Role of NR2B/ERK signaling in the neuroprotective effect of dexmedetomidine against sevoflurane induced neurological dysfunction in the developing rat brain

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Dexmedetomidine (DEX) is a potent α-2 adrenergic receptor agonist and has been widely applied in clinic. The present study explored the protective effect of DEX on sevoflurane-induced learning and cognitive impairment and examined its underlying mechanism. Sprague-Dawley rat pups were exposed to 0.85% sevoflurane for 6 h and injected with DEX in different doses. The Morris water maze test was performed to evaluate the learning and memory function of rats. Western blot was used for the measurement of protein levels. The water maze results indicated that sevoflurane treatment increased the escape latency but reduced the time spent in the original quadrant of rats. The protein levels of NR2B, phosphorylated ERK were significantly influenced by sevoflurane. Ifenprodil administration alleviated sevoflurane-induced neurological impairment. DEX treatment reversed the effect of sevoflurane on both escape latency and time in original quadrant in a dose manner, and pretreatment with DEX had the most dramatic effect. DEX regulated the NR2B/ERK signaling in sevoflurane treated rats. NR2B/ERK signaling is involved in sevoflurane induced neurological impairment. DEX may protect against sevoflurane induced neurological dysfunction in the developing rat brain *via* regulating the NR2B/ERK signaling.

Key words: dexmedetomidine, sevoflurane, learning and memory function, NR2B/ERK

INTRODUCTION

Sevoflurane is one of the most comment used inhalational anesthetics, because it helps rapid induction and recovery (Du et al., 2020). It is most widely applied during surgical operations and cesarean deliveries (Xie et Wang, 2018). Sevoflurane has a neurotoxic effect in central nervous system, especially for developing brain (Wang et al., 2016; Wang et al., 2012; Wang WY et al., 2013). Emerging evidence from an animal experiments has demonstrated that volatile anesthetics at clinical concentrations can lead to neuronal apoptosis and impair the learning ability and cognitive function in neonatal and aged rodents (Kodama et al., 2011; Li XM et al., 2014; Sun et al., 2016). Previous retrospective studies have reported that the application of anesthesia in children may influence the progression of behavior and cognitive function, especially children younger than 3 years old (DiMaggio et al., 2009; Wilder et al., 2009; Servick, 2014). Because of the common use of sevoflurane in anesthetics application for childbirth and surgeries, it is urgent to explore effective therapy to prevent anesthesia-induced neurotoxicity.

Dexmedetomidine (DEX) is a potent highly selective α2 adrenergic receptor agonist. It has been widely applied in the clinic, as a result of its ability to provide sedation without risk of respiratory depression (Feng et al., 2018). In recent years, DEX has drawn widespread attention for its neuroprotective effects (Wu et al., 2018). In a study of subarachnoid hemorrhage (SAH), DEX is determined to play a neuroprotective effect in the hippocampus of vasospastic SAH rabbits (Cosar et al., 2009). Additionally, DEX is also suggested to prevent against propofol exposure-induced neurotoxicity in the fetal brains, and play neurocognitive protection in the offspring rats (Li J et al., 2016). Clinically, DEX is more and more widely used in patients undergoing general anesthesia (Yu et al., 2019). Although DEX is previously suggested to have a neuroprotective effect in various brain injuries, it is still unknown whether DEX can directly protect against cognitive dysfunction induced by sevoflurane exposure, especially in developing brain. Notably, in the depression model of rats treated by chronic unpredictable mild stress, DEX is suggested to protect against learning and memory impairment caused by electroconvulsive shock in depressed rats, with the involvement of the NMDA receptor subunit 2B (NR2B)-ERK signaling pathway (Gao et al., 2016). In light of the above results, whether NR2B-ERK signaling is involved in the neuroprotective effect of DEX against sevoflurane induced cognitive dysfunction attracts the interest.

