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Dexmedetomidine mitigates sevoflurane-induced cell cycle arrest in hippocampus.

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Abstract

Background: Epidemiologic studies suggest the possibility of a modestly elevated risk of adverse neurodevelopmental outcomes in children exposed to anesthesia during early childhood. Sevoflurane is widely used in paediatric anaesthetic practice. It is urgent to search for neuroprotective strategies.
Materials and methods: In our study, the hippocampal neuron cells were isolated from new-born neonatal rats and cultured *in vitro*. We performed immunocytochemistry with anti-MAP-2 and anti-GFAP antibodies to examine the purity of neurons. Cell cycle distribution was examined by flow cytometry. The protein levels of BDNF and TrkB were analysed *via* Western blot.
Results: Immunocytochemistry results showed that the purity of neurons>94%, which provided a good model for neural pharmacology experiments. The exposure of sevoflurane induced cell cycle arrest at S phase and suppressed the expression of BDNF and TrkB, and the exposure duration influenced the role of sevoflurane. In our study, the addition of DEX partly relieved the cell cycle arrest and the inhibitory of BDNF and TrkB expression induced by sevoflurane. What's more, the function of DEX was in dosage-dependent manner and suppressed by a α2 adrenergic receptor blocker yohimbine.
Conclusion: Taken together, sevoflurane suppressed neurons cell proliferation *via* regulating the expression of BDNF and TrkB, and DEX relieved the neurotoxicity induced by sevoflurane *via* α2 adrenergic receptor.

Keywords: Anesthetics, Sevoflurane, Neuroprotection, Dexmedetomidine, Brain-derived neurotrophic factor-tyrosine kinase B.

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Introduction

Volatile anaesthetics are used in millions of young children every year during surgical procedures and imaging studies around the world [1]. The potential for commonly used anesthetics and sedatives to cause neurodegenerative changes in the developing mammalian brain has become evidence in animal studies over the past 15 years [2]. Epidemiologic studies suggest the possibility of a modestly elevated risk of adverse neurodevelopmental outcomes in children exposed to anesthesia during early childhood [3]. Sevoflurane is an inhaled anaesthetic that is widely used in paediatric anaesthetic practice [4] because of its rapid induction and lower pungency [5]. Some reports showed that sevoflurane led to the long-term cognitive impairment in young rats [1]. Sevoflurane significantly reduced the number of primary dendrites and the number of branching points [6]. Numerous studies in small rodents have demonstrated a widespread increase in brain apoptosis shortly after exposure to a variety of inhaled anesthetics, including isoflurane [7] and sevoflurane [8]. Considering that more than 3 million children are exposed to inhaled anesthetics every year [9], there is an urgent need to search for neuroprotective strategies.

The selective α2-adrenoreceptor agonist Dexmedetomidine (DEX) was shown to exert neuroprotective effects in various ischemic and hemorrhagic brain injury models of animals [10]. It was reported that neuroprotective effect of DEX attenuated isoflurane-induced cognitive impairment through antioxidant, anti-inflammatory and anti-apoptosis in aging rats [11]. However, it is not clear that whether DEX protected against sevoflurane-induced neuroapoptosis during brain development.

In our study, we selected neonatal rats and isolated hippocampal neuron cells for *in vitro* culture. After continuous culture for 7 d, the nerve cells started to aggregate with the increase of the three-dimensional sensation and the obvious halo. The purity of hippocampal neurons reached 94% determined by a neuronal marker Microtubule-Associated Protein 2 (MAP-2) stains, which provided a good model for neural pharmacology investigation. Brain-Derived Neurotrophic Factor (BNDF) regulated and maintained the development and function of the nervous system by promoting the proliferation and differentiation of nerve cells *via* binding

to Tyrosine Kinase B (TrkB) receptors [12]. Sevoflurane exposure inhibited cell proliferation and suppressed the expression of BDNF and TrkB. The addition of DEX affected the neuron cell proliferation and the expression of BDNF and TrkB induced by sevoflurane exposure.

