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Original Article

Enhanced Histone Lysine Methyltransferase G9a Might Contribute to Repeated Sevoflurane Exposure-Induced Apoptosis and Cognitive Impairment in the Developing Brain

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ABSTRACT

Background: Early exposure to sevoflurane induces neurodegeneration in the developing brain and subsequent long-term neurobehavioral abnormalities in animals and humans. However, the underlying molecular mechanisms remain unclear. We explored whether hippocampal histone lysine methyltransferase G9a plays a role in sevoflurane-induced cognitive impairment in neonatal rats.

Methods: The anesthesia was induced with 6% sevoflurane for 3 minutes and maintained with 3% sevoflurane for 1 hour and 57 minutes. The anesthesia was applied for three successive days from postnatal day 5 (P_5) to P_7. The inhibitor of G9a (Bix-01294, Bix) or vehicle was given 15 minutes before the sevoflurane exposure, respectively. The rats were allocated into four groups: control + vehicle (Con + Veh) group, control + Bix (Con + Bix) group, sevoflurane + vehicle (Sev + Veh) group, and sevoflurane + Bix (Sev + Bix) group. Cognitive performance was evaluated by the open field, fear conditioning, and Morris water maze tests at P_{35}, $P_{39–41}$, and $P_{49–55}$, respectively. After the last sevoflurane exposure or before the open field test, the brain tissues were harvested for further analysis.

Results: The freezing time to context in the fear conditioning test and the time spent in the target quadrant in the Morris water maze test were significantly decreased in the Sev + Veh group compared with the Con + Veh group. Sevoflurane exposure increased cleaved caspase-3, enhanced G9a activity, and subsequently upregulated dimethylation lysine 9 of histone H3 (H3K9me2) in the hippocampus. By contrast, Bix treatment could rescue these changes.

Conclusion: Our results demonstrated that the G9a-mediated enhancement of H3K9me2 expression might be involved in the sevoflurane-induced neuroapoptosis and cognitive impairment in the developing brain.

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Each year, millions of children receive surgical operation with general anesthesia (1). Accumulative evidence has suggested that children exposed to anesthesia, especially with multiple exposures, less than 4 year old, have an increased risk for the long-term diminution of language abilities and cognition (2-4). Sevoflurane is one of the most commonly used anesthetics for children due to its sweet-smelling, nonflammable, fast acting, and short recovery time. Although recent studies have shown that sevoflurane can induce neuroapoptosis in developing brain and subsequently cause long-lasting learning and memory deficits later in life (5, 6), the precise mechanisms underlying the neurotoxicity of general anesthesia remain largely to be determined.

Epigenetic mechanisms, including DNA methylation, posttranslational his-

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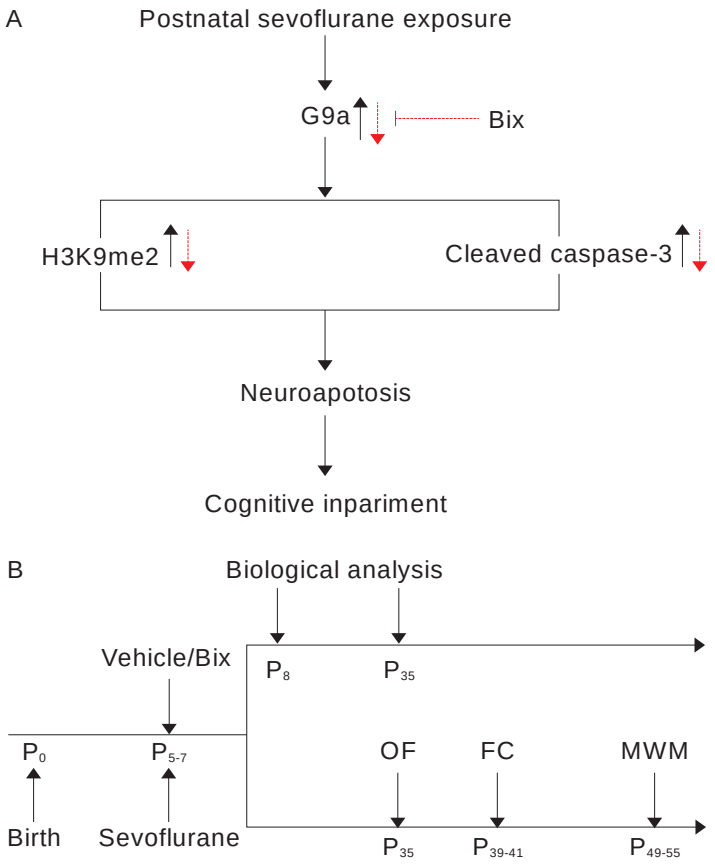

Figure 1. The Schematic Timeline of the Experimental Procedure and the Schematic Representation of Proposed Mechanism of Action by Which G9a-Mediated Histone Methylation Regulates Sevoflurane-Induced Apotosis in Neonatal Rats and Cognitive Impairment in Adolescent Rats.

