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Brief communication

Developmental neurotoxicity in the context of multiple sevoflurane exposures: Potential role of histone deacetylase 6

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ABSTRACT

Animal studies have demonstrated that multiple exposures to sevoflurane during the postnatal period lead to impaired synaptogenesis and cognitive deficits in adulthood. However, the underlying mechanisms remain unclear. Histone deacetylase 6 (HDAC6), a unique isoform of class II histone deacetylases (HDACs), mediates diverse cellular processes such as cell survival, inflammation, intracellular trafficking and protein degradation. Varieties of literature suggest the importance of HDAC6 in memory formation and abnormal neurodegenerative diseases. The aim of this study was to investigate potential roles of HDAC6 in sevoflurane-induced developmental neurotoxicity. Postnatal day 7 (P7) rat pups were randomly assigned to control group and sevoflurane group (n = 6 for each group). They were exposed to 60% oxygen and 40% nitrogen with or without 3% sevoflurane for 2 h daily for three consecutive days (P7, P8 and P9). Immediately after the last exposure, both hippocampi were harvested for detection of HDAC6 expression and activity. Next, P7 rat pups were divided into control group, sevoflurane group, sevoflurane + Tubastatin A, and Tubastatin A groups (n = 6 for each group in molecular experiments; n = 16 for each group in behavioral testing). A dose of 25 mg/kg body weight of Tubastatin A (a selective HDAC6 inhibitor) were administrated intraperitoneally 30 min prior to each sevoflurane exposure. After treatments, expression levels of synaptophysin and postsynaptic density 95 protein (PSD95) were quantified using Western blot, and synaptic ultrastructure was evaluated by transmission electron microscopy. Additional pups were raised until P49 to measure cognitive performance using the Morris water maze test. Our results demonstrated that multiple sevoflurane exposures enhanced HDAC6 expression and activity in hippocampi of the developing brain. Tubastatin A ameliorated sevoflurane-induced decreases in synaptophysin and PSD95 expression during development, as well as synaptic ultrastructural damage and cognitive deficits in adulthood. In conclusion, HDAC6 is involved in the developmental neurotoxicity caused by multiple sevoflurane exposures and its inhibition may prevent related damage.

1. Introduction

Two pioneering animal studies first reported that exposure of the developing brain to general anesthetics leads to neurodegenerative changes and long-term cognitive deficits (Ikonomidou et al., 1999; Jevtovic-Todorovic et al., 2003). These findings establish an increasingly pressing need to determine if children receiving general anesthesia have unfavorable neurodevelopmental effects. Although some epidemiological studies show a mild association between exposure to anesthesia and behavioral and developmental disorders in young children (Backeljauw et al., 2015; DiMaggio et al., 2009, 2011; Ing et al., 2012), other retrospective studies (Bartels et al., 2009; Bong et al., 2013; Hansen et al., 2011, 2013; Ing et al., 2014; Ko et al., 2014, 2015; Poor Zamany Nejat Kermany et al., 2016), as well as recent high-qualified cohort studies and randomized controlled trials (Davidson et al., 2016; Ing et al., 2017; McCann et al., 2019; Sun et al., 2016; Warner et al., 2018), provide strong evidence that a single exposure to general anesthesia for a short time does not increase the risk of neurodevelopmental deficits in children. In contrast, most, but not all, clinical studies find that children with multiple exposures to general anesthesia prior to the age of three years are more likely to develop adverse neurodevelopmental outcomes (Flick et al., 2011; Hu et al., 2017; Sprung et al., 2012; Sun et al., 2016; Warner et al., 2018; Wilder et al., 2009). Sevoflurane is the least pungent of the inhalation anesthetics, with relatively quick induction and emergence. An increasing number of studies have demonstrated that multiple postnatal exposures to sevoflurane result in impaired synaptogenesis and long-term cognitive deficits in young rodents and nonhuman primates (Alvarado et al.,

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2017; Lu et al., 2017; Makaryus et al., 2018; Shen et al., 2013b; Tao et al., 2014). However, the underlying mechanism remains largely unknown.

