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Original Article

MicroRNA-181 inhibitor protects against sevoflurane-induced hippocampal apoptosis and memory impairment

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Abstract: We aimed to investigate whether miR-181 was associated with sevoflurane-induced neurodegeneration, as well as to elucidate the potential regulatory mechanism of miR-181 in hippocampus. We established a model by administering 2% sevoflurane into rats from 9:00 AM to 3:00 PM. We then conducted quantitative real-time polymerase chain reaction (qRT-PCR) to determine the expression of miR-181. We then used lentivirual tools to down-regulate the expression of miR-181 in hippocampus, and discovered the effect of miR-181 inhibition on sevoflurane-induced hippocampal function and apoptosis after sevoflurane exposure using Morris water maze and hippocampal terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) staining. Besides, the expression levels of PSD95, total CREB and p-CREB were determined by western blot. miR-181 was significantly upregulated in hippocampus following general anesthesia by sevoflrane. In addition, sevoflrane could induce hippocampal apoptosis and memory impairment, and miR-181 inhibition could alleviate these negative effects induced by sevoflrane to some extent. Besides, miR-181 inhibition increased the expression of PSD95 and p-CREB/CREB that was significant decreased after sevoflrane exposure in hippocampus. Our findings indicate that miR-181 negatively modulated sevoflrane-induced hippocampal apoptosis and memory impairment possibly through regulating the levels of PSD95 and p-CREB/CREB ratio. MiR-181 inhibitor may effectively protect against sevoflurane-induced hippocampal impairments.

Keywords: MicroRNA-181, sevoflurane, hippocampus, apoptosis, memory impairment

Introduction

Sevoflurane is one of the most common general anesthetics widely used in pediatric anesthesia [1]. However, emerging evidences have demonstrated that sevoflurane can cause neuronal apoptosis, reduce neurogenesis, induce neurodegeneration and lead to memory impairment in the developing brain [2-4]. Unfortunately, the molecular mechanisms involved in sevoflurane-induced hippocampal neurodegeneration or memory impairment are largely unknown to data. Therefore, a better understanding of the molecular mechanisms will help to improve sevoflurane-induced negative effects.

MicroRNAs (miRNAs) are shown to be abundantly expressed in brain and may play critical roles in many aspects of brain development, such as neurogenesis [5], axon regeneration [6] and neurodegeneration [7]. Several miRNAs have been reported to be involved in the development of central nervous system (CNS) and neurodegenerative disorders [8]. Recently, the essential roles of microRNA-181 (miR-181) in neuropathology have aroused more the more attention. Overexpression of miR-181 is shown to regulate the expression of c-Fos and SIRT-1 which are involved in synaptic plasticity, thus contributes to Alzheimer's disease (AD) neuropathology [9]. Delay et al. also demonstrated that miR-181 was an AD-specific miRNA to provide essential functions in this disease [10]. Moreover, miR-181 is reported to regulate neuronal vulnerability and neurogenesis in neuroinflammatory responses of astrocytes [11]. Besides, miR-181 is identified to function as a therapeutic target in neurodegenerative diseas-

es [12]. Notably, sevoflrane anesthesia can alter miRNA expression patterns in healthy rat liver [13]. However, whether the expression miR-181 can be altered by sevoflurane in rat hippocampus and the crucial role of miR-181 in sevoflurane-induced neurodegeneration and memory disability have not been fully investigated.

In the present study, we aimed to examine whether miR-181 was involved in the process of sevoflurane-induced neurodegeneration, as well as to elucidate the potential regulatory mechanism of miR-181 in hippocampus. To confirm whether sevoflrane anesthesia affected the expression of miR-181 in hippocampus, we conducted quantitative real-time polymerase chain reaction (qRT-PCR) on hippocampal tissues following general anesthesia induced by sevoflrane. We then used lentivirual tools to down-regulate the expression of miR-181 in hippocampus, and discovered the effect of miR-181 inhibition on sevoflurane-induced hippocampal function and apoptosis after sevoflurane exposure using Morris water maze and hippocampal terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) staining. Besides, the expression levels of neurodevelopmental proteins were determined by western blot to further explore the potential regulatory mechanism of miR-181 in hippocampus.