Therefore, the present study explored the protective effect of DEX on sevoflurane-induced learning and cognitive impairment. Furthermore, the underlying mechanism of the neuroprotective effect was further examined, particularly, involving the NR2B-ERK signaling pathway.

METHODS

Animals and ethic statement

Postnatal day 7 (P7) Sprague-Dawley rat pups were purchased from the Shanghai animal center. All rats were housed in an environment with a temperature of 20-25°C, a humidity of 40%-70%, a light/dark cycle of 12/12 h, and free access to food and water. The study design was conducted with the approval of the Animal Ethics Committee of the Second Affiliated Hospital of Shandong First Medical University.

Animal grouping

In addition to the control group, rats in other groups were exposed to 0.85% sevoflurane for 6 h as previously described (Ma et al., 2017). 30 min before sevoflurane treatment, rats were injected with 25, 50, 75 µg/kg Dex or normal saline intraperitoneally.

All rats were randomly divided into 8 groups (n=20 in each group): control group: rats received regular air inhalation for 6 h; Sevo group: rats were exposed to 0.85% sevoflurane for 6 h; Sevo + vehicle group, 30 min before sevoflurane treatment, rats were injected with 0.5 ml/kg vehicle (dimethylsulfoxide (DMSO)), then exposed to 0.85% sevoflurane for 6 h; Sevo + Ifen group, 30 min before sevoflurane treatment, rats were injected with 5 mg/kg ifenprodil (Ifen), then exposed to 0.85% sevoflurane for 6 h; Sevo+NS group: 30 min before sevoflurane treatment, rats were injected with saline (S), then exposed to 0.85% sevoflurane for 6 h; Sevo + DEX25 group: 30 min before sevoflurane treatment, rats were injected with 25 µg/kg Dex, then exposed to 0.85% sevoflurane for 6 h; Sevo + DEX50 group: 30 min before sevoflurane treatment, rats were injected with 50 µg/kg Dex, then exposed to 0.85% sevoflurane for 6 h; Sevo + DEX75 group: 30 min before sevoflurane treatment, rats were injected with 75 µg/kg Dex, then exposed to 0.85% sevoflurane for 6 h. After the experiment finished, rats were euthanized by decapitation, and the hippocampi tissues were collected for further experiments.

Neurological function test

The P7 rats were fed until P14, then a modified neurological severity score was performed to assess the neurological severity of rats in each group through evaluating the behavior and motor changes, including balance, touch, vision, abnormal behavior, sensation, and movement, according to Loga et al protocol (Lan et al., 2014). The score ranged from 0 to 18, and 0 point indicated a normal brain system while 18 points indicated the most severe neurological dysfunction.

Morris water maze test

The P7 rats were fed until P14, then their learning and memory function was evaluated by the Morris water maze (MWM) test. The water maze consisted of a 120 cm circular pool with a depth of 50 cm filled with about 450 L warm water. A hidden circular platform was placed 1.5 cm below the water surface. Each rat was placed in the maze from four random points of the tank and released to find the hidden platform for 2 min. The time of rat taken to reach the hidden platform (latency) was recorded. If the rat did not

reach the platform within 2 min, the rat was gently placed on the platform and left for the 20 s. All rats have received training trials for four consecutive days. On the fifth day, the platform was removed from the circular toll and the probe trial was performed, the time spent in the original quadrant and escape latency was recorded.

Western blot

The total proteins were lysed by using RIPA lysates with protease and phosphatase inhibitors in ice, and then the protein concentration was detected by the BCA protein assay kit. Then protein samples were mixed with a 10% SDS buffer in a 1:1 ratio and boiled at 95°C for 5 min. The protein was then concentrated and separated by 10% gel-electrophoresis, and the separated protein was transferred to the PVDF membrane. Subsequently, the PVDF membrane was sealed with a 5% BSA solution for 2 h, and the primary antibody was incubated at 4°C overnight. After cleaning with TBST 3 times, the proteins were detected with anti-rabbit or anti-mouse secondary antibody for 2 h at room temperature. Finally, the results are displayed by the imaging system.