Epigenetics refer to the alteration of gene expression without affecting DNA sequence. At early stages of brain development, neuronal circuits are created and rapidly remodeled. Various environmental agents could influence this process through epigenetic modification of target gene expression to alter subsequent neurodevelopmental outcomes (Fagiolini et al., 2009; Karpova et al., 2017; Rudenko and Tsai, 2014). Recent mechanistic investigations reveal that exposure of the developing brain to general anesthetics initiates aberrant activation of DNA methylation and histone deacetylation, and decreases transcriptional levels of synapse-related genes (Bhattacharya et al., 2017; Jia et al., 2016; Ju et al., 2016; Liang and Fang, 2016; Sen and Sen, 2016; Wu et al., 2016; Zhong et al., 2015). Jia et al. (2016) reported that repeated exposures of neonatal rats to 3% sevoflurane for 2 h increased hippocampal histone deacetylases 3 (HDAC3) and 8 (HDAC8), and acetylated histones H3 and H4. Treatment with sodium butyrate, an HDAC inhibitor, could rescue sevoflurane-induced behavioral abnormalities, loss of dendritic spine density and decreased expression of synaptic proteins including c-fos, brain-derived neurotrophic factor (BDNF), postsynaptic density 95 protein (PSD95), microtubule-associated protein 2 (MAP2), synapsin 1, phosphorylated cAMP response element binding protein (p-CREB), and CREB binding protein. However, the mechanism by which HDAC3 and HDAC8 transcriptionally regulated expression of these important proteins was not well elucidated. In addition, this study only investigated the role of class I HDACs (1–3 and 8) in the developmental neurotoxicity of sevoflurane. As such, the involvement of other categories of HDACs in this process had not been clarified.

In contrast to other HDACs, HDAC6 (a class II HDACs) has two catalytic domains and a C-terminal zinc finger domain (Seidel et al., 2015). This unique structure enables HDAC6 to deacetylate both nuclear and cytoplasmic substrates. Moreover, HDAC6 has the ability to bind ubiquitinated proteins and subsequently degrade them. Physiologically, HDAC6 is abundantly distributed in the brain, where it is required for synaptic plasticity and memory (Perry et al., 2017). It controls neurotransmitter release and memory formation by regulating microtubule-dependent trafficking of BDNF-containing vesicles and GluA1 receptor (Bhattacharya et al., 2017; Xu et al., 2014). Abnormal HDAC6 activity is associated with many neurodegenerative diseases including Alzheimer's disease, Parkinson's disease and Huntington's disease (Simoes-Pires et al., 2013). As HDAC6 can exert tremendous effects that mediate various physiological and pathological activities in the central nervous system, the present study investigated potential roles of HDAC6 in sevoflurane-induced impairment of synaptogenesis and cognitive decline in adulthood.

2. Materials and methods

2.1. Animals

All animal studies were approved by the Ethics Committee for the Care and Use of Laboratory Animals of Shanghai Jiao Tong University. Postnatal day 7 (P7) Sprague-Dawley rats (including mother rats) provided by SLAC Laboratory Animal Co., Ltd. (Shanghai, China) were housed in a temperature- and humidity-controlled room under a 12-h light/dark cycle with food and water available *ad libitum*. For detecting changes of HDAC6 expression and activity after multiple sevoflurane exposures, rat pups (both genders) from one litter was randomly assigned to control group and sevoflurane group. For investigating roles of HDAC6 in sevoflurane-induced impairment of synaptogenesis and cognitive deficits, rat pups were randomly assigned to control group, sevoflurane group, sevoflurane + Tubastatin A (TBA), and TBA groups. The number of rat pups used in molecular experiments was six in each group, while in behavioral testing the final number was sixteen in each group. We attempted to minimize the number of animals used and their suffering in experiments.

2.2. Sevoflurane exposure and drug administration

P7 rat pups were placed in a chamber pre-flushed with 60% oxygen and 40% nitrogen with or without 3% sevoflurane for 2 h daily for three consecutive days (P7, P8 and P9), similar to previous studies (Lu et al., 2017; Shen et al., 2013b; Tao et al., 2014). Concentration monitoring of sevoflurane, CO_2, O_2, and N_2 was conducted using a gas analyzer (Datex-Ohmeda, Madison, WI, USA). We did not perform blood gas analysis because previous studies demonstrated that this sevoflurane exposure protocol did not cause significant changes in blood gas parameters (Ji et al., 2017; Xia et al., 2017). Tubastatin A (TBA; Selleck Chemicals, Houston, TX) was dissolved in dimethylsulfoxide (DMSO) and diluted with 2% Tween-20 in normal saline. TBA was chosen because that it is a potent and selective HDAC6 inhibitor with an IC50 values of 15 nm and 1000-fold selectivity against all other isoforms (Butler et al., 2010). TBA is also a neuroprotective agent, as previous studies have reported that it could rescue neuronal cell death in various neurodegenerative models (Guedes-Dias et al., 2015; Selenica et al., 2014; Xu et al., 2014; Zhang et al., 2014). To inhibit HDAC6 activity, a dose of 25 mg/kg body weight was injected intraperitoneally 30 min before each sevoflurane exposure according to previous studies and our preliminary experiment (Jian et al., 2017; Selenica et al., 2014; Wang et al., 2016). Rat pups in the control group received an equivalent volume of solvent. To reduce the influence of maternal separation, rat pups in treatment and control groups were sent back to their cages after each exposure and raised by mother rats.