Materials and Methods

Reagents

Dulbecco's Modified Eagle Medium (DMEM), Fetal Bovine Serum (FBS), propidium iodide; 4', 6-Diamidino-2-Phenylindole (DAPI), Radioimmunoprecipitation Assay (RIPA) buffer, (Sigma, USA); Papain (Solarbio Science and Technology Co., Ltd. Beijing, China); Neurobasal medium (Sigma, USA); Matrigel basement membrane (BD Biosciences, San Jose, CA); Cytosine arabinoside (Sunray Pharmaceutical Co., Ltd. Suzhou, China); Rabbit anti-MAP-2 antibody (Epitomics, USA); Mouse anti-glial fibrillary acidic protein (GFAP) (BD, USA); Dylight 594 labeled sheep anti-rabbit IgG (KPL,USA); Dylight488 labeled sheep anti-mouse IgG (KPL,USA); Dylight 594-conjugated mouse anti-rabbit IgG (KPL, USA); Bicinchoninic Acid kit (BCA) and Horseradish Peroxidase (HRP) labeled IgG secondary antibody (Beyotime Institute of Biotechnology, Shanghai, China); Polyvinylidene Difluoride (PVDF, Millipore, Billerica, MA); Enhanced Chemiluminescence substrate (ECL, Pierce; Thermo Fisher Scientific, Inc., Waltham, MA, USA).

Cell culture and grouping

Sprague-Dawley (SD) rats born within 24 h were provided by the Experimental Animal Center of Hebei Medical University (Certificate NO. 1409001). Rats were housed at a temperature of 24 ± 1°C and relative humidity of 40-50% in a clean environment under 12:12 h light and dark cycle. Rats had free access to food and purified water was made available randomly. All procedures were approved by the Animal Ethic Committee of the Second Hospital of Hebei Medical University. Rats born within 24 h were placed in 75% ethanol for 2 min. We removed the skin and skull of the rats' head and took out the whole brain. With the help of an anatomical microscope, we removed the olfactory bulb, septum, thalamus, and hypothalamus. We then peeled off the dural and choroid plexus and took out the hippocampus tissue under the cerebral cortex into pre-cold sterile phosphate buffered saline. The hippocampus was cut into pieces with ophthalmology scissors, and then digested with 40 μg/ml papain at 37°C for 5 min to prepare single cell suspension. After removing the supernatant by centrifugation, the cell precipitate was suspended in DMEM containing 10% FBS and seeded into 24 well plate wrapped with matrigel basement membrane at the density of 1×10^6 cells/ml in 5% CO_2 incubator at 37°C. After 6 h, cells were incubated with serum-free neurobasal medium supplemented with 2% B27 and 1% N2. After 2 d, neuronal cells were cultured with cytosine arabinoside at the final concentration of 10 μM to inhibit the over growth of non-neuronal cells.

Neurons were randomly divided into four groups: Control (C), F3, F6 and F12 groups. C group referred that neurons were exposed to atmosphere containing 5% CO_2; the neurons were exposed to 1 Minimum Alveolar Concentration (MAC) sevoflurane (4%) with a total gas flow of 6 L/min using air as a carrier for 3 h (F3), 6 h (F6) or 12 h (F12), respectively.

Neurons were randomly divided into 9 groups: Control (C), F, D, FD1, FD2, FD3, Y1, Y2 and Y3 groups. Neurons in Y1, Y2 and Y3 groups were pretreated with 2 μM yohimbine for 30 min, and neurons in C, F, D, FD1, FD2 and FD3 groups were treated with the same amount of physiological saline. Then neurons in C and D groups were exposed to air, and neurons in other groups were exposed to 1 MAC sevoflurane using air as a carrier. After 6 h, neurons were incubated with DEX at the concentration of 0.1 μM (D, FD1 and Y1), 1 μM (FD2 and Y2) or 10 μM (FD3 and Y3), and neurons in other groups were treated with the same amount of physiological saline. After 12 h incubation, cells were collected for further experiments.