Figure A showed the proposed mechanism of action: sevoflurane exposure at P_{5-7} enhances hippocampal G9a expression and activity (→) leads to hippocampal caspase-3 activation and the increase of H3K9me2 (→) leads to cognitive impairment in adolescent rats. Inhibition of G9a/GLP activity by Bix (- - -) prevents sevoflurane-induced increase in H3K9me2 and rescues neuroapotosis (cleaved caspase-3) in neonatal hippocampus. Figure B showed the schematic timeline.

tone modification (acetylation, phosphorylation, and methylation, et al), and small noncoding RNAs, play important roles in human neurodegenerative diseases including Alzheimer's disease, Parkinson's disease, and postoperative cognitive dysfunction (7-11). Methylated DNA induces gene silencing via recruiting methyl CpG-binding proteins. However, the histone modification can result in activation or repression of gene transcription. For example dimethylation lysine 9 of histone H3 (H3K9me2) is correlated with transcriptional silencing, whereas trimethylation lysine 9 of histone H3 (H3K4me3) is linked to active transcription (9). Especially, histone lysine methylation plays a critical role in psychiatric disorders, including schizophrenia, depression, and posttraumatic stress disorder (12, 13). G9a and G9a-like protein (GLP), a class of histone lysine methyltransferases, are primary enzymes for H3K9me2 (14) that suppress the expression of genes (15). More importantly, G9a is also reported to be essential for neuronal function during brain development (16).

In the present study, we hypothesized that sevoflurane exposure at postnatal day (P_{5-7}) may enhance hippocampal G9a expression and then lead to the hippocampal caspase-3 activation and the increase of H3K9me2, which may induce cognitive impairment in adolescent rats (Figure 1A). So in this study, we aimed to explore the relationship between the G9a-mediated H3K9me2 and the sevoflurane-induced long-term neurobehavioral abnormities by the inhibition of G9a/GLP activity.

MATERIALS AND METHODS

Animals

The study protocol was approved by the Ethics Committee of Jinling Hospital, Nanjing University and the experiments were performed in accordance with the Guideline for the Care and Use of Laboratory Animals from the National Institutes of Health, USA. Male Sprague-Dawley rats aged from P_5 to P_{55} days were used for sevoflurane anesthesia and subsequent analyses (Figure 1B). The pups after birth were housed with the dam until weaning in the P_{21}. All rats were housed in a standard condition under a 12:12 hour light/dark cycle with lights from 7:00 to 19:00, with the room temperature of 23 ± 1℃, and free access to food and water.

We used three different sets of rats for the following experiments. The first set of rats (N=36) were used to study the neurotoxicity of sevoflurane (the 0, 4, 8 and 24 hours after the end of last anesthesia) and the suitable dose of Bix (Bix-01294, 2-(Hexahydro-4-methyl-1H-1, 4-diazepin-1-yl)-6, 7-dimethoxy-N-[1-(phenylmethyl)-4-piperidinyl]-4-quinazolinamine trihydrochloride) (Cayman, Ann Arbor, MI, USA) to improve the detrimental effect of sevoflurane exposure. The second set of rats (N=36) were used

to study the effect of Bix on the sevoflurane anesthesia at P_8 and P_{35}. The third set of rats (N= 32) were used for behavioral tests. The second and third sets of rats were equally randomized into one of the four groups: control + vehicle (Con + Veh) group, control + Bix (Con + Bix) group, sevoflurane + vehicle (Sev + Veh) group, or sevoflurane + Bix (Sev + Bix) group.

Sevoflurane Anesthesia and Drug Treatment

Anesthesia was induced with 6% sevoflurane by 100% of 2 L/minute O_2 for 3 minutes and maintained with 3% sevoflurane by 100% of 2 L/minute O_2 for 1 hour and 57 minutes in a thermostatic chamber daily for three successive days from P_5 to P_7. Previously, we have shown that this anesthesia protocol could cause the neurobehavioral abnormalities without respiratory depression (6). The rats breathed spontaneously, and concentrations of anesthetic and oxygen were measured continuously using a calibrated Datex side stream analyser that sampled from the interior of the chamber. Temperature of the rats was maintained at 37±0.5℃ during anesthesia by heating lamps and heating pads. The pups returned to their mother cage after recovery from anesthesia. Accordingly, Bix was diluted with vehicle (normal saline) to final 0.2 mg/ml and subcutaneously injected with 0.25, 0.5, and 1 mg/kg 15 minutes before each sevoflurane exposure, respectively.

Behavioral Tests

All behavioral tests were conducted in a sound-isolated room with the instruments (Shanghai Softmaze Information Technology Co. Ltd., Shanghai, China). The rats used for behavioral tests were not analyzed in further biochemical experiments.

Open Field (OF) Test

At P_{35}, the rat was placed into the OF apparatus (XR-XZ301, 100 cm × 100 cm × 40 cm black plastic box) for 10 minutes. The behaviors of each rat were traced by a camera for further analysis. At the end of each test, the surface of the arena was thoroughly cleaned with 75% alcohol to avoid the presence of olfactory cue.

Fear Conditioning (FC) Test

The FC test was performed by using the FC paradigm (XR-XC404, 30 cm × 30 cm × 45 cm soundproof chamber) at P_{39-41}. The FC test was a simple and sensitive test of hippocampus-dependent and hippocampus- independent memory function. During the training, the rat was allowed to explore the training chamber for 180 seconds. Within the last 30 seconds, the rat was subjected to a tone (30 seconds, 70 dB, 3000 HZ) with a terminating 2 seconds electrical foot-shock (0.5 mA), followed by an additional 30 seconds without any stimulation. The rat was placed in the training chamber for 5 minutes (context association testing, without tone or foot-shock) after 24 hours. Then, the cue conditioning test was conducted on the next day in a novel training chamber with a radically changed context. A 3 minutes exploration of the new context was followed by 3 minutes of tone presentation (70 dB, 3000 HZ, without foot-shock). Fear response was reflected by the time of freezing.