2.3. Western blot

Western blot analysis was conducted following our previously described methods (Li et al., 2015). Briefly, both hippocampi were harvested immediately after the last sevoflurane exposure and homogenized with ice-cold RIPA buffer. Protein concentration was measured by the BCA method. Fifty to one hundred micrograms of proteins from each sample were isolated on an SDS-PAGE gel and transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA). After blocking with 5% nonfat milk for 2 h, membranes were incubated overnight at 4 °C with primary antibodies (HDAC6, synaptophysin, PSD95, and GAPDH, 1:1000, Cell Signaling Technology, Danvers, MA) and subsequently with species-specific secondary antibodies conjugated with horseradish peroxidase. Immunoreactive protein bands were visualized using an ImageQuant LAS 4000 Mini system (GE Healthcare Bio-Sciences, Pittsburgh, PA) and analyzed with ImageJ software (US National Institutes of Health, Bethesda, MD). Protein levels of HDAC6, synaptophysin, and PSD95 were normalized to GAPDH.

2.4. Real-time PCR

After completion of three sevoflurane treatments, hippocampal tissues were immediately isolated and total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA). Next, RNA was reverse transcribed into cDNA using a cDNA Synthesis Kit (TaKaRa, Shiga, Japan). Amplification reactions were performed in triplicate to quantify mRNA expression of HDAC6 with a SYBR Green Select Master Mix kit (Thermo Fisher Scientific, Waltham, MA) on a 7500 Real Time PCR System (Applied Biosystems, Foster City, CA). All procedures followed the manufacturer's instructions. GAPDH was used as an internal control for gene expression. Relative expression of each gene was calculated using the $2^{-\Delta\Delta CT}$ method.

2.5. HDAC6 activity assay

The deacetylase activity of HDAC6 after multiple sevoflurane

exposures was detected with an HDAC6 fluorometric activity assay kit (K466, BioVision, Milpitas, CA) according to the manufacturer's instructions. The experiment was repeated three times.

2.6. Transmission electron microscopy

Changes in synaptic ultrastructure in the CA1 area of hippocampi were observed by transmission electron microscopy (TEM) following the protocol stated in our previous study (Tao et al., 2016). Briefly, rat pups were transcardially perfused with 2.5% glutaraldehyde in 0.1 M cacodylate buffer (CB) after the last sevoflurane exposure. The hippocampi of both hemispheres were isolated, cut into 1-mm^3 cubes, and fixed in 2.5% formaldehyde (4 °C) for 2 h. After washing in CB three times for 15 min each, tissues were post-fixed in 1% osmium tetroxide for 2 h and dehydrated in a graded series of ethanol at room temperature. Next, tissues were infiltrated in a mixture of acetone and resin with increasing resin concentrations (1:1, 1:2, and 1:3; 60 min each, 100% resin overnight). Polymerization was performed in an oven at 45 °C for 12 h, and then 60 °C for 48 h. Ultrathin (70-nm) sections were cut using an ultramicrotome (Leica UC6, Wetzlar, Germany) and stained with 3% uranyl acetate and 0.5% lead citrate. The synaptic ultrastructure of hippocampi was observed under a transmission electron microscope (PHLIP-CM-120, Holland). Six images of the CA1 area were acquired for each ultrathin section (six pups per group) and analyzed using ImageJ software. Synaptic density was calculated as the average number of synapses per unit volume. Synaptic width was expressed as average values for all synapses in all images. The TEM observer was blind to experimental conditions.

2.7. Morris water maze

Spatial learning and memory were measured by an experienced observer blinded to experimental conditions using the Morris water maze test, as described in our previous study (Li et al., 2015). In brief, a black painted pool (180-cm diameter, 50-cm height) located in a separate room was filled with water and divided into four quadrants. The temperature of the water was maintained at 25 ± 1 °C. A hidden 10-cm diameter platform was placed 1.5 cm below the surface of the water in the middle of a quadrant. The motions of rats were tracked with a video recording device above the pool. The place trial was performed four times daily from P49 to P53, while the probe trial was conducted on P54. In the place trial, rats were allowed to search for the platform for 60 s. Otherwise, rats were gently guided to the platform. All rats were permitted to stay on the platform for 20 s. Swim speed and latency to find the platform were recorded. In the probe trial, rats were introduced to the quadrant opposite to where the platform was located and allowed to swim freely for 60 s after removing the platform. The number of target crossings, percentage of time in the platform quadrant to total time, and percentage of distance in the platform quadrant to total distance were recorded. After each trial, rats were placed on a heater plate for 5 min before returning to the home cage.

