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SAHA attenuates sevoflurane-induced learning and memory impairments in fetal mice

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ABSTRACT. Previous studies have found that children with multiple exposures to anesthesia at an early age are at increased risk of learning and memory impairment. Sevoflurane is the most commonly used inhalational anesthetic for general anesthesia in children. Multiple exposures to sevoflurane have been shown to induce neuroinflammation, inhibit neurogenesis, and cause subsequent learning and memory impairments in fetal mice. Histone-tail acetylation has been implicated in memory formation. In this study, we employed suberanilohydroxamic acid (SAHA), an inhibitor of histone deacetylases, to treat sevoflurane-

induced learning and memory impairments. Six-day-old C57BL/6 mice were exposed to sevoflurane for 2 h daily for 3 days. Morris water maze test performed to evaluate learning and memory impairments and the expression of genes related in to synaptic remodeling/plasticity, or regulated by neuronal activity or the cell cycle were detected by real-time PCR. We found that SAHA attenuated sevoflurane-induced learning and memory impairments in fetal mice. Our findings suggest that SAHA may have potential as a therapeutic agent for preventing or treating the neurotoxicity associated with anesthesia.

Key words: Suberanilohydroxamic acid; Memory impairment; Sevoflurane

INTRODUCTION

Children with multiple exposures to general anesthesia and surgery at an early age may develop learning disabilities (Wilder et al., 2009; Sun, 2010; Flick et al., 2011). Sevoflurane is the most commonly used inhalational anesthetic for general anesthesia in children. In previous studies, sevoflurane has been shown to inhibit the proliferation of mouse neural progenitor cells, decrease the capacity of neural stem cells for self-renewal, and induce neuroinflammation in microglial cells (Nie et al., 2013; Ye et al., 2013; Zhang et al., 2013a,b). Moreover, in animal studies, multiple exposures to sevoflurane have induced neuroinflammation and inhibited neurogenesis in the brain tissue of 6-day-old fetal mice, and caused subsequent learning and memory impairments 3 weeks later (Lei et al., 2013; Shen et al., 2013). Therefore, the neurotoxicity of sevoflurane is drawing increasing attention in the context of children exposed to inhalational general anesthetics for surgery.

Chromatin modifications, especially histone-tail acetylation, have been implicated in memory formation (Kurdistani and Grunstein, 2003; Goldberg et al., 2007). Acetylation and deacetylation of nucleosomal core histones are important for modulation of chromatin structure and regulation of gene expression (Alarcón et al., 2004). Upregulation of HDAC may result in aberrantly low histone acetylation and abnormal expression of genes critically involved in learning and memory impairments (Korzus et al., 2004). Increased histone-tail acetylation induced by inhibitors of HDAC has been shown to facilitate learning and memory in wild-type mice as well as in mouse models of neurodegeneration (Kumar et al., 2005; Park et al., 2013).

Suberanilohydroxamic acid (SAHA) is a member of a larger class of compounds that inhibit HDAC and is a clinically approved agent. It has been used to treat Sézary syndrome, a type of lymphoma closely related to cutaneous T cell lymphoma (Hockly et al., 2003). A recent study suggesting that SAHA also exerts some activity against recurrent glioblastoma multiforme reported a median overall survival of 5.7 months, compared to the values 4-4.4 months reported in earlier studies (Iwamoto et al., 2013). SAHA can cross the blood-brain barrier, as shown indirectly by measuring changes in histone acetylation in the brain and directly by measuring the amount of SAHA in the brain (Tsankova et al., 2006). We, therefore, tested whether SAHA could ameliorate sevoflurane-induced learning and memory impairments in fetal mice.

In this study, we used 6-day-old mice as a model for investigating the effects of SAHA on the learning and memory impairments induced by sevoflurane. We found that SAHA attenuated sevoflurane-induced learning and memory impairments in these mice.