Immunocytochemistry

After 7 d, neuronal cells were washed with PBS for three times and fixed with fresh cold 4% paraformaldehyde at 4°C for 15 min, and permeabilized with 0.2% Triton-X100 for 15 min. After being washed with PBS, cells were blocked with BSA for 30 min and incubated with rabbit anti-MAP-2 (1:100 dilution) and mouse anti-GFAP (1:100 dilution) antibodies at 4°C overnight. The next morning neurons were washed with PBS for three times, and incubated with Dylight 594 labeled sheep anti-rabbit IgG (1:100 dilution) and Dylight488 labeled sheep anti-mouse IgG (1:100 dilution) for 2 h at 37°C in the dark. After being washed with PBS, cells were stained with DAPI at the final concentration of 1 μg/ml for 10 min in the dark. At least five non-overlapped signals were captured with Fluorescence inverted microscope.

Cell cycle analysis with flow cytometry

Neurons were fixed with 70% ethanol at -20°C for 30 min, and then stained with propidium iodide for 30 min in the dark. The neuronal cells were analysed by Fluorescence-Activated Cell Sorting (FACS) by Epics-XL II flow cytometry (Beckman Coulter, USA) with excitation wavelength of 488 nm. The resulting histograms were analysed by Muticycle AV software (Beckman Coulter, USA) for cell distribution in cell cycle phase. Proliferative Index (PI) of neurons were calculated according to the formula: $PI=(S+G_2/M)/(G_0/G_1+S+G_2/M) \times 100\%$, where G_0/G_1, S, and G_2/M represented the fraction of cells in each phase of the cell cycle.

Western blot

Proteins were extracted with RIPA buffer for western blot. The concentration of protein was detected by bicinchoninic acid kit. Equal amount of proteins were subjected into 12% SDS-PAGE and subsequently transferred onto PVDF membrane. The membrane was blocked with 5% non-fat milk for 1 h at room temperature, and incubated overnight at 4°C with primary

antibodies against CRMP-2, BDNF or TrkB (all in 1:100 dilution). After being washed with TBST (20 mM Tris, 140 mM NaCl, 0.2% Tween-20, pH 7.5), blots were probed with the HRP labeled IgG secondary antibody (1: 1,000 dilution) at 37°C for 2 h. After being washed with TBST for three times, the membranes were incubated with an ECL kit to detect the proteins expression. The band intensities were determined normalization to β-actin using the Quantity One software version 4.1 (Bio-Rad Laboratories, Inc., Hercules, CA, USA) with three independent repeats.

Statistical analysis

The data were performed using the SPSS 19.0 statistics package (SPSS Inc., Chicago, IL, USA), and expressed as mean ± SD. Statistical differences were assessed using Student's t-test or one-way Analysis of Variance (ANOVA) by Tukey's post hoc test, where appropriate among groups. A P-value<0.05 was considered statistically significant.

Results

The purity of neurons was verified by immunocytochemical staining

Hippocampal neurons were isolated from SD rats born within 24 h, and the neuron cell morphology was observed in Figure 1A. After 2 d incubation, the neuronal cells displayed 2-3 neurites, and a few smooth ploygliall cells disappeared. At 3 d, the classical fusiform neuronal cells disappeared, and the cell processes connected into networks. At 6 d and 7 d, the typical neuronal cells displayed aggregation, the increase of the three-dimensional sensation and the obvious halo. To further verify the purity of neuronal cells after 7 d incubation, we performed immunocytochemistry assay using anti-MAP-2 and anti-GFAP antibodies. In Figure 1B, the neurons were marked with anti-MAP-2 antibody in red and the astrocytes were stained in green with anti-GFAP antibody. By observing the ratio of the number of MAP-2-positive cells to the total number of cells marked with DAPI, its purity was found to be 94.81% ± 1.90%. That's to say, neurons were successfully cultured *in vitro* and provided a good model for further study.