Morris Water Maze (MWM) Test

The MWM task was carried out at P_{49-55}. In the spatial discrimination trials, the rat was placed in a round tank (XR-XM101, diameter 120 cm, height 50 cm) filled with opaque water containing an escape platform (height 22 cm) submerged 2 cm below the surface of water. The platform remained in the same location relative to the four visual cues in the tank. The rat received four swims per day for six consecutive days using a 15 seconds interval on platform between swims. Every training session, the rat was placed carefully in the water next to the maze wall and allowed to swim until finding the platform or for 60 second (whichever came first). The time taken to climb the platform (latency) was recorded. In the probe trials (on the seventh day), the rat was placed in the tank for 60 seconds during which the escape platform was removed from the tank, the time spent in swimming in the target quadrant and the swimming route were recorded. The water temperature was maintained at 22 ± 1℃. After finished the per day swimming task, the rat was removed from the tank, dried with a clean towel, and placed in a holding box that is warmed with a temperature- controlled heating pad placed beneath it.

Western Blot

The Western blot analysis was performed previously (17). The rat was sacrificed by 2% sodium pentobarbital (70 mg/kg, intraperitoneal [i.p.]) at the indicated time points (0, 4, 8, and 24 hours [P_8] after the end of last sevoflurane exposure and P_{35}), and the hippocampus was dissected, frozen, and stored at -80℃. On the day of analysis, the brain tissues were allowed to equilibrate to a temperature of 4℃. Tissue protein was collected by using the protein extraction kit (Nanjing KeyGEN Biotech, Nanjing, China). The protein concentrations were determined using the QuantiPro BCA Assay kit (Nanjing KeyGEN Biotech, Nanjing, China). Protein extracts (70 μg protein/lane) were separated using polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene fluoride membranes with an electrophoretic transfer system. Membranes were blocked with 5% nonfat-dried milk in tris-buffered saline tween (TBST) for 1 hour and then incubated with the primary antibodies: anti-rabbit cleaved caspase-3 (polyclonal, #9661, 1:1000, Cell Signaling Technology, Danvers, MA, USA), anti-rabbit-G9a (monoclonal, #3306, 1:1000, Cell Signaling Technology, Danvers, MA, USA), anti-rabbit H3K9me2 (monoclonal, #4658, 1:1000, Cell Signaling Technology, Danvers, MA, USA) and anti-rabbit β-actin (polyclonal, #YM3214, 1:3000, ImmunoWay, New Jersey, USA) overnight in a room at 4℃. After thorough washing, membranes were incubated in 5% nonfat-dried milk in TBST with the secondary antibody (goat anti-rabbit, #AP132P, 1:5000, Millipore, Darmstadt, Germany) for 2 hours at room temperature. Enhanced chemiluminescent and the Image Quant Software (Syngene, Cambridge, United Kingdom) were used to visualize and quantitate the immunoreactivity, respectively.

Immunocytochemistry

The immunohistochemistry was performed as we described previously (6). Briefly, the rats were anesthetize with 2% sodium pentobarbital (70 mg/kg, i.p.) at P_8, and perfused with phosphate buffered saline (PBS), followed by freshly prepared 4% paraformaldehyde. Sections of paraffin-embedded tissue (4 μm) were cut and mounted on Vectabond adhesive-coated slides (RM2016, Shanghai Leica Instrument Co. Ltd., Shanghai, China). Sections were deparaffinized in xylene and rehydrated in ethanol to water, then were washed in PBS, and incubated with the primary antibody anti-rabbit cleaved caspase-3 (polyclonal, #9661, 1:1000, Cell Signaling Technology, Danvers, MA, USA) at 4℃ for 24 hours. The sections were washed three times for 5 minutes with PBS and incubated in the secondary antibody (goat anti-rabbit, #AP132P, 1:500, Millipore) for 50 minutes, following three washes in PBS and staining using the 3, 3'-diaminobenzidine chromogenic liquid. The cells of brown were considered to be positive. An investigator who blinded to the treatment conditions counted the number of cleaved caspase-3^+ cells by using the Image Quant Software.

Statistical Analysis

The statistical analyses were performed using the Prism software (GraphPad, San Diego, CA, USA). The data were presented as the mean ± standard error of the mean (SEM) and statistically analyzed by one-way analysis of variance (ANOVA) or two-way ANOVA with Bonferroni's post hoc test. Pearson's rank correlation coefficient method was used for correlation analysis. In all of the comparisons, $P<0.05$ was considered to indicate statistical significance.

RESULTS

The Effect of Repeated Sevoflurane Exposure on the Cleaved Caspase-3 and G9a Expressions in the Neonatal Hippocampus

Hippocampal cleaved caspase-3 was increased at 24 hours after the last sevoflurane exposure ($P<0.01$, Figure 2A). Consistently, the results of immunohistochemistry showed significantly increased apoptotic cells in the hippocampus in the Sev + Veh group compared with the Con + Veh group ($P<0.01$, Figure 2B). Hippocampal G9a expression including the 165 kDa G9a-L and 140 kDa G9a-S isoforms was also increased at 24 hours after the last sevoflurane exposure ($P<0.01$, Figure 2C). The expression of hippocampal cleaved caspase-3 was positively correlated with the expression of G9a at different time points ($r=0.8696$, $P<0.0001$, Fig-