Materials and methods

Animals and grouping

This protocol was approved by the local Animal Research Committee. Male Sprague-Dawley (7-days-old) rats, weighing 250 ± 10 g, were acquired form Vital River Laboratory Animal Technology Co. Ltd. (Beijing, China). Before experiments, all rats were housed in an animal room under a controlled 12 h light-dark cycle with free access to food and water for 1-week. Afterwards, rats were randomly assigned into 2 groups: sevoflurane group and sham group (n = 8 each group). Rats in the sevoflurane group were administered 2% sevoflurane (AbbVie Inc., North Chicago, IL, USA) from 9:00 AM to 3:00 PM. Rats in sham group were kept in the same anesthesia box for 6 h and the tail vein of these rats was inserted a catheter to deliver normal saline at 1 mL/h during treatment. Animal care and treatment were conducted according to the standards for ethical treatment of laboratory animals as published by the National Institutes of Health.

Lentivirus production

Lentivirual conduction was used to inhibit miR-181 in hippocampus. The coding oligonucleotides of miR-181 inhibitor and scramble control were purchased from RiboBio (Shanghai, China) and then were cloned into Pcdh-CMV-MCS-EF1-copGFP (SBI, USA), a feline immunodeficiency virus (FIV) based lentivirus expression vector. Pcdh-CMV-MCS-EF1-copGFP was then co-expressed with pPACK packaging system in 293 TN cells to produce viral particles of miR-181 antisense inhibitor (miR181-I) and scramble RNA. Twenty-four hours before anesthesia, lentiviruses (miR181-I and scramble RNA) were injected to rat hippocampus on the right side of the brain. With the aid of a surgical microscope, total volume of 2 μL lentiviruses were injected from a tiny hole (0.05 mm in diameter) on the right cortex just above hippocampus into dorsal hippocampus (from anterio-posterior, -1.8 mm; medio-lateral, +2.4 mm; bregma: dorso-ventral, -2.0 mm) with a Hamilton syringe.

Morris water maze

Morris water maze was performed to evaluate hippocampal function 14 days after sevoflurane exposure. Each rat was putted into each of four quadrants in a pool (150 cm in diameter and 50 cm in height) which were colored with black ink. Then rats were trained to find a hidden circular platform (10 cm in diameter, 2 cm beneath the water). The time to locate the original platform was recorded to evaluate the memory ability of the rats. In addition, the average swimming speed to find the platform was recorded and analyzed with SLY-WMS 2.0 software (Beijing Sunny Instruments).

Hippocampal TUNEL staining

At 14 days after anesthetic exposures, rats in each group were sacrificed and hippocampal slices (300 μm) were prepared by a vibratone (Leica, Germany). TUNEL staining was performed using the TACS 2 TdT®-DAB In Situ Apoptosis Detection Kit (TREVIGEN®) to detect cell apoptosis. The principle of this assay is that DNA fragmentation resulting from apoptotic

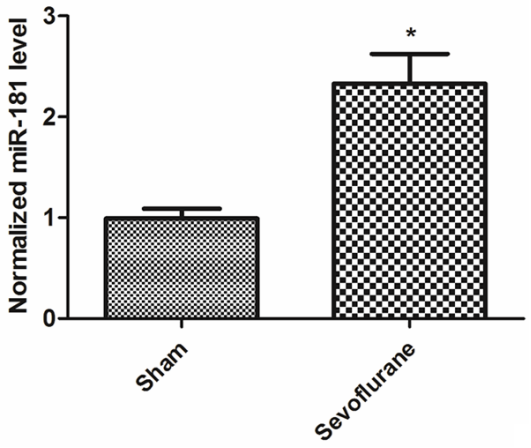

Figure 1. RT-PCR analysis displayed the expression of miR-181 in hippocampus. Error bars indicate means ± SD and *indicates significant difference compared with sham group ($P < 0.05$).

signaling cascades can be identified with terminal deoxynucleotidyl transferase. This enzyme can catalyze the addition of dUTPs which were labeled with a secondary antibody conjugated to horseradish peroxidase to generate a brown coloration when reacted with DAB. Finally, positive cells on 5 randomly selected horizons in 10 hippocampal slices of each rat were counted.

qRT-PCR analysis

Total RNA of hippocampus was firstly extracted using Trizol (Invitrogen, Carlsbad, CA) in accordance with manufacturer's protocols. The quality of the total RNA was assessed by an Agilent 2100 Bioanalyzer. Then cDNA was synthesized with the High-Capacity cDNA Reverse Transcription Kits (Applied Biosystems, USA). qRT-PCR was then performed with a SYBR Green PCR kit (Applied Biosystem, USA) according to the following reactions: 95°C 10 min; 40 cycles of 95°C 15 sec, 69°C 30 sec and 72°C 30 sec; 72°C 5 min. Endogenous U6 was used as internal standards to normalize the expression of miR-181. The relative expression of miR-181 was calculated using the $2^{-\Delta\Delta CT}$ method as previously described [14].