Statistical analysis

All data were compared between groups by using student's t-test and one-way ANOVA analysis followed by Turkey's test. All data analysis was carried out by using GraphPad Prism 7.0 software. The data were expressed as the mean ± standard deviation (SD). P values less than 0.05 were considered statistically significant.

RESULTS

Sevoflurane impaired the neurological function in rats

As shown in Fig. 1, the learning and memory function of rats was assessed by MWM test. During the training time, the escape latency of rats in each group decreased gradually. On the fourth day, the rats in sevoflurane group showed longer latency time compared to the control group (t_8=9.316, P<0.001, Fig. 1A). But there was no significant difference in swimming speed between the two groups (P>0.05, Fig. 1B). On the fifth day, the learning and memory were evaluated by a probe trial. As shown in Fig. 1C-D, sevoflurane treatment increased the escape latency time
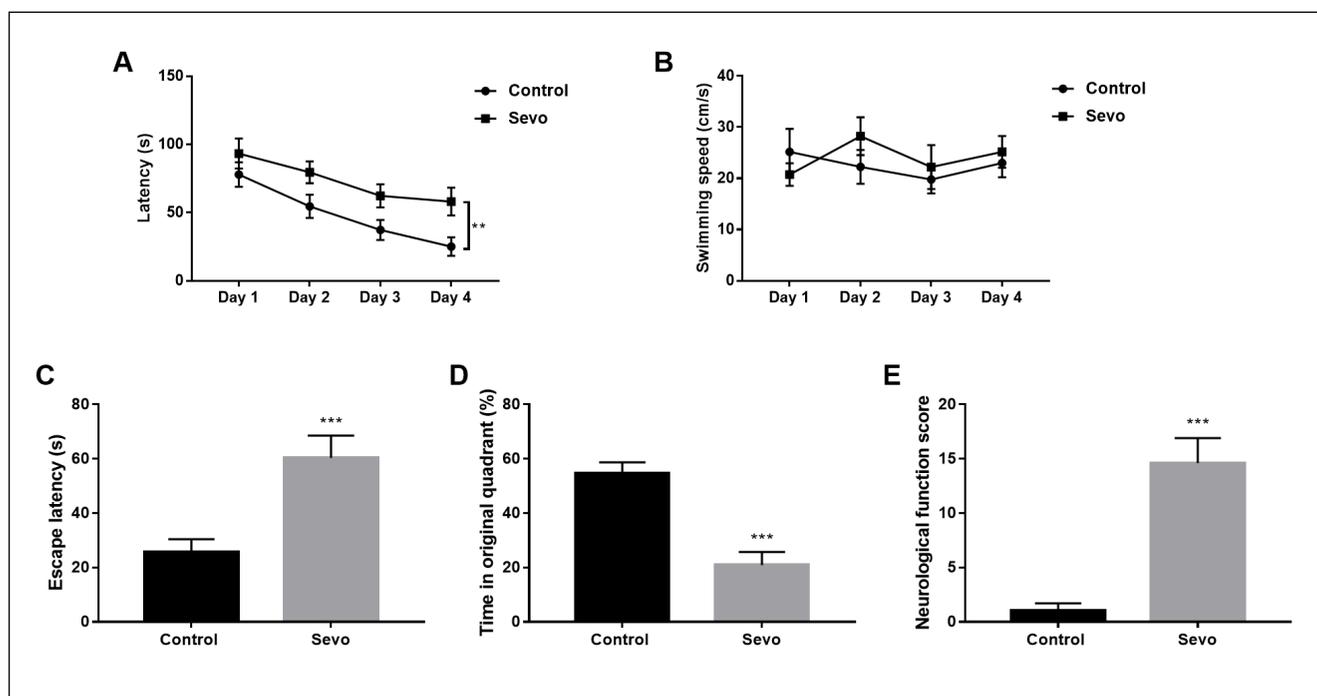

Fig. 1. Sevoflurane impaired the neurological function in rats. (A) During the training time, the escape latency of rats in each group decreased gradually, and the latency of rats in the sevoflurane group was significantly higher than that in the control group on the fourth day. (B) There was no significant difference in swimming speed between the two groups. (C–D) Sevoflurane treatment increased the escape latency but reduced the time spent in the original quadrant. (E) Sevoflurane treatment significantly increased the neurological function score. ** P<0.01; *** P<0.001.