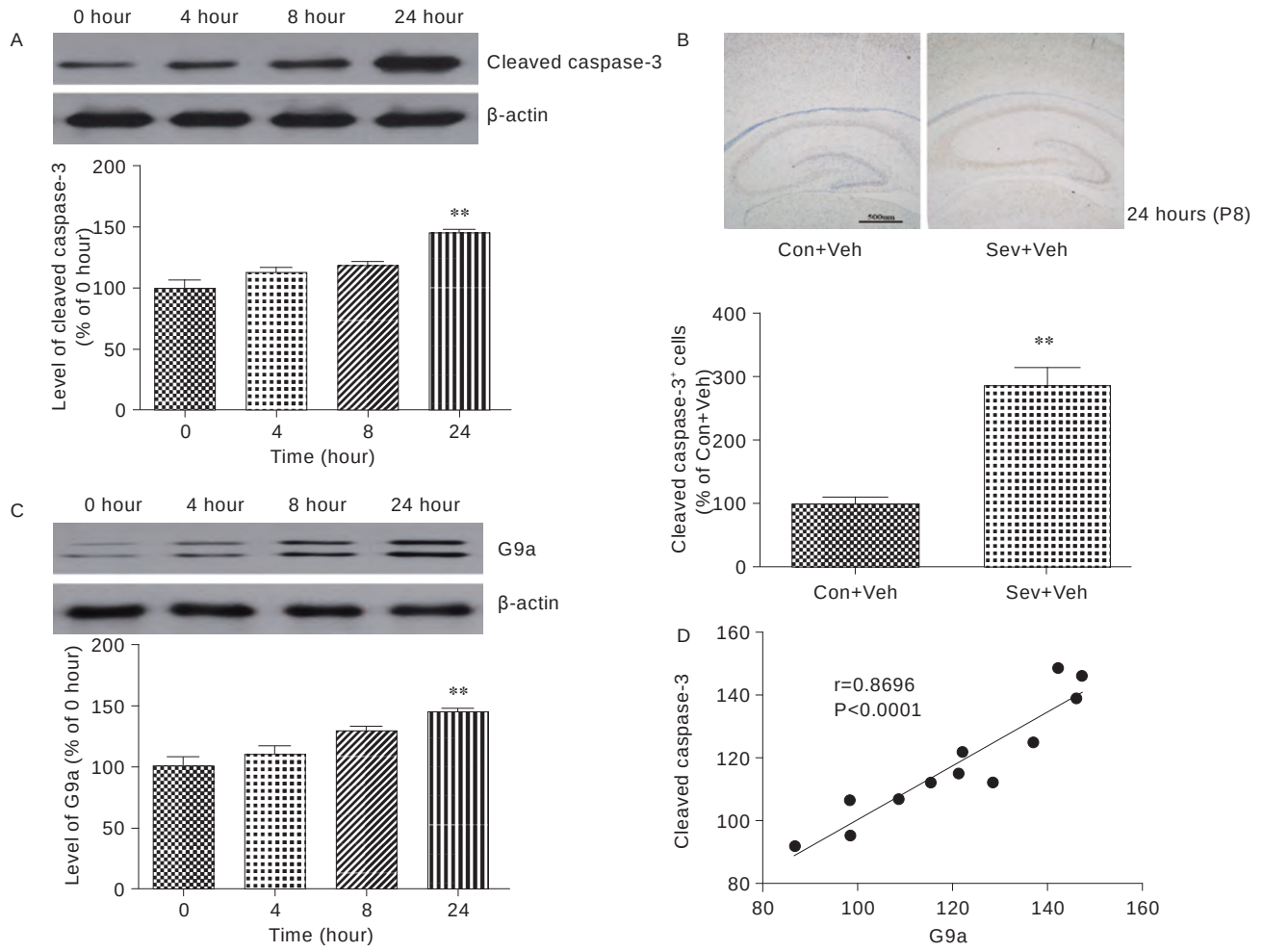

Figure 2. The Effect of Repeated Sevoflurane Exposure on the Cleaved Caspase-3 and G9a Expressions in the Neonatal Hippocampus.

Figure A showed the hippocampal level of cleaved caspase-3 at 0, 4, 8 and 24 hours after the last sevoflurane exposure. Figure B showed the hippocampal cleaved caspase-3$^+$ cells 24 hours after the last sevoflurane exposure. Figure C showed the hippocampal G9a protein including the 165 kDa G9a-L and 140 kDa G9a-S isoforms levels at 0, 4, 8 and 24 hours after the last sevoflurane exposure. Figure D showed the hippocampal level of cleaved caspase-3 at 0, 4, 8 and 24 hours after the last sevoflurane exposure. Scale bars=500 μm. Each data point represent mean ± the SEM (N=3, *P<0.05 or **P<0.01 versus the Con + Veh group or the 0 hour after the last sevoflurane exposure).

ure 2D).

Dose Response of Bix on the Cleaved Caspase-3 Expression after Repeated Sevoflurane Exposure in the Neonatal Hippocampus

Because the repeated sevoflurane exposure- induced neurotoxity was most obvious at 24 hours after the last sevoflurane exposure in our previous experiment, we chose this time point to study the effect of Bix treatment on neuro-apoptosis. Our results indicated that Bix could dose- dependently inhibit sevoflurane- induced hippocampal caspase- 3 activation (Figure 3), and 1 mg/kg of Bix had the maximal effect. Thus, we chose the dose of 1 mg/kg in our subsequent experiments ($P>0.05$).