METHODS

Animals

Six-day-old mice were obtained from the specific pathogen free (SPF) animal center at the Shanghai East Hospital affiliated with Tongji University. All animal experiments were approved by the Animal Care Committee of the East Hospital affiliated with Tongji University.

The mice were administered sevoflurane at postnatal day 6 (P6) or from P6 to P8, and then behavioral testing was performed from P31 to P35 or P37. The mice were administered anesthetic (sevoflurane) plus 60% oxygen (balanced with nitrogen) as performed in previous studies. The 60% oxygen maintains sufficient partial pressure of oxygen in the mice during anesthesia. Control groups received 60% oxygen at an identical flow rate in similar chambers. No significant difference was observed in learning and memory functions between mice that received 60% oxygen and those that received 21% oxygen (data not shown). The concentrations of the anesthetic and oxygen were measured continuously (Ohmeda, GE Healthcare, Tewksbury, MA, USA). The temperature of the anesthetizing chamber was controlled to maintain a rectal temperature of 37° ± 0.5°C in the mice. Previous studies (Shen et al., 2013; Zhang et al., 2013a,b) have shown that anesthesia with 3% sevoflurane for two hours did not significantly change the values of pH, partial pressure of oxygen, or partial pressure of carbon dioxide in anesthetized mice compared with the control group. Furthermore, compared with control mice, the anesthetized mice did not show significant changes in behavior after anesthesia (e.g., eating, drinking, general activity, and body weight). Mortality rate for mice in these studies was less than 1%. For the intervention studies, SAHA (SML0061; Sigma-Aldrich Shanghai Trading Co. Ltd., Shanghai, China) was dissolved in DMSO as a stock solution and diluted in saline just before injection. SAHA (25 mg/kg) was administered to mice via intraperitoneal injection 1 h before each dose of sevoflurane anesthesia for three days.

Morris water maze test (MWM)

The MWM was conducted in a circular tank (diameter 1.8 m) filled with opaque water. A platform (11 x 11 cm) was submerged below the water surface in the center of the target quadrant. The swimming path of the mice was recorded using a video camera, and analyzed using the Videomot 2 software (TSE). For each training session, the mice were placed into the maze consecutively from four random points in the tank. Mice were allowed to search for the platform for 60 s. If the mice did not find the platform within 60 s, they were gently guided towards it. Mice were allowed to remain on the platform for 15 s. Two training trials were given every day; the latency for each trial was recorded for analysis. During the memory test (probe test), the platform was removed from the tank, and the mice were allowed to swim in the maze for 60 s.

Real-time polymerase chain reaction (PCR)

Real-time PCR was carried out using SYBR-Green-based reagents (EXPRESS

SYBR® GreenER™, Invitrogen) using a CFX96™ Real-Time PCR Detection system (Bio-Rad). The relative quantities of amplified product were calculated using the comparative Cycle threshold (Ct) method. The results obtained were compared to standard curves generated using serial dilutions of input DNA. Data were derived from three independent amplifications. Error bars represent standard deviations. The primer sequences used for real-time PCR are listed in Table 1. Beta-actin was used as a reference gene (Table 1).

Table 1. Primer sequences used for real-time PCR.

