the control level ($P > 0.05$; $n = 12-13$; J). Probe trial performance was unaffected by sevoflurane ($P > 0.05$) (D).

marker strictly associated with proliferation^[29]. During interphase, the Ki-67 antigen is exclusively detected within

the nucleus, whereas in mitosis most of the protein is re-located to the surface of the chromosomes. Ki-67 protein

is present during all active phases of the cell cycle (G1, S, G2, and mitosis), but is absent from resting cells (G0). The DG Ki-67 mRNA level was reduced by ~70% in sevoflurane-treated rats compared to control rats (Fig. 2E).

BrdU fluorescent staining was next done to evaluate *in situ* neurogenesis in the DG region. The development of BrdU immunohistochemistry to identify S-phase cells in the brain (Miller and Nowakowski, 1988) was an important advance over ³H-thymidine autoradiography; this new method reveals labeled cells throughout the relatively thick tissue sections required by stereologic techniques to determine the total number of S-phase cells within a brain region. We performed three groups of experiments. First, BrdU was administered to P7 pups 4 h before the scheduled sevoflurane anesthesia as a control experiment, since this dose of BrdU is available for <4 h^[30]. In this group, we did not observe any difference in the number of BrdU-positive cells per DG between the control and sevoflurane-treated groups (Fig. 2A, B) (BrdU⁺ cells/DG: control 37550 ± 1403 *versus* sevoflurane 35090 ± 1935; *P* > 0.05, *n* = 6). Second, we administered BrdU at the beginning of sevoflurane anesthesia and found that the number of BrdU-positive cells per DG was markedly lower in the sevoflurane-treated group than control (BrdU⁺ cells/DG: control 39430 ± 545.2 *versus* sevoflurane 22480 ± 1252; *P* < 0.05, *n* = 6; Fig. 2A, C), which indicated that sevoflurane has a negative effect on proliferation during anesthetic action. Third, we applied BrdU 4 days after sevoflurane administration. Interestingly, the proliferation was still inhibited in the sevoflurane-treated group (BrdU⁺ cells/DG: control 28480 ± 1050 *versus* sevoflurane 14120 ± 902; *P* < 0.05, *n* = 6; Fig. 2A, D). Collectively, these results showed that sevoflurane inhibits neuronal proliferation in the DG region of P7 rats upon the administration of anesthesia and this effect lasts for at least 4 days.

3.4 Sevoflurane induces delayed-onset neurocognitive deficits as measured by water maze spatial reference learning and memory retention tasks Both the sevoflurane and the control groups learned at a steady but slow rate when spatial reference memory was tested in the MWM on postnatal days 21–26 after 4 h of sevoflurane on

P7. At 2 weeks after anesthesia, there was no difference between groups in the performance of either the cued trials, in which the platform was hidden, or the probe trial, in which the platform was removed from the tank (Fig. 3A, B).

When the same animals were retested 6 weeks after the anesthesia, rats that had received sevoflurane at P7 spent significantly more time searching for the platform in the target quadrant during the cued trials. At the end of the cued trials, the two groups performed the same (Fig. 3C, E–J). There was no difference in platform-site crossings between the two groups on the probe trial (Fig. 3D).

4 Discussion

In this study, we reported for the first time that neonatally-administered sevoflurane induced deficits in hippocampus-dependent learning and memory in rats. The impairment caused by sevoflurane was twofold: acute neuronal damage at the cellular level and a delayed-onset neurocognitive malfunction.

The DG develops in three distinct phases and the peak of neuronal expansion occurs around P7^[15]. In the P7 rat brain, the newly-formed DG is critical to the development of episodic memory^[31]. Adequate and robust neurogenesis ensures hippocampal learning^[32], whereas decreased neurogenesis impairs it^[16,17]. Developing neurons from embryonic day 19 to P14 are most susceptible to anesthetic influences, as reported in several *in vivo* studies^[33,34]. In particular, the P7 rat is extremely sensitive to neurotoxic challenge^[5]. Therefore, we chose P7 as the time point in our studies to administer anesthesia in order to evaluate sevoflurane-induced neurotoxic effects.