(t_8=8.202, P<0.001) but reduced the time spent in the original quadrant (t_8=12.140, P<0.001). Consistent with the MWM test results, sevoflurane treatment also led to an increase in the neurological function score (t_8=2.630, P<0.001, Fig. 1E).

NR2B/ERK signaling is involved in sevoflurane-induced neurological impairment in rats

NR2B/ERK signaling-related proteins were detected *via* western bolt to investigate whether NR2B/ERK signaling is involved in sevoflurane-induced neurological impairment. As shown in Fig. 2A, high protein levels of NR2B ($F_{2,12}$=40.32, P<0.001), and low phosphorylated ERK levels ($F_{2,12}$=49.12, P<0.001) were detected in hippocampi tissues of rats exposed to sevoflurane, and the differences reached a significant level compared with the control group (P<0.001). To further verify the role of NR2B/ERK signaling, ifenprodil, the NMDAR antagonist, was selected to inhibit the NR2B subunit. It was found that ifenprodil administration reversed the effect of sevoflurane on neurological impairment induced by sevoflurane. The MWM results indicated that during the training time, the escape latency of rats decreased gradually in each group (Fig. 2B). But ifenprodil administration led to the decrease of the escape latency for sevoflurane treated rats compared with the sevoflurane group ($F_{2,12}$=39.78, P<0.001, Fig. 2B). The swimming speed showed no significant difference among different groups (P>0.05, Fig. 2C). According to the probe trial results, ifenprodil administration decreased the escape latency time ($F_{2,12}$=42.02, P<0.001, Fig. 2D) but reduced the time spent in the original quadrant of rats exposed to sevoflurane ($F_{2,12}$=54.64, P<0.001, Fig. 2E). Moreover, ifenprodil administration also decreased the neurological function score significantly ($F_{2,12}$=20.14 P<0.001, Fig. 2F).