2.8. Statistical analysis

Sample size was calculated following the methods described in previous studies (Charan and Kantharia, 2013; Richter et al., 2018). For experiments observing influences of multiple sevoflurane exposures on HDAC6 expression and activity, primary outcomes were HDAC6 mRNA and protein levels, and deacetylase activity of HDAC6. For experiments investigating roles of HDAC6 in sevoflurane-induced impairment of synaptogenesis, primary outcomes were synaptophysin and PSD95 protein levels, synaptic cleft width and synaptic density. In these biochemistry studies, a group size of six animals was required to detect a difference between means of 30% with an 80% power at a significance level of 0.05 (Tao et al., 2016). For behavioral testing, primary outcome was escape latency in place trial. Previous studies (Lu et al., 2017; Shen et al., 2013a; Tao et al., 2014; Xu et al., 2017) have demonstrated that sample size required to detect a difference in the behavioral changes at 80% power and a significance level of 0.05 was 10 animals per group. To strengthen reliability of behavioral results, a final group size of 16 was chosen in our study. A free software PowerSampleSize (http://www.powerandsamplesize.com/) was used to calculate sample size.

The normality of data was tested with the Shapiro–Wilk method. All normally distributed data were expressed as mean ± standard deviation, otherwise they were expressed as median with interquartile range. For normally distributed data, differences between two groups were analyzed using unpaired two-tailed Student's *t*-test, and differences among multiple groups were analyzed using one-way ANOVA followed by *post hoc* Bonferroni test. For non-normally distributed data, the Mann-Whitney *U* test was used to compare differences between two groups, and the Kruskal–Wallis test followed by Dunn's multiple comparison test was used to compare differences among three or more groups. Comparison of swim speed and latency to find the platform were performed using two-way repeated measures ANOVA analyses to evaluate interactions between treatment and time, and then a *post hoc* Bonferroni test was applied to show individual differences between two groups. $P < 0.05$ was considered to be statistically significant. Statistical analyses were performed using the SPSS 20.0 software (SPSS, Chicago, IL). Graphs were plotted using PRISM 5 software (GraphPad, La Jolla, CA).

3. Results

3.1. Multiple sevoflurane exposures enhanced HDAC6 expression and activity in the developing hippocampus

Three exposures to 3% sevoflurane for 2 h caused an increase in HDAC6 protein expression (Fig. 1A). Indeed, there was a significant difference between control and sevoflurane groups ($P = 0.0092$, Student's *t*-test) (Fig. 1B). Hdac6 mRNA expression was also increased significantly after sevoflurane treatments ($P < 0.001$, Student's *t*-test) (Fig. 1C). In addition, HDAC6 activity in the sevoflurane group was significantly upregulated compared with the control group ($P < 0.001$, Student's *t*-test) (Fig. 1D). These results indicated that multiple sevoflurane exposures enhanced HDAC6 expression and activity in the developing hippocampus.

3.2. Involvement of HDAC6 in cognitive deficits during adulthood caused by postnatal multiple sevoflurane exposures

As shown in Fig. 2A, there were no significant interaction effects between treatment and time in terms of swimming speed in the place trial ($F_{(8.6,\ 541.3)} = 0.69$, $P = 0.711$, two-way repeated measures ANOVA). For the main effect of treatment, no significant differences were observed ($F_{(3,\ 189)} = 1.675$, $P = 0.174$). However, there was a significant main effect of time ($F_{(4,\ 252)} = 18.292$, $P < 0.001$). Regarding the latency measurement, there were significant interaction effects between treatment and time ($F_{(9.2,\ 580.6)} = 3.508$, $P < 0.001$) (Fig. 2B). A *post-hoc* Bonferroni test showed that latency in the sevoflurane group was higher than in the control group on day 3 ($P = 0.02$). But it could be normalized by co-treatment of TBA ($P = 0.013$, sevoflurane + TBA group *versus* sevoflurane group; $P = 0.199$, sevoflurane + TBA group *versus* control group,). In the probe trial, significant differences were observed in terms of number of target crossings ($F_{(3,\ 60)} = 5.407$, $P = 0.003$, one-way ANOVA), percentage of time in the platform quadrant to total time ($F_{(3,\ 60)} = 42.399$, $P < 0.001$, one-way ANOVA), and percentage of distance in the platform quadrant to total distance ($F_{(3,\ 60)} = 167.038$, $P < 0.001$, one-way ANOVA) among control, sevoflurane, sevoflurane + TBA, and TBA groups (Fig. 2C, D, and E). A *post hoc* Bonferroni test showed that rats in the sevoflurane group had a lower number of target crossings ($P = 0.005$), percentage of time in the platform quadrant to total time ($P < 0.001$), and