The Effect of Bix on the Apoptosis after Repeated Sevoflurane Exposure in the Neonatal Hippocampus

As shown in figure 4, the cleaved caspase- 3 $^+$ cells in the hippocampal CA1 and CA3 areas ($P<0.01$) but not in the hippocampal DG areas ($P>0.05$) were significantly increased in the Sev

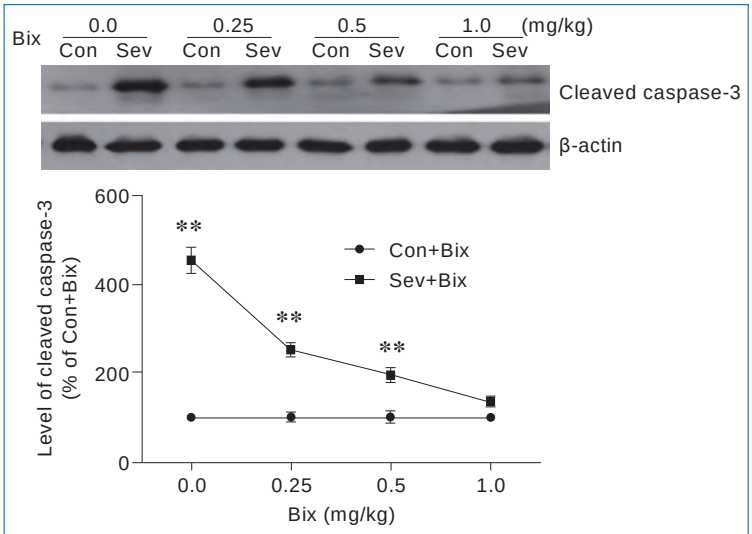

Figure 3. Dose Response of Bix on the Cleaved Caspase-3 Expression after Repeated Sevoflurane Exposure in the Neonatal Hippocampus.

Figure showed the hippocampal level of cleaved caspase-3 at different doses of Bix. Each data point represent mean ± the SEM (N=3, *P< 0.05 or **P<0.01 versus their corresponding concentrations of Bix, respectively).

+ Veh group compared with the Con + Veh group at P_8, whereas Bix could ameliorate this damage. Two- way ANOVA with Bonferroni's post hoc demonstrated significant effects of sevoflurane (vs. Con) (CA1, $F_{1,2}=23.784$, $P=0.040$; CA3, $F_{1,2}=157.465$, $P=0.006$; DG, $F_{1,2}=0.291$, $P=0.643$) and a significant interaction between sevoflurane and Bix (CA1, $F_{1,2}=21.789$, $P=0.035$; CA3, $F_{1,2}=33.631$, $P=0.028$; DG, $F_{1,2}=11.910$, $P=0.075$). These results demonstrated that the inhibition of G9a by Bix could decrease cleaved caspase-3^+ cells in the hippocampal CA1 and CA3 areas.

Bix Treatment Prevented Repeated Sevoflurane Exposure- Induced G9a and H3K9me2 Expressions in the Neonatal and Adolescent Hippocampus

Repeated sevoflurane exposure increased G9a expression in the hippocampus at P_8 and P_{35}, which was prevented by Bix treatment ($P<0.01$, Figure 5A and B). Two-way ANOVA with Bonferroni's post hoc demonstrated significant effects of sevoflurane (vs. Con) (P_8, $F_{1,2}=92.778$, $P=0.011$; P_{35}, $F_{1,2}=180.233$, $P=0.006$). A significant interaction between sevoflurane and Bix was observed for the hippocampal level of G9a at P_8 and P_{35} (P_8, $F_{1,2}=80.997$, $P=0.012$; P_{35}, $F_{1,2}=164.320$, $P=0.006$).

Repeated sevoflurane exposure increased hippocampal H3K9me2 level at P_8 and P_{35}, whereas Bix only decrease hippocampal H3K9me2 level at P_{35} ($P<0.05$, Figure 5D). However, the hippocampal H3K9me2 level in the Sev + Bix group was similar when compared with the Con + Veh group at P_8 ($P>0.05$, Figure 5C). Furthermore, two- way ANOVA for the hippocampal H3K9me2 level showed a significant interaction between sevoflurane and Bix (P_8, $F_{1,2}=21.196$, $P=0.044$; P_{35}, $F_{1,2}=209.452$, $P=0.005$). These results suggested that G9a mediated- increased H3K9me2 expression may be one mechanism responsible for repeated sevoflurane exposure- induced apoptosis in the hippocampus.

Bix Prevented Repeated Sevoflurane Exposure-Induced Behavioral Abnormalities in Adolescent Rats

In the OF test, repeated sevoflurane exposure had no effect on the total distance compared with the Con + Veh group ($P>0.05$, Figure 6A). Two- way ANOVA demonstrated no significant interaction between sevoflurane and Bix ($F_{1,7}=0.220$, $P=0.635$). As shown in figure 6B and C, in the FC test, the freezing time to context was significantly decreased in the Sev + Veh group compared with the Con + Veh group ($P<0.01$), which was rescued by Bix treatment ($P<0.01$). Two-way ANOVA for the freezing time to context presented a significant effect of sevoflurane (vs. Con) ($F_{1,7}=16.139$, $P=0.005$) and a significant interaction between sevoflurane and Bix ($F_{1,7}=6.640$, $P=0.037$). There was no significant difference in the freezing time to tone among the groups ($P>0.05$) and no significant interaction between sevoflurane and Bix ($F_{1,7}=1.331$, $P=0.278$). In the MWM training phase, there was no significantly difference in latency spent to find the underwater platform among the groups ($P>0.05$, Figure 6D). In the testing phase, the time spent in the target quadrant of the Sev + Veh group was significantly increased than that in the Con + Veh group ($P<0.01$, Figure 6E), which was reversed by Bix treatment ($P<0.05$, Figure 6E). In addition, two- way ANOVA with Bonferroni's post hoc indicated a significant effect of sevoflurane (vs. Con) ($F_{1,7}$ =