Effect of DEX on sevoflurane-induced neurological impairment in rats

Rats were given DEX with different concentrations to explore the role of DEX in sevoflurane-induced neurological impairment. According to the MWM results, the escape latency of rats in each group decreased gradually among different groups, and DEX treatment reduced the escape latency in a dose-dependent manner (Fig. 3A). On the fourth day, rats pretreated with
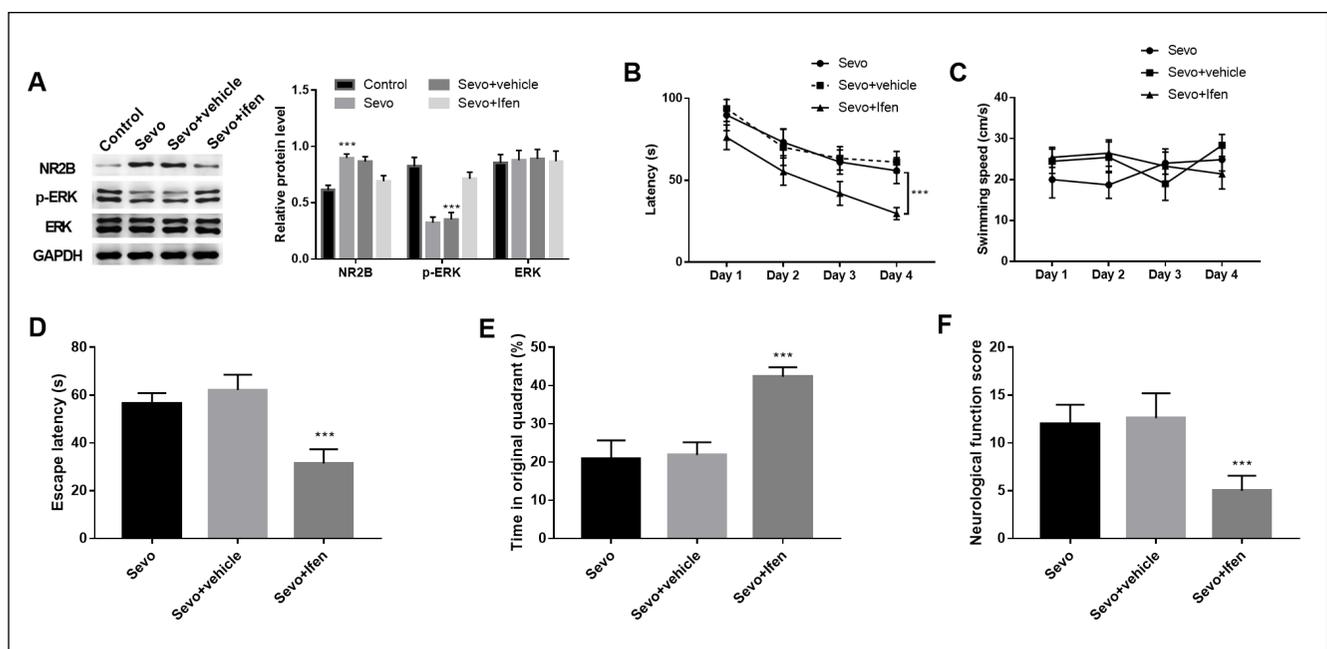

Fig. 2. The role of NR2B/ERK signaling in sevoflurane-induced neurological impairment in rats. (A) The protein levels of NR2B, phosphorylated ERK were significantly influenced by sevoflurane compared with the control group, but the influences were reversed by ifenprodil administration. (B) during the training time, the escape latency of rats decreased gradually, and on the fourth day, ifenprodil administration decreased the escape latency of sevoflurane treated rats compared with the sevoflurane group. (C) The swimming speed showed no significant difference among different groups. (D–E) On the fifth day, ifenprodil administration decreased the escape latency time but reduced the time spent in the original quadrant of rats compared with the sevoflurane group. (F) ifenprodil administration also decreased the neurological function score significantly. *** P<0.001.
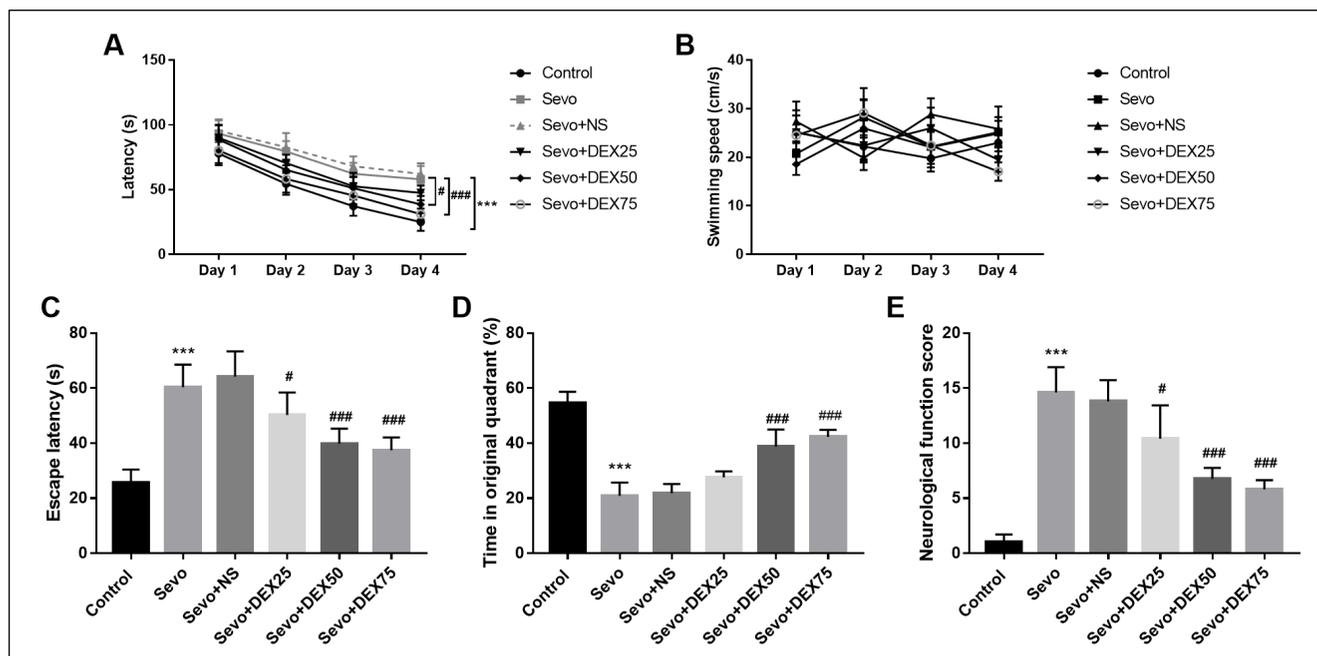
Fig. 3. Effect of DEX on sevoflurane-induced neurological impairment in rats. (A) The escape latency of rats in each group decreased gradually among different groups, and DEX treatment reduced the escape latency in a dose-dependent manner. (B) There was no significant difference in swimming speed among different groups. (C–D) DEX treatment reversed the effect of sevoflurane on both escape latency and time in the original quadrant, and pretreatment with 75 μg/kg DEX had the most dramatic effect. (E) Sevoflurane treatment significantly increased the neurological function score, which was decreased by DEX pretreatment in a dose-dependent manner. *** $P<0.001$, compared with control group; # $P<0.01$, ### $P<0.001$, compared with sevoflurane group.