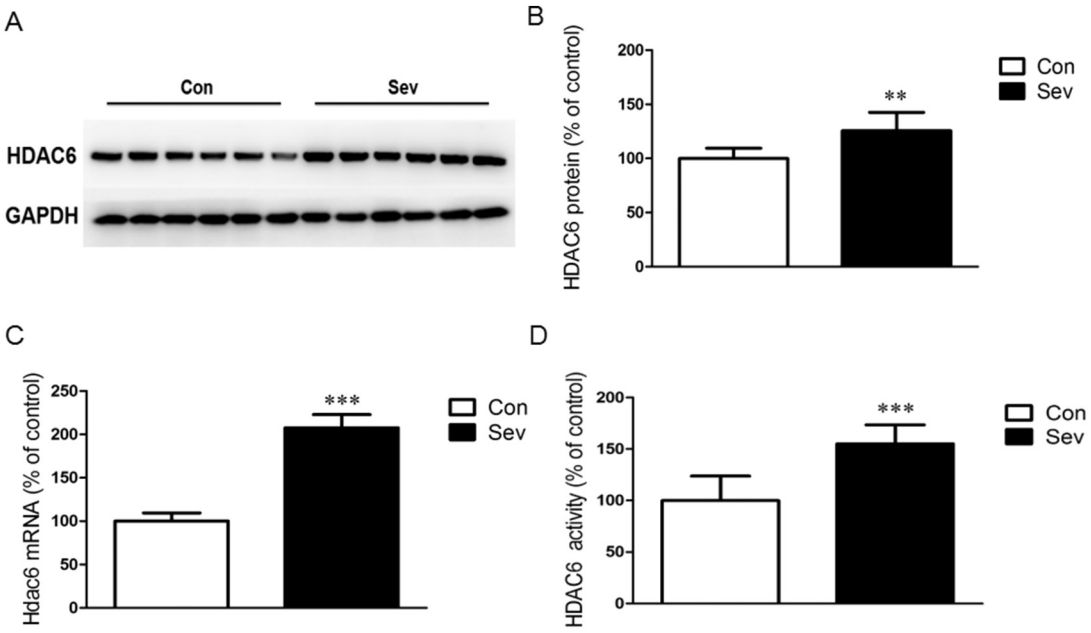

Fig. 1. Effects of multiple sevoflurane exposures on HDAC6 expression and activity in the developing hippocampus. (A) Postnatal day 7 rat pups were exposed to 3% sevoflurane for 2 h daily for three consecutive days. Immediately after the last exposure, both hippocampi were harvested to measure protein level of HDAC6 by Western blot. (B) The histogram summarizes the experiments as shown in (A). Densities of protein bands are normalized to GAPDH. The percentages of control group are calculated and compared. (C) Changes in HDAC6 mRNA level after multiple sevoflurane exposures were detected by real-time PCR. (D) Differences in HDAC6 activity between control and sevoflurane groups were analyzed with a fluorometric assay kit. All data are presented as mean ± standard deviation, n = 6 for each group, $^{**}P < 0.01$, $^{***}P < 0.001$ *versus* Con group. Con: control, Sev: sevoflurane.

percentage of distance in the platform quadrant to total distance ($P < 0.001$) compared with the control group. However, these effects were ameliorated by administration of the HDAC6 inhibitor TBA ($P = 0.016$, $P < 0.001$ and $P < 0.001$ respectively, sevoflurane + TBA group *versus* sevoflurane group). There was no significant difference between TBA and control groups ($P > 0.05$). These results suggested that sevoflurane impaired cognitive performance partly through HDAC6 activation.

3.3. Involvement of HDAC6 in sevoflurane-induced impairment of synaptogenesis

As shown in Fig. 3A and B, there were significant differences in the expression of synaptophysin ($F_{(3,\ 20)} = 9.110$, $P = 0.006$, one-way ANOVA) and PSD95 ($F_{(3,\ 20)} = 10.158$, $P = 0.004$, one-way ANOVA) among control, sevoflurane, sevoflurane + TBA, and TBA groups. A *post hoc* Bonferroni test indicated that multiple sevoflurane exposures reduced synaptophysin and PSD95 expression in the hippocampus ($P = 0.01$ and $P = 0.025$, respectively, sevoflurane group *versus* control group). However, TBA pretreatment attenuated sevoflurane-induced decreases in synaptophysin and PSD95 expression ($P = 0.032$ and $P = 0.004$ respectively, sevoflurane + TBA group *versus* sevoflurane group). There were no significant differences between TBA and control groups ($P > 0.05$). Further synaptic ultrastructure analysis revealed significant differences among the four groups in terms of synaptic cleft width ($F_{(3,\ 20)} = 40.004$, $P < 0.001$, one-way ANOVA) and synaptic density ($F_{(3,\ 20)} = 44.163$, $P < 0.001$, one-way ANOVA) (Fig. 3C–F). Indeed, both synaptic cleft width and synaptic density were significantly decreased in the sevoflurane group ($P < 0.001$, sevoflurane group *versus* control group), but were drastically increased in the presence of the HDAC6 inhibitor TBA ($P < 0.001$, sevoflurane + TBA group *versus* sevoflurane group). There were no significant differences between TBA and control groups ($P > 0.05$). These results indicated that HDAC6 participated in the impairment of synaptogenesis induced by multiple sevoflurane exposures.