We first used FJ staining to assess the neuronal apoptosis induced by sevoflurane. FJ is in general comparable to silver staining^[24,25]. We found that just like isoflurane^[35,36], sevoflurane significantly increased the numbers of FJ-positive cells in the DG immediately after exposure, and that this effect was sustained for at least 4 days. Apoptosis was further confirmed by Western analysis of cleaved caspase-3. However, despite the clear signs of apoptosis, we did not conclude that apoptosis is the only cause of the

neurocognitive deficits shown by our MWM results. If apoptosis were the only cause, deficits should be evident immediately after the cell loss. But in our study, the MWM deficit occurred 6 weeks but not 2 weeks after sevoflurane application.

We next examined the effect of sevoflurane on DG neurogenesis, since disrupted neurogenesis in the DG region has been suggested as a candidate mechanism for anesthesia-induced long-term neurocognitive dysfunction^[28]. We found that BrdU-positive cells in the DG in the anesthesia group were significantly fewer than in the control group both immediately after anesthesia and 4 days later. This indicates that neurogenesis in the DG was reduced by anesthesia, and that this effect lasted for at least 4 days. In addition, we performed qPCR to assess the mRNA level of Ki-67, which is a cell-cycle regulator gene. Sall *et al.*^[37] showed that the Ki-67 mRNA level in cultured hippocampal neural precursor cells is significantly reduced by isoflurane exposure. Consistent with their findings, Ki-67 mRNA was also drastically decreased in the DG tissue from rat pups exposed to sevoflurane in our studies. Decreases in the number of BrdU⁺ cells and the mRNA level of Ki-67 suggested that DG neurons in the experimental pups either were in growth phase arrest or had exited the cell cycle, as would occur with differentiation.

The MWM was chosen to evaluate the effect of sevoflurane on cognitive behavior in the rats since it is a reliable measure of hippocampus-dependent spatial navigation and reference memory^[38]. Interestingly, we found that the learning curves of the rats that received sevoflurane or vehicle for 4 h on P7 were similar at P21. However, 6 weeks after anesthesia, the latencies shown by the rats receiving sevoflurane at P7 were significantly longer than those receiving vehicle. These results are consistent with the findings from other groups^[13]. However, the uniqueness of our study is that there was no longer any difference in latency between the two groups at the end of the basic acquisition training. The probe test showed that the time spent in reaching the target quadrant was similar to the other quadrants. The numbers of platform-site crossings were also the same in the two groups. We interpret this to mean

that the worsening of performance in the MWM caused by 4-h sevoflurane in P7 rats developed progressively so that no deficit was observed at 2 weeks but the deficit was evident at 6 weeks after anesthesia. However, what caused this delay in the onset of the cognitive deficit? As we noted previously, it is unlikely that cell death is the only consequence of anesthesia that contributes to this cognitive deficit. As shown by Keith *et al.*, the hippocampal deficit caused by cell death alone in the DG indeed improves over 6 weeks^[28].

Both the type of deficit caused by sevoflurane and its onset between 2 and 6 weeks suggest that the decrease in neurogenesis may be the key mechanism. Decreased proliferation in neonates could lead to cognitive dysfunction by permanently disrupting the architecture of the hippocampus during a critical period of development or by depleting the pool of precursor cells present for the duration of the animal's life^[37]. In our study, we found no difference between control and sevoflurane groups in the MWM at 2 weeks after anesthesia. Although the latency shown by the rats receiving sevoflurane was significantly longer than the controls when tested 6 weeks after anesthesia, they managed to catch up with the controls after 5 days of training, and the performance of both groups in the probe trial was comparable. These suggest that the long-term memory impaired by sevoflurane can be retrieved by training, in other words, stimulation.

Environmental stimuli may either enhance^[39] or suppress^[40] stem/progenitor cell proliferation, depending on the relative predominance of enriching or noxious qualities^[41]. Anesthesia may alter the environmental stimuli, including neuronal signaling, and have age-dependent effects on cell proliferation in the DG^[13]. It is possible that the retrieval of hippocampal learning after training is because the adult hippocampus integrates new neurons into its circuitry with learning^[42,43]. It should be noted that we did not assess the progenitor proliferation and degeneration beyond any time point after 4 days post-anesthesia. It is also not known whether the cognitive function would improve or deteriorate after 6 weeks post-anesthesia. Further studies are needed to address these concerns.

In summary, our study showed that sevoflurane significantly inhibited progenitor cell proliferation and enhanced degeneration in developing brain, and these effects led to subtle, progressive deficits in hippocampal-dependent learning and memory. Further studies are needed to determine whether the decreased proliferation is due to exit from the cell cycle or cellular differentiation. In addition, how sevoflurane affects the shaping of synapses in the DG is of great interest.

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