Sevoflurane induced neuronal cell arrest at S phase and inhibited BDNF and TrkB expression

To examine the effect of sevoflurane on neuronal cell proliferation, we incubated neurons with sevoflurane for 3, 6 and 12 h and performed flow cytometry assay. In Table 1, there was no significant difference in the percentage of G_0/G_1-, S- and G_2/M-phase cells between C group and F3 group (P>0.05). However, compared with C group, the percentage of cells in the G_0/G_1 phases of the cell cycle in F6 and F12 groups were significantly decreased (P<0.01), and the frequency of S-phase and PI in F6 and F12 groups was obviously increased (P<0.01), and the increase was in exposure duration-dependent manner. These data showed that neurons cell cycles were arrested at S phase induced by sevoflurane and sevoflurane exposure of 1 MAC for 6 h was a suitable model for further investigation.

To explore the mechanism by which sevoflurane affected cell cycle distribution, we examined the protein levels of BDNF and TrkB after sevoflurane exposure. In Figure 2, no markedly differences on the protein levels of BDNF and TrkB were found between C and F3 groups (P>0.05). Nevertheless, the expression of BDNF and TrkB in F6 and F12 groups was significantly suppressed than that in C group (P<0.01), and the decrease was in exposure duration-dependent manner. These results revealed that sevoflurane inhibited the expression of BDNF and TrkB in neurons.

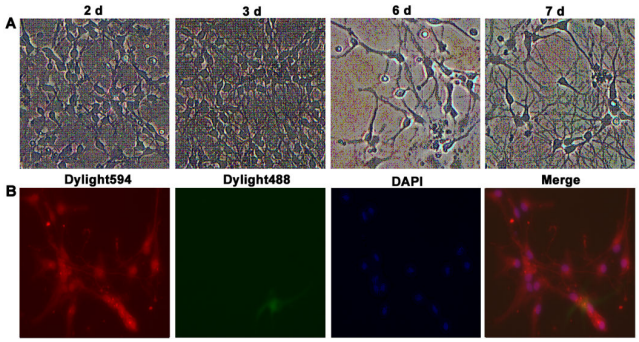

Figure 1. *The purity of neurons was verified by immunocytochemical staining. A. Neuronal morphology was observed after 2, 3, 6 or 7 d of incubation in vitro. Neurons were isolated from SD rats born within 24 h and cultured with serum-free Neurobasal medium supplemented with 2% B27 and 1% N2. B. The purity of neurons after 7 d of incubation in vitro was verified via immunocytochemistry. Dylight594 stood for neurons incubated with rabbit anti-MAP-2 antibody marked in red; Dylight488 referred to astrocytes probed with mouse anti-GFAP antibody marked in green; Nuclear was stained in blue with DAPI.*

The combination of sevoflurane and DEX affected neurons cell cycle and the expression of BDNF, TrkB and CRMP-2

To investigate the role of DEX on sevoflurane-incubated neurons cell cycle arrest, we incubated neurons with DEX and sevoflurane and examined cell cycle distribution *via* flow cytometry. In Table 2, the percentage of G_0/G_1-phase cells in F group was significantly suppressed than that in C group (P<0.01), however the frequency of S-phase in F group and PI were obviously upregulated (P<0.01). There was no significant difference on cell cycle distribution between C and D groups (P>0.05). Compared with C group, the combination of sevoflurane and DEX (0.1 μM) in FD1 group partly decreased the percentage of G_0/G_1-phase cells and PI, and upregulated the percentage of S-phase cells. Compared with FD1 group, higher concentration of DEX (FD2: 1 μM; FD3: 10 μM) enhanced the frequency of G_0/G_1-phase, and reduced the percentage of S-phase and PI, suggesting that DEX relieved neuron cell cycle arrest at S phase induced by sevofluvane in concentration-dependent manner. Then we employed a α2 adrenergic receptor blocker yohimbine to block the role of DEX. In Table 2, compared with C group, the addition of

yohimbine partly decreased the percentage of G_0/G_1-phase cells, and upregulated the percentage of S-phase cells and PI. There was no significant difference on cell cycle distribution among Y1, Y2 and Y3 (P>0.05). Taken together, these data showed that sevoflurane blocked cell arrest at S phase, single DEX had no significant effect on cell cycle, and the combination of sevoflurane and DEX partly relieved the cell cycle arrest at S phase induced by sevoflurane. What's more, the addition of yohimbine blocked the role of DEX on cell cycle and neurons proliferative activity.