Primer	Sequence (5'-3')
Creb	5'-CTACACCAGCTTCCCCGGT-3'
	5'-ACGGAAACAGCCGAGCTC-3'
Cbp	5'-CGGGCAGGGGATGAG-3'
	5'-GCGAGCCAGCGAGGA-3'
Nrxn 1	5'-CAGGGCCTTTGTCCTGAATA-3'
	5'-GCTTTGAATGGGGTTTTGAG-3'
Nrxn 3	5'-ACTGAGAGCTAGCCACCCAGAC-3'
	5'-TTGCCCATTTGTGAATTTGA-3'
Pgk1	5'-ACATTTTGGCAACACCGRGAG-3'
	5'-GAAGTAGCACGTCTCACTAGTCTCGTG-3'
Atf4	5'-GTGATAACCTGGCAGCTTCG-3'
	5'-GGGGTAACTGTGGCGTTAGA-3'
p21	5'-CCACAGTTGGTCAGGGACAG-3'
	5'-CCCTCCCCTCTGGGAATCTA-3'
Egr1	5'-GTGCCCACCACTCTTGGAT-3'
	5'-CGAATCGGCCTCTATTTCAA-3'
Egr2	5'-GGCTGCAAATCGTTCCTG-3'
	5'-TCGGAGTATTTATGGGCAGGT-3'
c-Fos	5'-GAAAGCCTGGGGCGTAGAGT-3'
	5'-CCTCAGCTGGCGCCTTTAT-3'
CamKIIα	5'-GACCTGGATGCTGACGAAG-3'
	5'-AGGTGATGGTAGCCATCCTG-3'
Cpg15	5'-GCGAGATTTCGTTGAGATCG-3'
	5'-GGGATGACACGGATTGATTTT-3'
Agrin	5'-TTGTAACCAACAGGGGTTGC-3'
	5'-AGTTGTGGCTAGGGGAGCAC-3'
Psd95	5'-CCCCTACCCCTCCTGAGAAT-3'
	5'-GAGGGGAAGGAGAAGGTTGG-3'
Homer1	5'-CTGCCTGAGTGTCGTGGAAG-3'
	3'-ATGATTTCACTCGCGCTGAC-3'
Cdk5	5'-CGCAGCCTGTTGGACTTTGT-3'
	3'-GCGTTGCAGAGGAGGTGGTA-3'
Shank3	5'-TTTTCCAGGTCCCAGTGGTG-3'
	5'-CCTGCCCACAGTGTCACTCC-3'
Svp	5'-CTAGCCTCCCGAATGGAATG-3'
	5'-CAGCAGCAGCATCAGCAATG-3'
Arc	5'-CAGCATAAATAGCCGCTGGT-3'
	5'-GAGTGTGGCAGGCTCGTC-3'
Synapsin2	5'-GGCTTTCCTTCCCTCCACAC-3'
	5'-TGTTAGCGAGGGAGCAGTGG-3'
SNK	5'-TTTCCCACGTCCAAAGTCAG-3'
	5'-GCAGCGAAGCTTTAAATACGC-3'
NR2A	5'-TCGGCTTGGACTGATACGTG-3'
	5'-AGGATAGACTGCCCCTGCAC-3'
NR2B	5'-CCTTAGGAAGGGGACGCTTT-3'
	5'-GGCAATTAAGGGTTGGGTTC-3'
GLUR-1/AMPA 1	5'-GGAGGAGAGCAGAGGGAGAG-3'
	5'-TTCCTGCAATTCCTTGCTTG-3'
GLUR-2	5'-GCGGTGCTAAAATCGAATGC-3'
	5'-ACAGAGAGGGGCAGGCAGT-3'
β-actin	5'-CCCATCGCCAAAACTCTTCA-3'
	5'-GGCCACTCGAGCCATAAAAG-3'

Statistical analysis

Data for biochemical changes are reported as means ± SD. Data for changes in escape latency are reported as means ± SE. The data for platform crossing times were not distributed normally, and therefore are expressed as median and interquartile range. Two-way repeated measures ANOVA with interactions of factors time and group was used to analyze the differences in learning curves (based on escape latency) for the MWM. The Mann-Whitney U test was used to determine the differences in platform crossing times. A Student two-sample *t*-test was used to determine the differences in gene expression from real-time PCR results. Values of $P < 0.05$ were considered statistically significant. The SAS software version 9.2 (SAS Institute Inc., Cary, NC, USA) was used to analyze the data.