4. Discussion

Numerous animal studies have demonstrated that postnatal exposure to sevoflurane three or more times can induce cognitive deficits in adulthood. The involved mechanism has attracted great attention in recent years. In the present study, we observed for the first time that multiple postnatal sevoflurane exposures promoted HDAC6 expression and activity in the developing hippocampus. More importantly, we demonstrated that HDAC6 is involved in the impairment of synaptogenesis and memory decline induced by multiple sevoflurane exposures. Moreover, HDAC6 inhibition could ameliorate developmental neurotoxicity of sevoflurane.

We found that multiple sevoflurane exposures resulted in increased HDAC6 expression and activity in hippocampi of the developing brain, consistent with previous studies reporting that general anesthetics increased HDAC expression and disturbed histone acetylation (Jia et al., 2016; Sen and Sen, 2016; Wu et al., 2016). A previous study reported that expression levels of HDAC3 and HDAC8 increased when neonatal rats were repeatedly exposed to sevoflurane (Jia et al., 2016). Accordingly, acetylation of H2K14, H3K9/14, and H4K5/12 was decreased in hippocampal CA1 and dentate gyrus regions. In addition, two studies reported that isoflurane, an inhalational anesthetic comparable to sevoflurane, induced HDAC2 and HDAC4 upregulation and decreased hippocampal H4K12 acetylation (Sen and Sen, 2016; Wu et al., 2016). Findings from the present study enhanced this association between abnormal HDAC expression and developmental neurotoxicity resulting from general anesthetics.

The current study further demonstrated the involvement of HDAC6 in sevoflurane-induced cognitive deficits in adulthood. The HDAC6 inhibitor TBA protected rat pups against sevoflurane-induced cognitive deficits. These results are at least partly in line with several studies reporting that pan-HDAC inhibitors, such as suberanilohydroxamic acid, trichostatin A, and sodium butyrate, could attenuate inhalational anesthetic-induced cognitive impairment (Jia et al., 2016; Lin et al., 2014; Zhong et al., 2015). However, these pan-HDAC inhibitors lack specificity. Our study focused on HDAC6 protein and revealed the