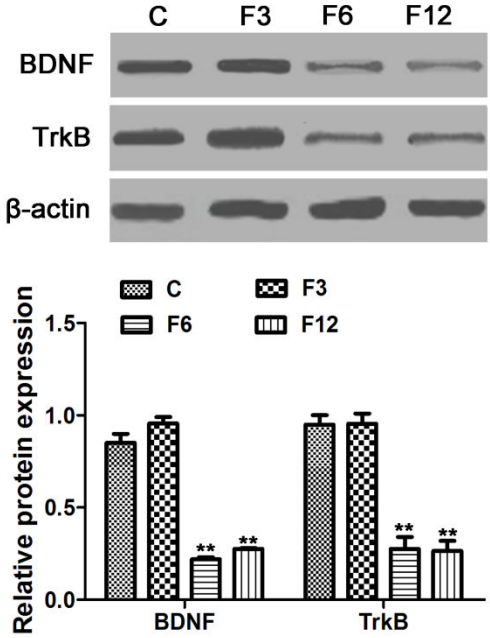

Figure 2. Sevoflurane suppressed BDNF and TrkB expression in time-dependent manner. Neurons were divided into four groups: C, F3, F6 and F12 groups. C referred to neurons exposed to air for 3 h; F3, neurons were exposed to 1MAC sevoflurane for 3 h with a total gas flow of 6 L/min; F6, neurons were exposed to 1 MAC sevoflurane for 6 h; F12, neurons were exposed to 1 MAC sevoflurane for 12 h. Proteins were isolated from four groups for Western blot. The relative density of band was calculated by β-actin with Quantity One software. $^{*}P<0.05$, $^{**}P<0.01$.

To explore the molecular basis of different drugs on cell cycle distribution, we examined the expression of BDNF, TrkB and CRMP-2. In Figure 3, compared with C group, the application of sevoflurane suppressed the expression of BDNF, TrkB and CRMP-2 (P<0.01), while DEX had no obvious effect on the protein levels of BDNF, TrkB and CRMP-2 (P>0.05). Compared with C group, the combination of sevoflurane and DEX partly blocked the expression of BDNF, TrkB and CRMP-2, and the effect of DEX was in concentration-dependent manner. Compared with F group, the combination of sevoflurane and DEX partly upregulated the expression of BDNF, TrkB and CRMP-2. The protein levels of BDNF, TrkB and CRMP-2 in Y1 group were lower than those in C group, higher than those in F group and partly lower than those in FD1 group. What's more, there was no significant difference among Y1, Y2 and Y3. Taken together, sevoflurane blocked the expression of BDNF, TrkB and CRMP-2, and the addition of DEX partly increased the protein levels of BDNF, TrkB and CRMP-2 reduced by sevoflurane, and the application of yohimbine partly blocked the function of DEX.