RESULTS

Multiple exposures of sevoflurane in fetal mice induced learning and memory impairments

Clinical studies have shown that children who undergo three exposures, but not one exposure, to anesthesia are at risk of developing learning and memory impairments. Therefore, we treated six-day-old mice with sevoflurane for 2 h daily over 1 or 3 days. This animal model conceptually mimics single versus multiple exposures of anesthesia and allows us to study anesthesia-induced learning and memory impairments. The fetal mice were treated with 3% sevoflurane anesthesia for 2 h daily for 3 days from P6-P8 and tested in the MWM from P30-P34. A comparison of the time that each mouse took to reach the platform during reference training (escape latency) showed that there was a statistically significant interaction of time and group based on escape latency in the MWM between the control group and the sevoflurane anesthesia group (Figure 1A) ($P = 0.0257$, two-way repeated measures ANOVA). A comparison of the number of times that each mouse crossed the location of the absent platform at the end of reference training (platform crossing times) indicated that the sevoflurane anesthesia group crossed the platform fewer times compared with the control group (Figure 1B). There was no significant difference in mouse swimming speed between the mice in the sevoflurane anesthesia group and the mice in the control group (data not shown). The expression of genes related to synaptic remodeling/plasticity, or regulated by neuronal activity or the cell cycle, was decreased after sevoflurane treatment, including *Egr1*, *c-Fos*, *Cpg15*, *CamKIIα*, *Creb*, *Cbp*, *Nrxn3*, genes encoding got the NMDA receptor subunits, *p21*, *Atf4*, and *Pgk1* (Table 2). These data suggest that multiple exposures of sevoflurane in fetal mice may induce learning and memory impairments in the fetal mice.

SAHA attenuated sevoflurane-induced learning and memory impairments in fetal mice

SAHA is a clinically approved agent that has been shown to improve learning and memory. Therefore, we tested whether SAHA could ameliorate the sevoflurane-induced learning and memory impairments in the fetal mice. SAHA was administered to mice via intraperitoneal injection 1 h before each of the three doses of sevoflurane anesthesia. The mice were

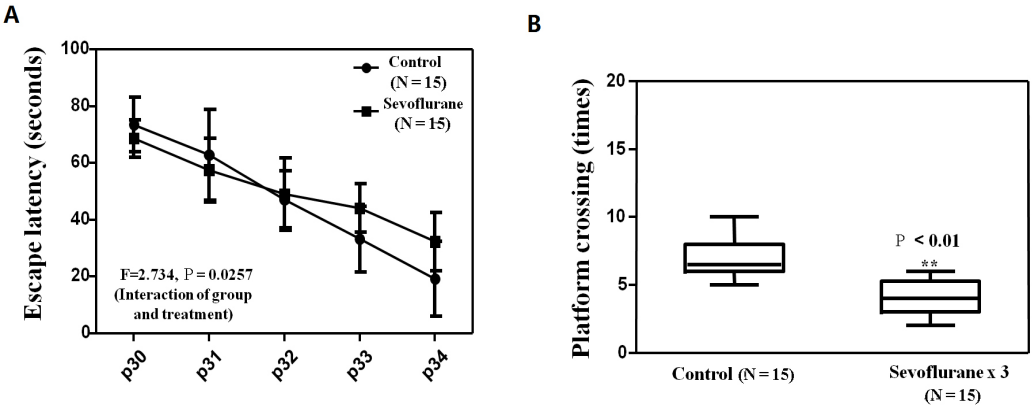

Figure 1. Multiple exposures to sevoflurane in fetal mice induced learning and memory impairments. **A.** Anesthesia with 3% sevoflurane for 2 h daily for 3 days in P6 mice increases the escape latency of mice swimming in the MWM as compared with the control condition (tested from P30-P34) (control, N = 15; sevoflurane, N = 15). Two-way repeated measure ANOVA shows that there is a statistically significant interaction of time and group based on escape latency of MWM between the control group and the sevoflurane anesthesia group. **B.** Anesthesia with 3% sevoflurane for 2 h daily for 3 days in P6 mice decreases the number of platform crossings (platform crossing times) of mice swimming in the MWM compared with the control group tested at P34 (control, N = 15; sevoflurane, N = 15). *P < 0.05, **P < 0.01; MWM = Morris water maze.