75 μg/kg DEX had the lowest escape latency time than other concentrations ($F_{5,24}=19.83$, $P<0.001$, Fig. 3A). But there was no significant difference in swimming speed among different groups ($P>0.05$, Fig. 3B). On the fifth day, the learning and memory were evaluated by a probe trial., The results indicated that DEX treatment reversed the effect of sevoflurane on both escape latency ($F_{5,24}=21.90$, $P<0.001$, Fig. 3C) and time spent in the original quadrant ($F_{5,24}=53.21$, $P<0.001$,

Fig. 3D), and pretreatment with 75 μg/kg DEX had the most dramatic effect. Additionally, the neurological function score was also detected in different groups. Consistent with the MWM test results, sevoflurane treatment significantly increased the neurological function score, which was decreased by DEX pretreatment in a dose-dependent manner ($F_{5,24}=39.56$, $P<0.001$, Fig. 3E). It was concluded that DEX alleviated sevoflurane-induced neurological impairment in rats.
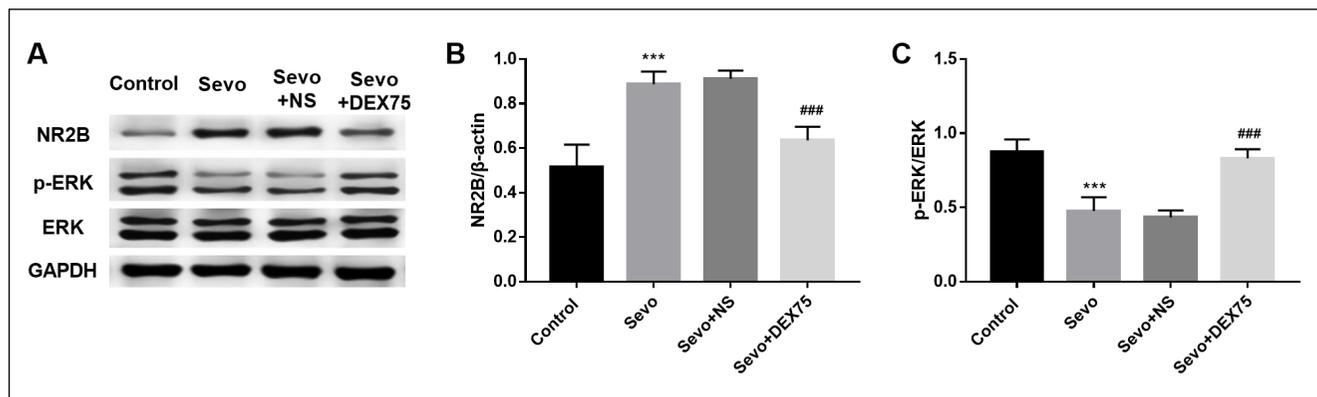