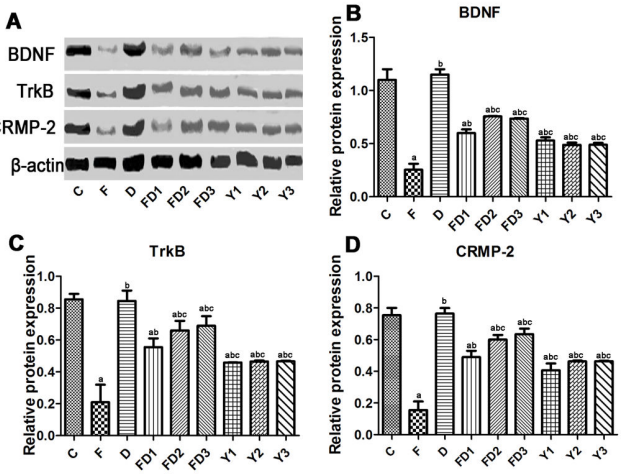

Figure 3. The addition of DEX relieved the inhibitory of the levels of BDNF, TrkB and CRMP-2 induced by sevoflurane. Neurons were randomly divided into 9 groups. C, neurons were treated with physiological saline and exposed to air; F, neurons were exposed to 1 MAC sevoflurane for 6 h using air as a carrier; D, neurons were incubated with 0.1 μM DEX for 12 h; Neurons were exposed to 1 MAC sevoflurane for 6 h and incubated with DEX at the concentration of 0.1 μM (FD1), 1 μM (FD2) or 10 μM (FD3) for 12 h; Neurons were pretreated with 2 μM of yohimbine for 30 min, exposed to 1MAC sevoflurane for 6 and incubated with DEX at the concentration of 0.1 μM (Y1), 1 μM (Y2) or 10 μM (Y3) for 12 h; Proteins were extracted for Western blot (A). The relative expression of BDNF (B), TrkB (C) and CRMP-2 (D) was normalized to β-actin. Compared with C group, $^{a}P<0.01$; Compared with F group, $^{b}P<0.01$; Compared with group FD1, $^{c}P<0.01$.

Table 1. Effects of sevoflurane on the cell cycle phase distribution in neurons in vitro.

Groups	Phases of cell cycle (%)			PI
	G_0/G_1	S	G_2/M	
C	85.7 ± 1.15	8.6 ± 0.89	5.6 ± 0.35	14.2 ± 1.09
F3	83.7 ± 0.93	9.9 ± 0.58	6.3 ± 0.52	17.1 ± 0.85
F6	66.4 ± 1.65**	29.2 ± 1.13**	4.6 ± 0.67	33.7 ± 2.31**
F12	54.1 ± 3.57**	44.3 ± 1.77**	4.8 ± 0.36	47.6 ± 1.86**

C: Control group, neurons were exposed to atmosphere; F3: neurons were exposed to 1 MAC sevoflurane for 3 h with a total gas flow of 6 L/min; F6: neurons were exposed to 1MAC sevoflurane for 6 h; F12: neurons were exposed to 1MAC sevoflurane for 12 h; G_0/G_1, S, and G2/M represented the fraction of cells in each phase of the cell cycle; PI: Proliferation Index; Compared with C group, $^{*}P<0.05$, $^{**}P<0.01$.

Table 2. Effects of sevoflurane and DEX on the cell cycle phase distribution in neurons in vitro.

Groups	Phases of cell cycle (%)			PI
	G_0/G_1	S	G_2/M	
C	85.10 ± 0.693	9.20 ± 0.601	5.70 ± 0.142	14.90 ± 0.693

F	66.70 ± 0.922[a]	27.43 ± 0.766[a]	4.87 ± 0.219	32.30 ± 1.01[a]
D	85.96 ± 0.417[b]	8.47 ± 0.161[b]	5.57 ± 0.268	14.03 ± 0.122[b]
FD1	77.66 ± 0.692[ab]	16.88 ± 0.291[ab]	5.46 ± 0.421	22.34 ± 0.728[ab]
FD2	81.26 ± 0.464[abc]	13.09 ± 0.671[abc]	5.65 ± 0.219	18.74 ± 0.473[abc]
FD3	81.77 ± 0.251[abc]	12.17 ± 0.417[abc]	6.06 ± 0.177	18.23 ± 0.262[abc]
Y1	71.79 ± 0.353[abc]	22.74 ± 0.284[abc]	5.46 ± 0.196	28.21 ± 0.348[abc]
Y2	71.68 ± 0.438[abc]	22.47 ± 0.359[abc]	5.84 ± 0.208	28.32 ± 0.433[abc]
Y3	72.44 ± 0.117[abc]	21.78 ± 0.061[abc]	5.79 ± 0.072	27.57 ± 0.115[abc]