Western blot analysis

Hippocampal tissues were homogenized and the supernatants were collected by centrifuge. The protein concentration was then determined by bicinchorinic acid assay (BCA). Equal amounts of protein were separated via SDS-PAGE electrophoresis and then transblotted onto polyvinylidene difluoride membranes (MilliporeCorp., Bedford, MA). The membranes were blocked and then incubated with appropriate primary antibodies overnight at 4°C. Primary antibodies specific for postsynaptic density-95 (PSD-95), total cyclic adenosine monophosphate (cAMP)-response element binding protein (CREB), and phospho-CREB (p-CREB) were obtained from Cell Signaling Technology (Danvers, MA, USA). Afterwards, membranes were incubated with horseradish peroxidase-conjugated secondary antibodies at room temperature for 1 h. Immunoreactive blots were visualized using a chemiluminescence reaction. The expression levels of these proteins were normalized to β-actin and analyzed using Quantity One software (Bio-Rad Laboratories Inc., Hercules, CA, USA).

Statistics analyses

All collected measurement data were presented as the mean ± SD and were firstly analyzed for the normal distribution with one-sample K-S test. The statistical analyses were analyzed with t-test (for two groups) or one-way ANOVA (for more than three groups) using SPSS 20.0 (SPSS Inc., Chicago, IL, USA). Post-hoc Tukey test was used for comparisons between-groups. A value of $P < 0.05$ represents statistically significant.

Results

Analysis of the expression of miR-181 in hippocampus

RT-PCR analysis displayed the expression of miR-181 in hippocampus. As shown in **Figure 1**, the expression level of miR-181 in sevoflurane group was significantly increased compared with that in sham group ($P < 0.05$).

Inhibition of miR-181 improved the anesthesia-induced memory impairment

We used Morris water maze to determine the effect of miR-181 inhibition on hippocampal function 14 days after sevoflurane exposure. As shown in **Figure 2A**, the time to locate the original platform that rats in sevoflurane group used was markedly increased compared with sham group ($P < 0.05$). However, the time to locate the original platform that rats in miR-