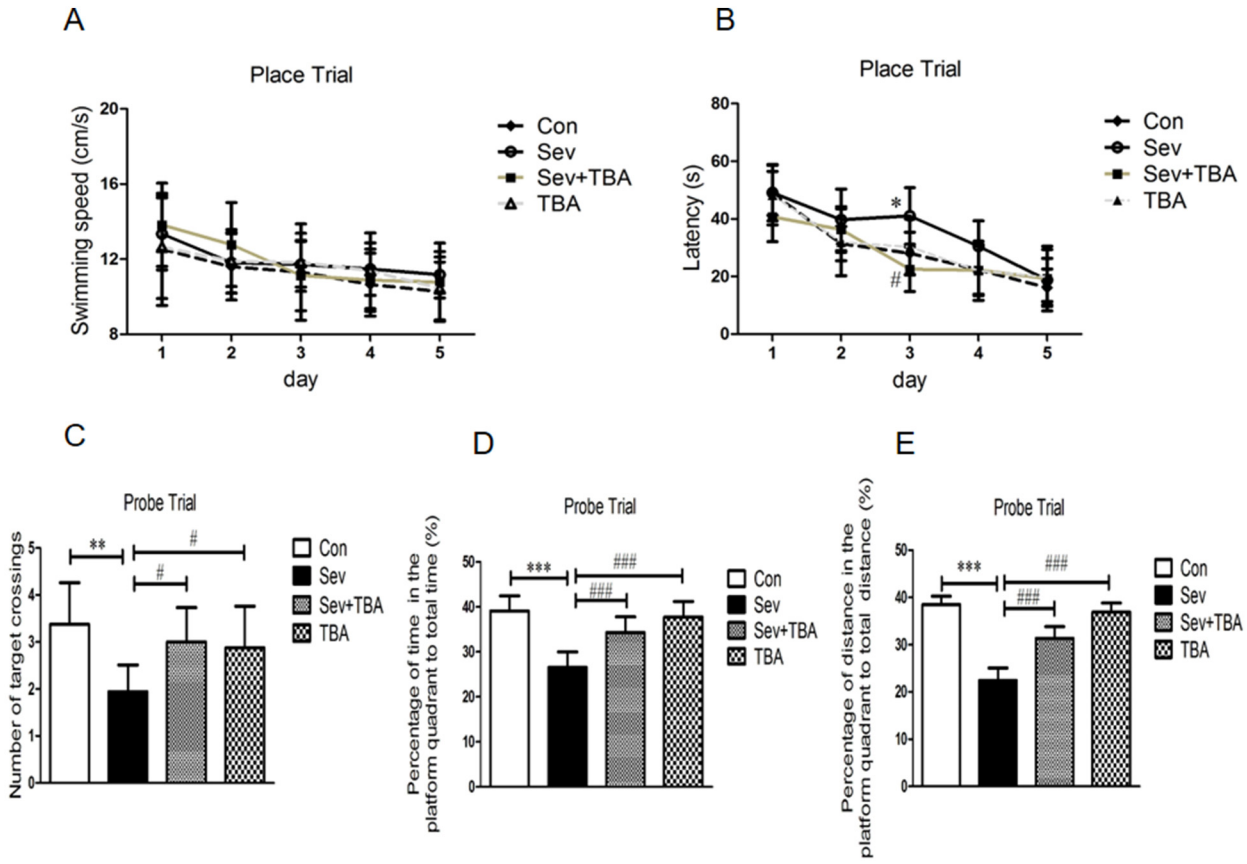

Fig. 2. Involvement of HDAC6 in memory decline during adulthood caused by multiple postnatal sevoflurane exposures. (A) and (B) A dose of 25 mg/kg body weight of Tubastatin A (a selective HDAC6 inhibitor) was injected intraperitoneally before each sevoflurane exposure. Rat pups were raised until P49 to measure spatial learning and memory using the Morris water maze test. Swimming speed and latency to find platform in the place trials among control, sevoflurane, sevoflurane + Tubastatin A and Tubastatin A groups were recorded and compared in five consecutive days. (C), (D) and (E) At the sixth day, the number of target crossings, percentage of time in the platform quadrant to total time and percentage of distance in the platform quadrant to total distance in the probe trial were collected and compared. All data are presented as mean ± standard deviation, n = 16 for each group, ** $P < 0.01$, *** $P < 0.001$ *versus* Con group, $^{\#}P < 0.05$, $^{\#\#\#}P < 0.001$ *versus* Sev group. Con: control, Sev: sevoflurane, TBA: Tubastatin A.

potential of an HDAC6 inhibitor to mitigate developmental neurotoxicity induced by sevoflurane. In fact, recent studies from other neurodegenerative models, such as Alzheimer's disease, have demonstrated a pivotal role for selective HDAC6 inhibitors in improving cognitive performance (Fan et al., 2018; Lee et al., 2018). Our findings provide another cue illustrating the comprehensive effects of HDAC6 inhibitors on reversing long-term cognitive deficits caused by sevoflurane and other general anesthetics.

The present study indicated that multiple sevoflurane exposures during the postnatal period caused impaired synaptogenesis, consistent with previous studies performed by us and others (Lu et al., 2017; Shen et al., 2013b; Tao et al., 2014, 2016). Moreover, new findings from the current study revealed a relationship between HDAC6 protein and this phenomenon, such that inhibition of HDAC6 could rescue impaired synaptogenesis in rat pups. Previously reports have described dramatic decreases in the transcriptional activities of genes involved in neurodevelopment and neuroplasticity, such as BDNF, PSD95, c-Fos, MAP2, synapsin 1, p-CREB/CREB, and CREB-binding protein following repeated sevoflurane anesthesia-activated HDAC3 and HDAC8 expression (Jia et al., 2016). Other studies concentrating on isoflurane neurotoxicity reported augmented HDAC2 and HDAC4 expression after exposure, which inhibited histone acetylation in the promoter region of BDNF exon IV, glutamate transporter-1, metabotropic glutamate receptor 1/5, and CREB, which led to insufficient synthesis of synaptic proteins (Liang and Fang, 2016; Sen and Sen, 2016; Wu et al., 2016). Our study found preliminarily that the HDAC6 inhibitor TBA could reverse sevoflurane-induced PSD95 expression reduction. However, whether HDAC6 augmentation results in impaired synaptogenesis by inhibiting the transcriptional activities of synapse-related genes requires further investigation.

In conclusion, our study demonstrated that multiple exposures of the developing brain to sevoflurane enhanced HDAC6 expression. Furthermore, HDAC6 was involved in the synaptogenesis impairment and cognitive deficits induced by sevoflurane. As such, HDAC6 inhibition may be a useful strategy to prevent developmental neurotoxicity induced by sevoflurane.

Transparency document

The Transparency document associated with this article can be found, in online version.

Declaration of Competing Interest

The authors declare that there are no conflicts of interest.