Table 2. Changes in hippocampal gene expression after sevoflurane treatment in mice (levels in the sevoflurane group/levels in the control group).

Gene name	Protein name	Fold Δ	P value
CREB	cAMP response element-binding protein	-3.275	<0.01
CBP	CREB-binding protein	-4.57	<0.05
Neurexin I	Neurexin I	-1.374	<0.05
Neurexin III	Neurexin III	-3.752	<0.05
PGK1	Phosphoglycerate kinase 1	-6.387	<0.01
ATF4	Activating transcription factor 4	-4.274	<0.01
CaMKIIA	Calcium/calmodulin-dependent protein kinase II alpha	-2.484	<0.05
P21	Cyclin-dependent kinase inhibitor 1A	-2.375	<0.05
EGR1	Early growth response 1	-3.573	<0.05
EGR-2	Early growth response 2	-4.754	<0.01
Agrin	Agrin	-5.356	<0.01
GluR-1	Glutamate receptor 1	-2.375	<0.05
GluR-2	Glutamate receptor 2	-3.756	<0.01
PSD95	Postsynaptic density protein 95	-4.586	<0.01
HOMER1	Homer protein homolog 1	-3.284	<0.05
CDK5	Cyclin-dependent kinase 5	-7.451	<0.01
SHANK3	SH3 and multiple ankyrin repeat domains 3	-3.485	<0.01
SVP	Short vegetative phase	-4.385	<0.01
Synapsin II	Synapsin II	-5.346	<0.01
ARC	Activity-regulated cytoskeleton-associated protein	-4.38	<0.01
FOS	FBJ osteosarcoma oncogene	-3.485	<0.01
CPG15	Cuticular protein glycine-rich 15	-2.385	<0.05
SNK	Serum-inducible protein kinase	-4.342	<0.01
NR2A	*N*-methyl-D-aspartate receptor subunit 2A	-6.483	<0.01
NR2B	*N*-methyl-D-aspartate receptor subunit 2B	-5.385	<0.01

Data are reported as means ± SD, N = 3.

treated with 3% sevoflurane for 2 h daily for 3 days (from P6-P8). Finally, the mice were tested in the MWM from P30-P34. There was no significant difference in escape latency and platform crossing times between the SAHA group and the control group (Figure 2A, B). Two-way

repeated measures ANOVA showed that there was a statistically significant interaction of time and group based on escape latency of MWM between the sevoflurane anesthesia group and the sevoflurane anesthesia plus SAHA group (Figure 2C) (P = 0.037). A comparison of the number of times that each mouse crossed the location of the absent platform at the end of reference training (platform crossing times) indicated that the sevoflurane anesthesia group crossed the platform fewer times compared with the sevoflurane anesthesia plus SAHA group (Figure 2D). The genes related to synaptic remodeling/plasticity, or regulated by neuronal activity or the cell cycle, were increased in the sevoflurane anesthesia plus SAHA group compared with the sevoflurane anesthesia group, including *Bdnf* promoter I/II, *Egr1*, *c-Fos*, *Cpg15*, *CamKIIα*, *Creb*, *Cbp*, *Nrxn3*, genes encoding the NMDA receptor subunits, *p21*, *Atf4*, and *Pgk1* (Table 3). These data suggested that SAHA attenuated sevoflurane-induced learning and memory impairments in fetal mice.