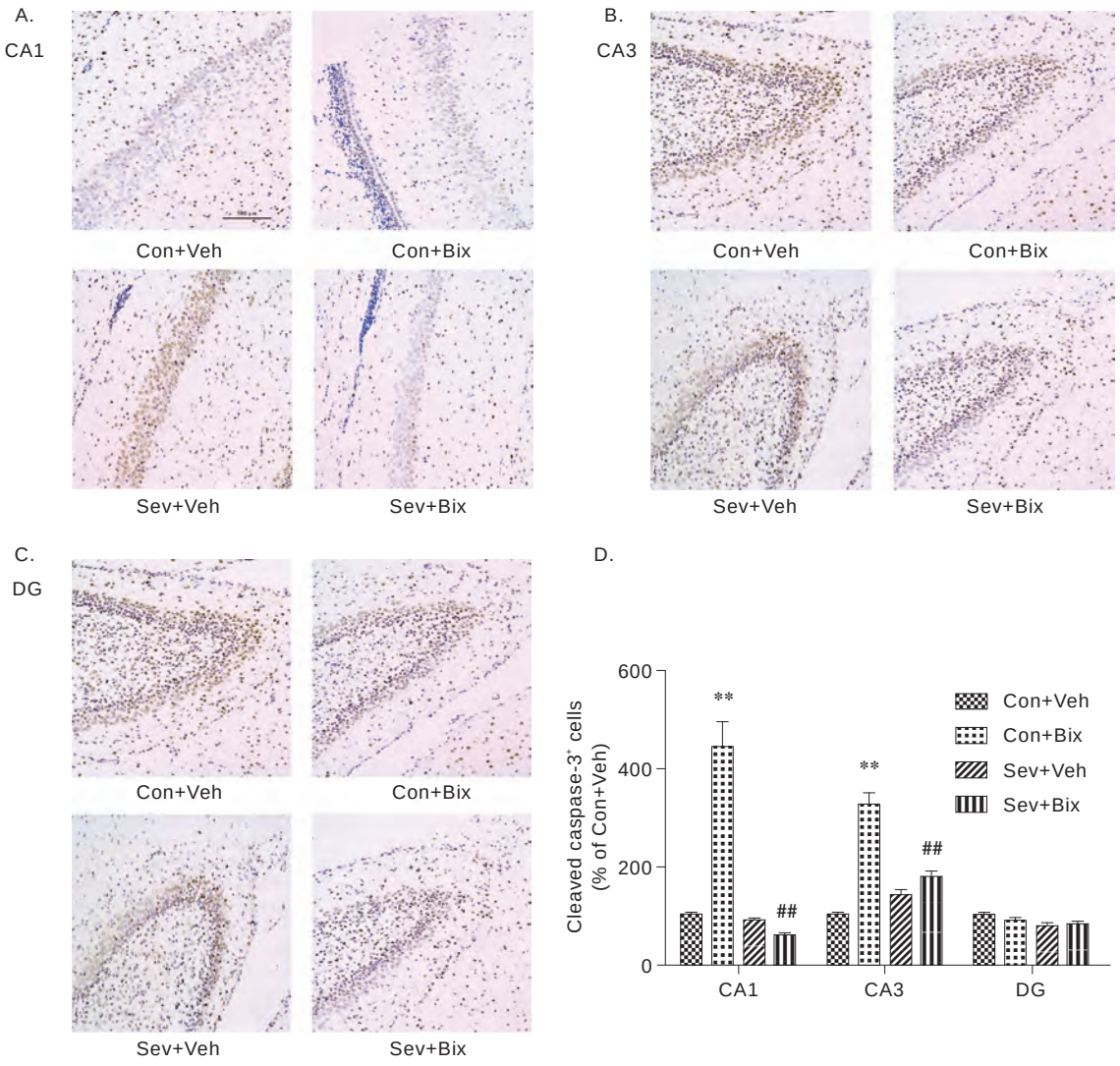

Figure 4. The Effect of Bix on the Apoptosis after Repeated Sevoflurane Exposure in the Neonatal Hippocampus.

Figure A-C showed the cleaved caspase-3^{+} cells in the CA1, CA3 and DG areas. Figure D showed the analysis of apoptosis in the different areas of hippocampus. Scale bars=100 μm. Each data point represent mean ± the SEM (N=3, *P<0.05 or **P<0.01 versus the Con + Veh group; #P<0.05 or ##P<0.01 versus the Sev + Veh group).

10.264, P=0.015) and a significant interaction between sevoflurane and Bix ($F_{1,7}$=18.079, P=0.004).

DISCUSSION

In the current study, we demonstrated that repeated sevoflurane exposure induced neurodegeneration and neurobehavioral abnormities in the developing brain, which was consistent with previous studies (18- 20). Furthermore, we found that sevoflurane-induced cognitive impairment was associated with G9a- mediated enhancement of H3K9me2. Notably, pharmacological inhibition of G9a activity with Bix prior to sevoflurane exposure could prevent the neuroapoptosis and cognitive impairment later in life (Figure 1A).