Fig. 4. DEX regulates the NR2B/ERK signaling in sevoflurane-treated rats. (A) Western blot results. (B) Sevoflurane treatment upregulated the protein level of NR2B, which was reduced by pretreatment with 75 μg/kg DEX. (C) phosphorylated ERK level was decreased by sevoflurane treatment, which was increased by pretreatment with 75 μg/kg DEX. *** $P<0.001$, compared with control group; ### $P<0.001$, compared with sevoflurane group.

DEX regulates the NR2B/ERK signaling in sevoflurane treated rats

To further investigate the mechanism of the protective effect of DEX against sevoflurane-induced neurological impairment, NR2B/ERK signaling-related proteins were detected *via* western bolt. As shown in Fig. 4, high protein levels of NR2B ($F_{3,16}$=41.23, *P*<0.001), and low phosphorylated ERK levels ($F_{3,16}$=48.51, *P*<0.001) were detected in rats exposed to sevoflurane, but the effect was reversed by DEX treatment *P*<0.001.

DISCUSSION

Sevoflurane is one of the most prominent inhalational anesthetics used in cesarean delivery and pediatric clinical application (Xie and Wang, 2018). Clinical studies have shown that sevoflurane exposure has certain harm on learning and cognitive functions, especially for children (Servick 2014; Wang et al., 2016). Therefore, in the current study, the P7 rat pups were recruited for animal experiments to investigate the role of sevoflurane in developing brain. It was found that sevoflurane exposure impaired the neurological function of rats. Additionally, western blot results suggested that sevoflurane treatment influenced the NR2B/ERK signaling-related proteins. Furthermore, ifenprodil, the NR2B subunit antagonist, reversed the effect of sevoflurane on neurological function in rats. It was concluded that NR2B/ERK signaling is involved in sevoflurane-induced neurological impairment in rats.

The N-methyl-D-aspartate receptor (NMDAR) is a subtype of ionotropic glutamate receptors which mediate the neuronal plasticity and memory or learning function in the mammalian central nervous system (Hu et al., 2016). It is observed that continued NMDAR activation can lead to neuronal injury (Alizadeh et al., 2015). Unbalance of NMDARs has been demonstrated to be involved in the occurrence and development of various central nervous system diseases, such as ischemic stroke, Alzheimer's disease, Parkinson's disease and so on (Hu et al., 2016; Schreiber et al., 2019). NR2B is a subtype of NMDAR, which is associated with the over-activation of NMDAR, followed by neuronal damage (Zhong et al., 2019). NR2B overexpression can trigger cell apoptotic pathways, which may be the pathology of the neuroprotective effect of NR2B antagonists (Shi et al., 2017). Notably, NR2B activation is also closely associated with the activation of ERK signaling (Evans et al., 2019). Moreover, NR2B-ERK signaling has been reported to regulate neuronal survival and participate in the development of neurological