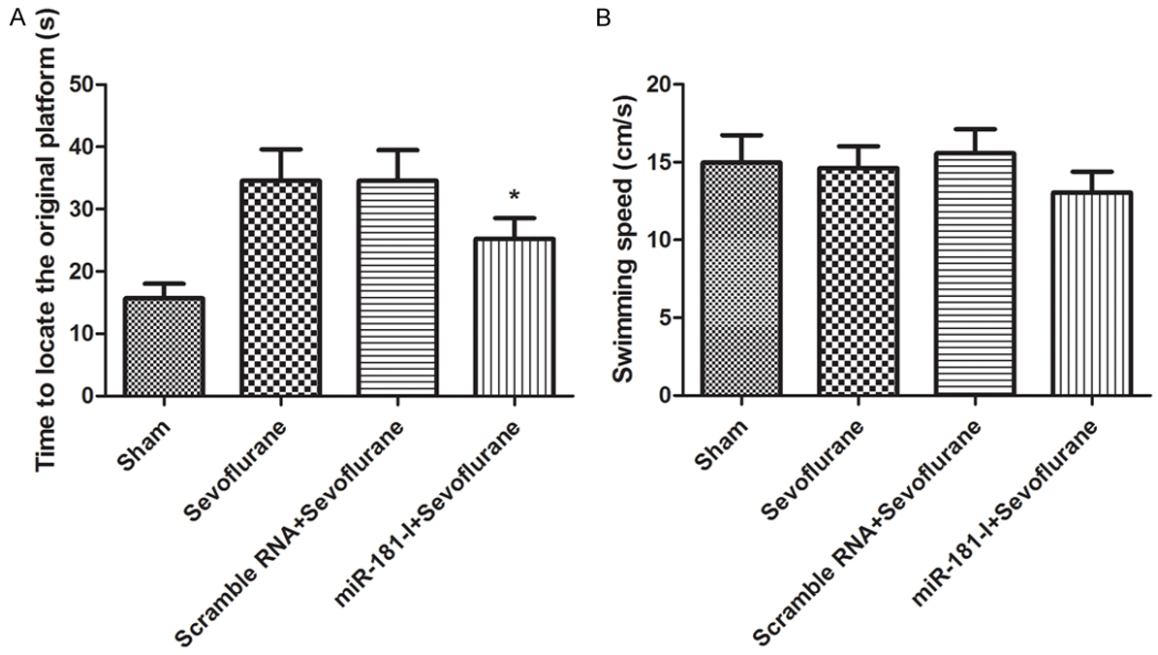

Figure 2. Morris water maze showed the effect of miR-181 inhibition on hippocampal function 14 days after sevoflurane exposure. A: The time to locate the original platform; B: Swimming speed of rats. Error bars indicate means ± SD and *indicates significant difference compared with sevoflurane group ($P < 0.05$).

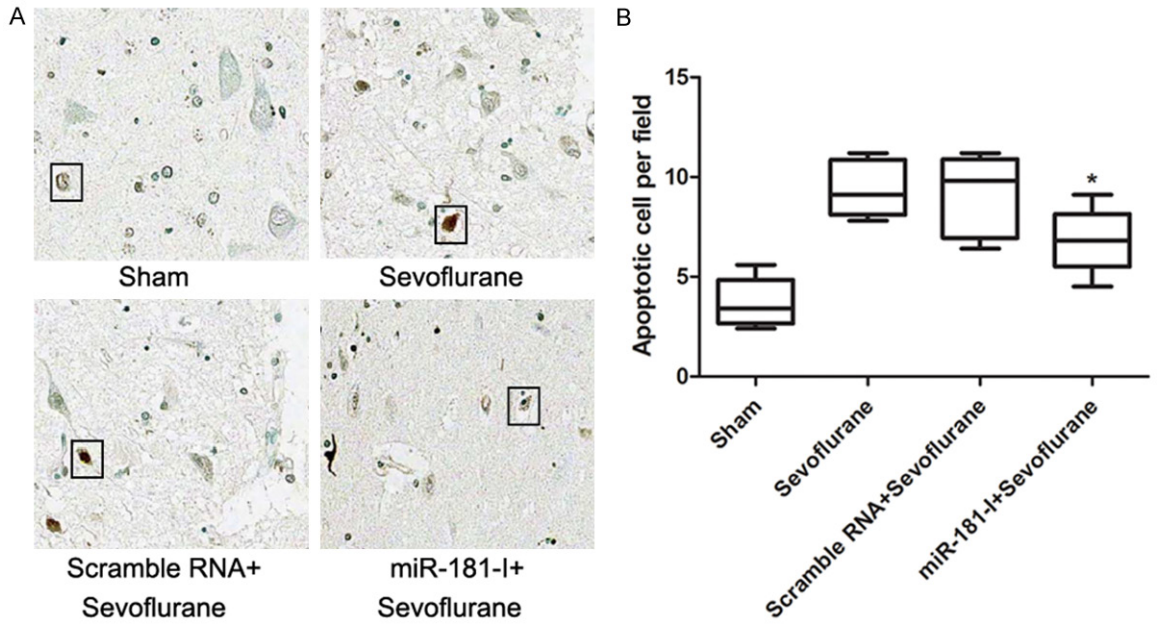

Figure 3. Hippocampal TUNEL staining displayed cell apoptosis 14 days after sevoflurane exposure. Error bars indicate means ± SD and *indicates significant difference compared with sevoflurane group ($P < 0.05$).

181-I + sevoflurane group used was significantly decreased compared with sevoflurane group ($P < 0.05$), indicating that miR-181 inhibition improved hippocampal memory impairment induced by sevoflurane to some extent. In addition, we also determine the effect of miR-181 inhibition on the swimming speed of rats at the same time (**Figure 2B**). The results showed that