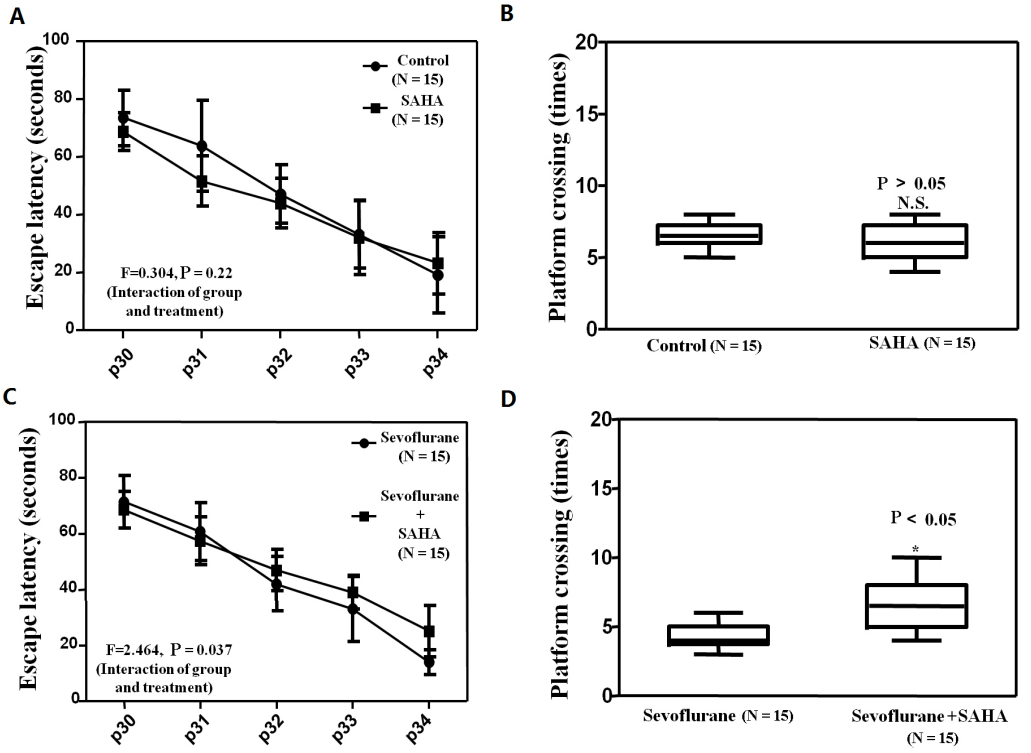

Figure 2. SAHA attenuated sevoflurane-induced learning and memory impairments in fetal mice. **A.** and **B.** There was no significant difference in escape latency and platform crossing times between the SAHA group and the control group. **C.** Two-way repeated measure ANOVA showed that there was a statistically significant interaction of time and group based on escape latency of MWM between the sevoflurane anesthesia group and the sevoflurane anesthesia plus SAHA group (P = 0.037). **D.** A comparison of the number of times that each mouse crossed the location of the absent platform at the end of reference training (platform crossing times) indicated that the platform crossing times in the sevoflurane anesthesia group were decreased compared with the sevoflurane anesthesia plus SAHA group. *P < 0.05, **P < 0.01; MWM = Morris water maze.

Table 3. Changes in hippocampal gene expression after sevoflurane and SAHA treatment in mice (levels in the sevoflurane + SAHA group/levels in the sevoflurane group).