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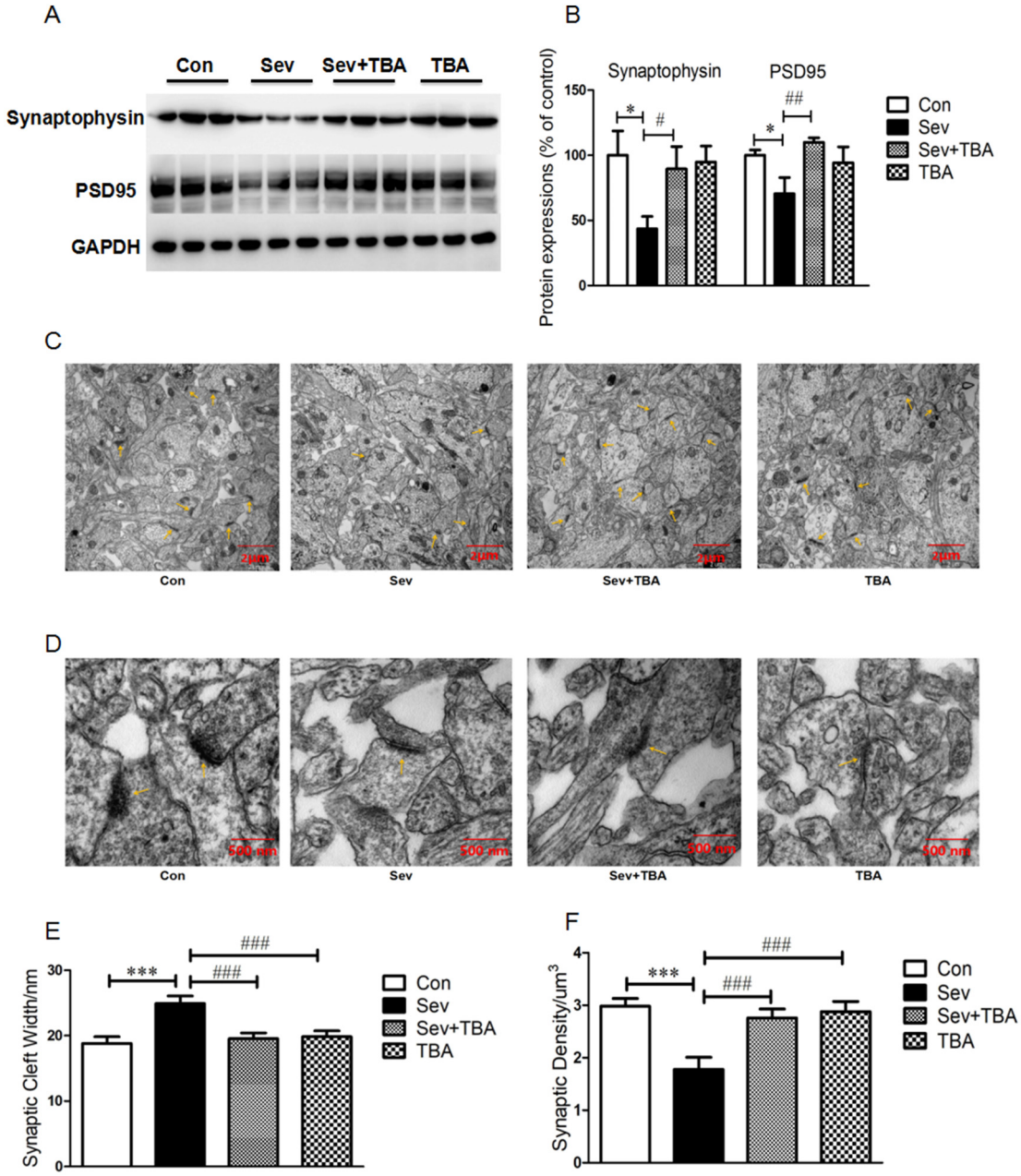

Fig. 3. Involvement of HDAC6 in sevoflurane-induced impairment of synaptogenesis. (A) A dose of 25 mg/kg body weight of Tubastatin A was administrated intraperitoneally prior to each sevoflurane exposure. After the last exposure, both hippocampi were harvested for determining expression levels of presynaptic protein synaptophysin and postsynaptic protein PSD95. (B) The histogram shows protein levels of synaptophysin and PSD95 in each group. Densities of protein bands are normalized to GAPDH. The percentages of control group are calculated and compared. (C) Synaptic ultrastructure in the CA1 area of hippocampus in each group was analyzed by transmission electron microscopy. Yellow arrows indicate synaptic linkages. Scale bar = 2 μm. (D) Representative high-magnification pictures of synaptic ultrastructure in each group. Scale bar = 500 nm. (E) and (F) the histogram shows differences of synaptic density and cleft width among four groups. All data are presented as mean ± standard deviation, n = 6 for each group, $^{*}P < 0.05$, $^{***}P < 0.001$ *versus* Con group, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ *versus* Sev group. Con: control, Sev: sevoflurane, TBA: Tubastatin A. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

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