diseases (Paul et al., 2010; H. Wang et al., 2013; Zhao et al., 2018). In the present study, sevoflurane treatment was suggested to increase the protein level of NR2B and decrease the level of phosphorylated ERK in the hippocampal tissues of rats, indicating that NR2B/ERK signaling might be involved in sevoflurane-induced neurological impairment in rats. Furthermore, ifenprodil, the NMDAR antagonist, was selected to inhibit the NR2B subunit, and further verify the role of NR2B/ERK signaling. As expected, ifenprodil administration was observed to alleviate sevoflurane-induced learning and memory dysfunction. The results confirmed that NR2B/ERK signaling is involved in sevoflurane exposure-induced neurological impairment in rats. Consistently, in a study about chronic intermittent hypoxia-hypercapnia, the ameliorative cognitive deficits caused by lovastatin are reported to be associated with the downregulation of NR2B expression and the increased expression of ERK signaling (Huo et al., 2014). Besides, NR2B-ERK pathway is also reported to be involved in the recall of morphine-associated contextual memory (Xu et al., 2012). All shreds of evidence reveal the crucial role of NR2B-ERK signaling in learning and memory function, which supported our present results.

DEX is a useful anesthetic adjuvant and has been widely used in the clinical area. Several studies have reported that DEX regulates memory formation in a dose-dependent (van Oostrom et al., 2010). In a study about drug-resistant depression, DEX is suggested to protect against learning and memory impairments caused by an electroconvulsive shock in depressed rats (Gao et al., 2016). Considering the neuroprotective role of DEX in various neurological diseases, the current study further explored the role of DEX in sevoflurane-induced neurological impairment. As expected, the results demonstrated that DEX pretreatment alleviated sevoflurane-induced neurological impairment in rats in a dose-dependent manner, and pretreatment with 75 μg/kg DEX had the most dramatic effect. As previously described, DEX is demonstrated to protect against anesthesia-induced neurotoxicity by several studies (Sanders et al., 2010). A recent study confirmed that DEX exerted neuroprotective effects by inhibiting sevoflurane-induced apoptosis, inflammation, and oxidative stress in P6 mice (Zhang et al., 2021). Neonatal injection of DEX can enhance spatial learning and memory in rat pups, potentially by promoting hippocampal neurogenesis and synaptic plasticity (Zhang et al., 2019). In addition, DEX is also reported to alleviate propofol-induced neurotoxicity and neurocognitive impairment *via* inhibiting activation of GSK-3β/CRMP2 and CDK5/CRMP2 pathways in the hippocampus of neonatal rats (Li et al., 2019). These findings supported our

present results about the protective role of DEX against sevoflurane-induced neurological impairment. Additionally, as the previous study reported, DEX at doses 10 and 25 μg/kg showed neuroprotective effects, and the dose of 25 μg/kg was more effective than 10 μg/kg (Gao et al., 2016). Therefore, the current study selected a higher dose of 50 and 75 μg/kg, and DEX at a dose of 75 μg/kg had a more powerful neuroprotective effect. Moreover, NR2B-ERK signaling is also reported to participate in the neuroprotective role of DEX in depressed rats (Gao et al., 2016). In consideration of the involvement of NR2B-ERK signaling in sevoflurane-induced neurological impairment, we suspected that whether NR2B-ERK signaling is involved in the neuroprotective effect of DEX in sevoflurane treated rats. It was found that DEX regulates the NR2B/ERK signaling in sevoflurane treated rats, we concluded that DEX may protect against sevoflurane-induced neurological dysfunction in the developing rat brain *via* regulating the NR2B/ERK signaling. However, other studies are needed to verify the present results, and thorough studies should be done for the mechanism exploration.

In conclusion, the present results demonstrated that NR2B/ERK signaling was involved in sevoflurane-induced neurological impairment. DEX might protect against sevoflurane-induced neurological dysfunction in the developing rat brain *via* regulating the NR2B/ERK signaling.

ACKNOWLEDGMENT

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