Accumulating evidence has suggested that neonatal exposure to anesthesia drugs can cause widespread neuroapoptosis and long-term cognitive impairment later in life (5, 19). It has been

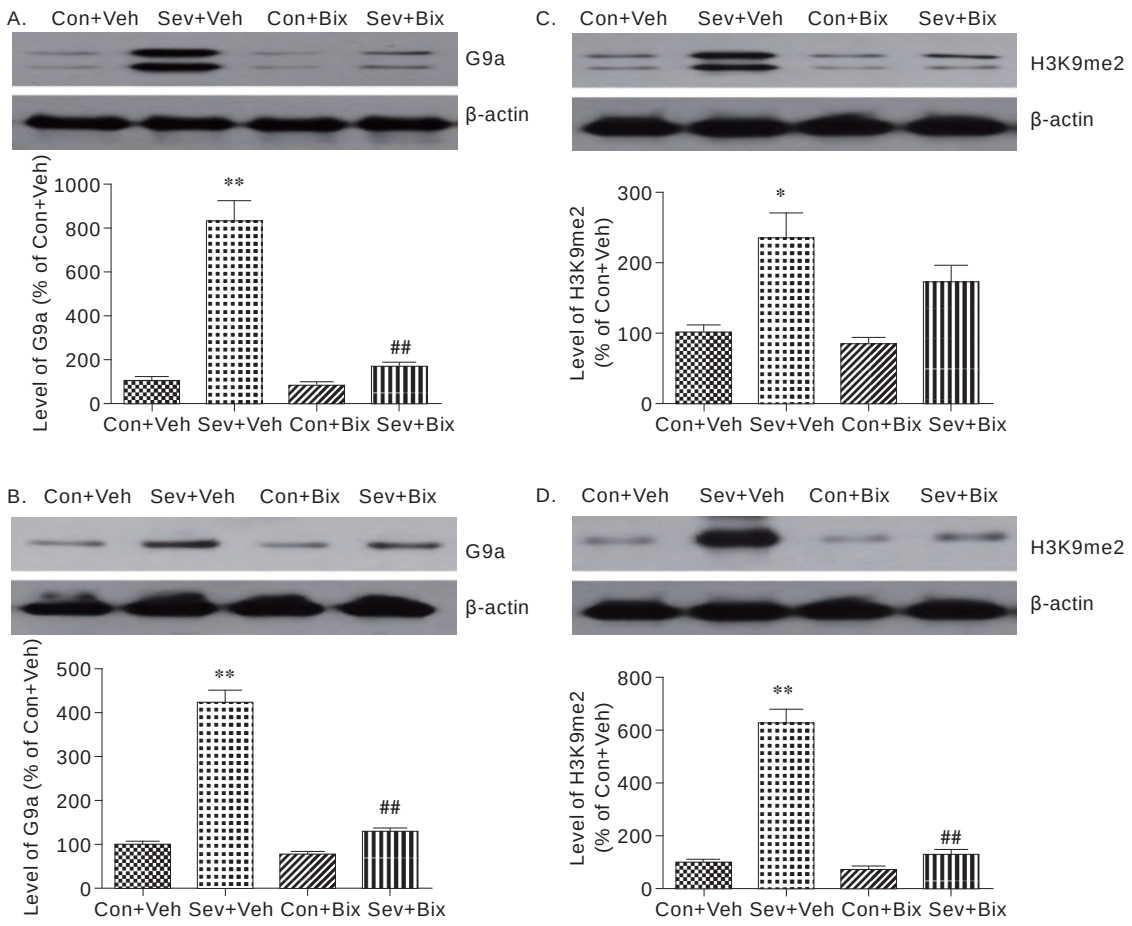

Figure 5. Bix Treatment Prevented Repeated Sevoflurane Exposure-Induced G9a and H3K9me2 Expressions in the Neonatal and Adolescent Hippocampus.
Figure A and B showed the hippocampal level of G9a including the 165 kDa G9a-L and 140 kDa G9a-S isoforms) at P_8 and P_{35}, respectively. Figure C and D showed the hippocampal level of H3K9me2 at P_8 and P_{35}, respectively Each data point represent mean ± the SEM (N=3, *P<0.05 or **P<0.01 versus the Con + Veh group; #P<0.05 or ##P<0.01 versus the Sev + Veh group).

proposed that 3% sevoflurane exposure to neonatal rodents can cause long-term cognitive impairment, but in another study comparing isoflurane to sevoflurane exposure, neither isoflurane nor sevoflurane was found to be associated with the impaired cognitive impairment (19, 21). These discrepancies might be attributed to the differences in methods of anesthetic exposure, animal species, anesthetic concentrations, anesthetic durations, and so on. Hippocampus is the primary region of the brain controlling the formation of memories and learned behaviors, which is discovered by the context associated FC test and the MWM test. In the present study, we found that sevoflurane-exposed rats had the decreased freezing time to context, but not the freezing time to tone in FC test. Furthermore, sevoflurane exposure induced significant spatial memory impairment as assessed in the MWM test. These results suggested that sevoflurane exposure impaired the hippocampus- dependent learning and memory in developing brain, which was supported by previous studies demonstrating that sevoflurane exposure influenced the hippocampus-dependent memory negatively (22-24). However, the molecular mechanism is not fully understood.