Gene name	Protein name	Fold Δ	P value
CREB	cAMP response element-binding protein	1.265	<0.05
CBP	CREB-binding protein	2.374	<0.01
Neurexin I	Neurexin I	1.465	<0.05
Neurexin III	Neurexin III	2.375	<0.05
PGK1	Phosphoglycerate kinase 1	5.375	<0.01
ATF4	Activating transcription factor 4	6.763	<0.01
CaMKIIA	Calcium/calmodulin-dependent protein kinase II alpha	4.375	<0.01
P21	Cyclin-dependent kinase inhibitor 1A	3.576	<0.05
EGR1	Early growth response 1	4.324	<0.01
EGR-2	Early growth response 2	2.485	<0.05
Agrin	Agrin	4.385	<0.01
GluR-1	Glutamate receptor 1	2.853	<0.01
GluR-2	Glutamate receptor 2	3.475	<0.05
PSD95	Postsynaptic density protein 95	2.475	<0.05
HOMER1	Homer protein homolog 1	3.285	<0.01
CDK5	Cyclin-dependent kinase 5	4.381	<0.01
SHANK3	SH3 and multiple ankyrin repeat domains 3	2.896	<0.05
SVP	Short vegetative phase	2.143	<0.01
Synapsin II	Synapsin II	1.472	<0.05
ARC	Activity-regulated cytoskeleton-associated protein	7.473	<0.01
FOS	FBJ osteosarcoma oncogene	3.462	<0.01
CPG15	Cuticular protein glycine-rich 15	1.879	<0.05
SNK	Serum-inducible protein kinase	4.774	<0.01
NR2A	*N*-methyl-D-aspartate receptor subunit 2A	5.783	<0.01
NR2B	*N*-methyl-D-aspartate receptor subunit 2B	2.785	<0.05

Data are reported as means ± SD, N = 3.

DISCUSSION

Previous studies have found that sevoflurane, the most commonly used anesthetic in children, can induce apoptosis, increases in β-amyloid levels, and neuroinflammation in the brains of fetal mice and caused learning and memory impairment 3 weeks later (Shen et al., 2013; Zhang et al., 2013a,b). Clinical studies have also found that children with multiple exposures to general anesthesia and surgery at an early age may develop learning disabilities (Sun, 2010; Flick et al., 2011). Therefore, the neurotoxicity of sevoflurane is becoming an important issue for parents and anesthetists in the context of children requiring general inhalational anesthesia for surgery.

Learning and memory formation and storage require alterations in gene expression. Chromatin remodeling, especially histone-tail acetylation, alters the compact structure of chromatin and changes the accessibility of DNA to regulatory proteins. Chromatin remodeling is emerging as a fundamental mechanism for regulating gene expression (Vecsey et al., 2007). Histone acetyltransferases add an acetyl group onto the ε-amino group of lysine, thereby reducing the positive charge on histones, which, in turn, decreases their ability to bind to negatively charged DNA; this leads to relaxed chromatin structure and increased accessibility for various transcription factors and other transcription components. Conversely, HDAC remove the acetyl group, thereby restoring the closed chromatin structure (Park et al., 2013). Recently, histone acetylation has been implicated in synaptic plasticity and learning behavior (Vecsey et al., 2007). Moreover, a non-selective HDAC inhibitor was found to reinstate learning ability and promote retrieval of long-term memory in mice, even after massive neuronal loss (Tsankova et al., 2006). Taken together, these observations indicate that HDAC inhibition may provide a potential remedy for memory impairments caused by neurodegenerative and other diseases.

In this study, we assessed the effects of multiple exposures to 3% sevoflurane on functional aspects of learning and memory, and brain levels of genes related to memory in mice. We treated 6-day-old mice with 3% sevoflurane anesthesia for 2 h daily for 3 days from P6-P8. The mice were tested in the MWM from P30-P34. MWM results showed increased escape latency and reduced platform-crossing times in the sevoflurane-treated group compared with the control group. Moreover, sevoflurane anesthesia downregulated the expression of memory-related genes in the brain tissue. SAHA, one of a larger class of compounds that inhibit HDAC, is used safely in clinical contexts. In our study, we found that SAHA rescued sevoflurane-induced learning and memory impairments and upregulated the expression of memory-related genes in the brain tissues of mice exposed to inhalational anesthesia. These data suggest that SAHA may be a potential therapeutic agent for treating sevoflurane-induced learning and memory impairments.

In conclusion, we found that anesthesia with 3% sevoflurane for 2 h daily for 3 days induced impairments in learning and memory in fetal mice. SAHA attenuated sevoflurane-induced learning and memory impairments and may be a potential therapeutic agent for preventing or treating neurotoxicity associated with anesthesia.

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