C: Neurons were treated with physiological saline and exposed to atmosphere; F: neurons were exposed to 1 MAC sevoflurane for 6 h using air as a carrier; D: neurons were incubated with 0.1 μM DEX for 12 h; Neurons were exposed to 1 MAC sevoflurane for 6 h and incubated with DEX at the concentration of 0.1 μM (FD1), 1 μM (FD2) or 10 μM (FD3) for 12 h; Neurons were pretreated with 2 μM of yohimbine for 30 min, exposed to 1 MAC sevoflurane for 6 and incubated with DEX at the concentration of 0.1 μM (Y1), 1 μM (Y2) or 10 μM (Y3) for 12 h; G_0/G_1, S, and G_2/M represented the fraction of cells in each phase of the cell cycle; PI: Proliferation Index; Compared with C group, [a]P<0.01; Compared with F group, [b]P<0.01; Compared with group FD1, [c]P<0.01.

Discussion

Hippocampus is an important part of the brain tissue, and its structure and boundary are relatively clear, enabling easy dissection and separation. These advantages make hippocampus an ideal tissue for neural pharmacology experiments [13]. In our study, we isolated hippocampal neurons from SD rat born within 24 h and labeled with the early neuronal marker MAP-2 and the astrocytic marker GFAP [14]. Our immunocytochemistry results showed that the purity of neurons was >94%, suggesting that hippocampal neurons were successfully obtained for further experiments.

The potential for anesthetic neurotoxicity is the most important clinical and research problem in the field of pediatric anesthesiology [15]. Some studies observed the association between exposure general anesthesia to an infant and later neurobehavioral problems in childhood [16,17]. There is mixed evidence from cohort studies that young children exposed to anaesthesia can have an increased risk of poor neurodevelopmental outcome [18]. Sevoflurane was widely used in anaesthesia of children and infants for general anesthesia [1]. It was reported that neonatal exposure to sevoflurane induced neurobehavioral abnormalities and long-lasting alterations in histone acetylation [19]. In our study, neurons exposed to 1 MAC sevoflurane for 3 h had no significant effect on cell cycle distribution; however after 6 or 12 h of exposure, neurons cell cycle was blocked at S phase significantly, suggesting that sevoflurane suppressed neuron cell proliferation depending on exposure duration, which was in agreement with previous report [20,21]. DEX was a potential neuroprotectant agent in anesthetic neurotoxicity [22]. In our study, we found that the addition of DEX significantly mitigated the cell cycle arrest at S phase induced by sevoflurane exposure, and the pretreatment of a α2 adrenergic receptor blocker yohimbine suppressed the function of DEX. These data showed that DEX functioned as a neuroprotective drug *via* α2A-adrenergic receptors, which was similar to previous study [23].

BDNF is an important neurotrophin that is involved in synaptic plasticity including hippocampal-dependent learning and memory [24] and neurogenesis [12]. BDNF-TrkB signaling caused the phosphorylation of downstream kinase signaling cascade to activate phosphoinositide 3-kinase/protein kinase B for cell proliferation [25] and normal morphology of neurons [26] in the hippocampus. So it is widely accepted that BDNF/TrkB complex is critical for neuronal survival, growth and proliferation [27]. CRMP-2 was expressed in the central nervous system and involved in neurite growth and the formation of axons [28]. In our study, we found that sevoflurane exposure significantly repressed the protein levels of BDNF, TrkB and CRMP-2. However the addition of DEX mitigated the downregulation of BDNF, TrkB and CRMP-2 induced by sevoflurane, which was in agreement with the previous results that the neuroprotective effect of DEX was achieved by upregulating the expression of BDNF [13]. And the pretreatment of yohimbine blocked the function of DEX, suggesting that DEX functioned in α2A-adrenergic receptors-dependent manner.

Taken together, sevoflurane suppressed the primary neurons cell proliferation *via* BDNF-TrkB signaling. And DEX functioned as a neuroprotective drug by regulating the expression of BDNF and TrkB and *via* α2A-adrenergic receptors-mediated signaling pathway.

Competing Interests

The authors declare that they have no competing interests.

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