Epigenetic modification of genome is a major point of regulation governing many developmental processes. Early-life experience can shape the developing brain with long-term implication for brain functions and behaviors, which is closely

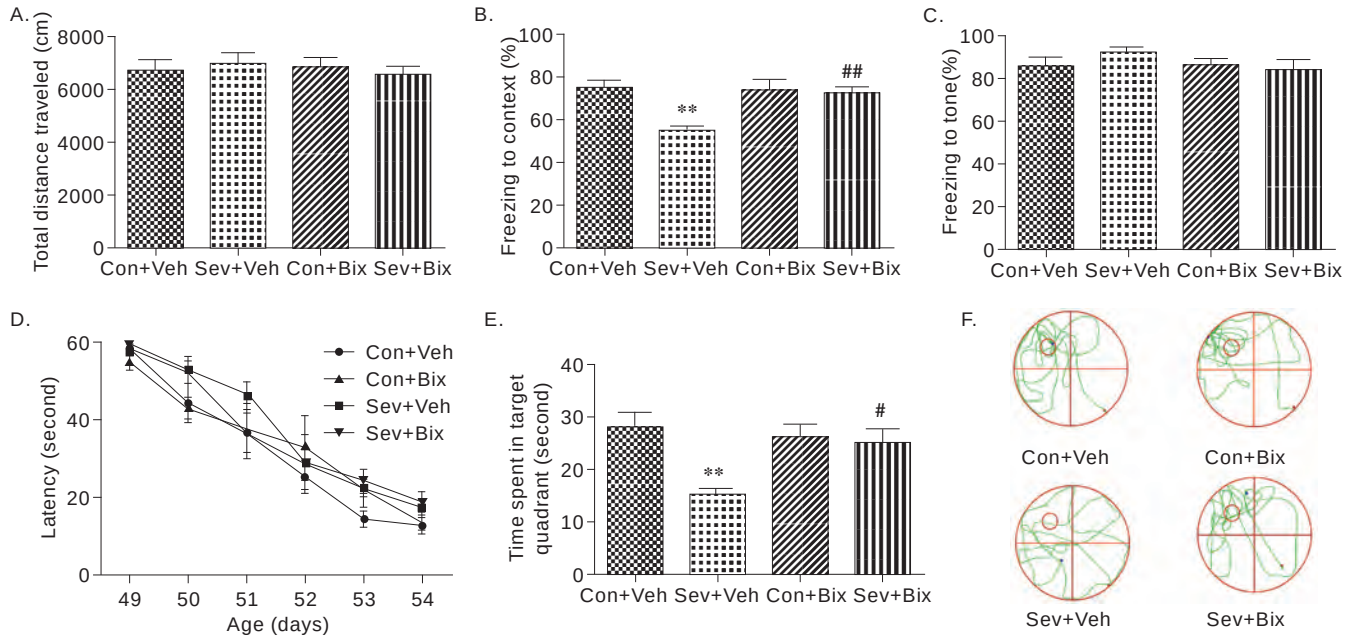

Figure 6. Bix Prevented Repeated Sevoflurane Exposure-Induced Behavioral Abnormalities in Adolescent Rats. Figure A showed the total distance traveled in the OF test. Figure B and C showed the percentage of freezing time in the FC test. Figure D showed the latency spent to find the underwater platform during training phase. Figure E and F showed the time spent in the target quadrant and the part of orbits in testing day. Each data point represent mean ± the SEM (N=8, *P<0.05 or **P<0.01 versus the Con + Veh group; #P<0.05 or ##P<0.01 versus the Sev + Veh group).

related to epigenetic modification (10). In eukaryotic nuclei, DNA with its nucleosome (two copies of H_2A, H_2B, H_3 and H_4 class histones) form the chromosomes by the tighter packaging of H_1 class histone. G9a has been reported to be critical for hippocampus-dependent long term memory (25). Likewise, GLP is originally described as a gene encoding a G9a-like protein. Although GLP and G9a independently exert histone lysine methyltransferase activity, many studies have demonstrated that they possess the same substrate specificities on histones (26, 27). GLP and G9a can form a complex and induce the increase of H3K9me2, which is associated with the suppressed transcription of genes encoding (28). For example, a recent study suggested that the inhibition of G9a/GLP in adult rats with the infusion of Bix inhibits the formation of H3K9me2 in the CA1 areas of the hippocampus and improves long-term memory (25). In another study, P_7 mice exposed to ethanol showed increased G9a/GLP-mediated H3K9me2 and decreased expression of histone H3, which may be associated with the increased cleaved caspase-3 (29). Thus, histone modification may be another potential mechanism of sevoflurane-induced apoptosis and cognitive impairment. Evidence suggests that the learning and memory deficits may be the result of the toxic effects of sevoflurane on CNS development. Caspase-3, a marker of apoptosis, is significantly increased after sevoflurane exposure (20), and our results were consistent with the previous study. Our study showed that repeated sevoflurane-exposure induced the increase of hippocampal G9a which was associated with the increased cleaved caspase-3. In our study, we found that G9a inhibitor Bix was able to decrease the levels of cleaved caspase-3, G9a, and H3K9me2 in the hippocampus as well as ameliorating the impaired cognitive performance. Bix pretreatment inhibited sevoflurane-induced cleaved caspase-3 activation in the hippocampus in a dose-dependent manner, and administration of Bix at the 1 mg/kg was more effective. It is possible that during the early brain development, sevoflurane-induced activation of G9a may suppress transcription of genes encoding for survival factors that induce a delay in neuronal development (30, 31). Increased hip-

pocampal G9a activity was associated with increased hippocampal H3K9me2, which suggested that the G9a-mediated hippocampal effects may be dependent on its catalytic activity on histones. Collectively, our results showed the importance of G9a-mediated H3K9me2 in the neurotoxicity of postnatal repeated sevoflurane exposure, which can possibly trigger neurological dysfunction later in life.

In conclusion, our results suggested that repeated sevoflurane exposure during the period of brain development can cause enhanced expression of hippocampal G9a and H3K9me2, which negatively affects neuronal survival and long-lasting cognition impairment in adolescent.

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