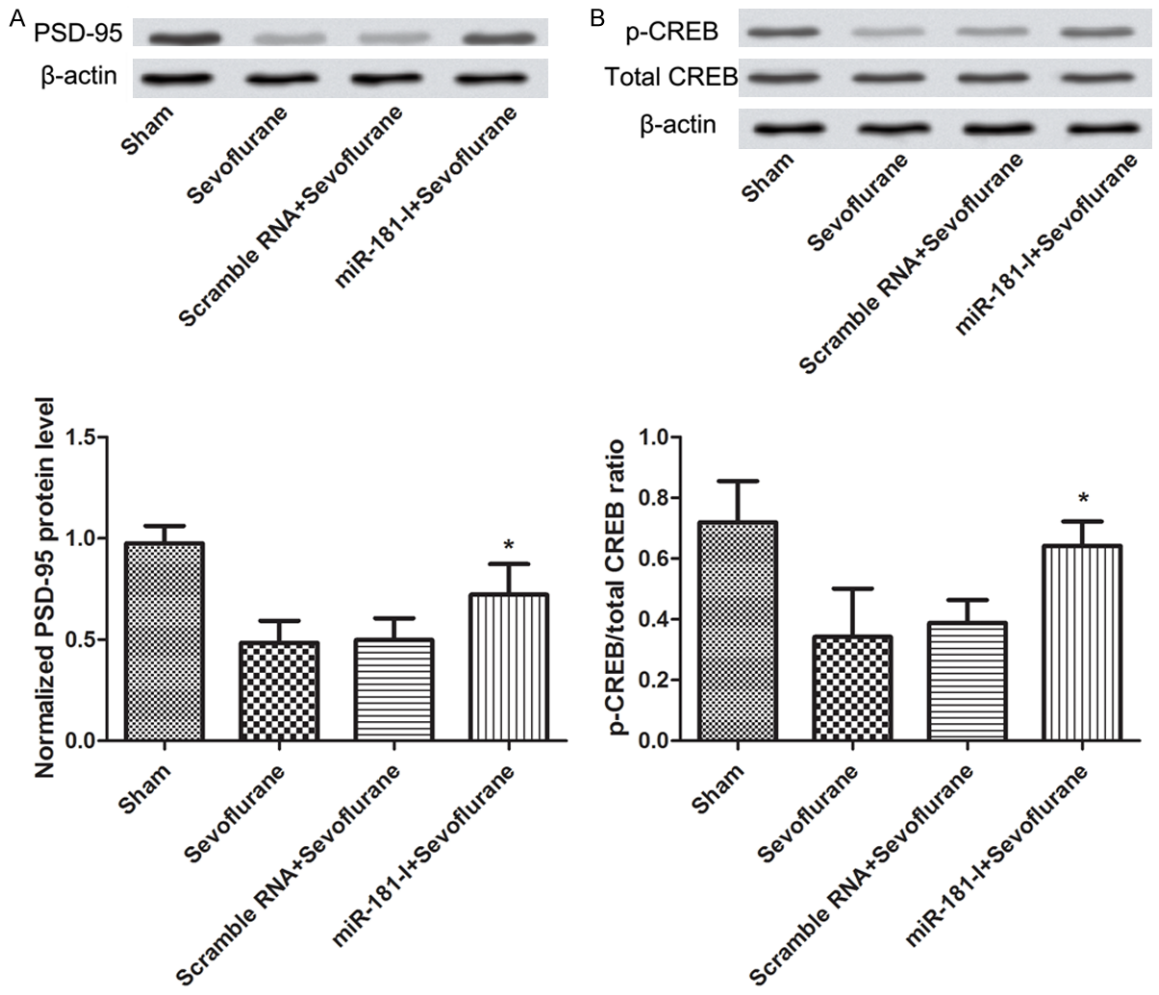

Figure 4. Western blot analysis displayed the expression of PSD95, total CREB and p-CREB. Error bars indicate means ± SD and *indicates significant difference compared with sevoflurane group ($P < 0.05$).

there were no significantly differences between groups in the average swimming speed.

Inhibition of miR-181 alleviated anesthesia-induced hippocampal apoptosis

Hippocampal TUNEL staining was used to analyze cell apoptosis after rats were treated with sevoflurane for 14 days (**Figure 3**). By counting the positive cells on 5 randomly selected horizons in hippocampal slices, we found that apoptotic cells of sevoflurane group were markedly increased compared with sham group. However, apoptotic cells of miR-181-I + sevoflurane group was significantly decreased compared with sevoflurane group ($P < 0.05$), indicating that miR-181 inhibition alleviated hippocampal apoptosis induced by sevoflurane to some extent.

Analysis the expression levels of neurodevelopmental proteins in hippocampus

To further explore the regulatory mechanism of miR-181 in hippocampus, the expression levels of neurodevelopmental proteins, such as PSD95, total CREB and p-CREB were determined by western blot (**Figure 4**). The results showed that the expression levels of PSD95 and p-CREB/CREB ratio in sevoflurane group were markedly decreased compare with sham group. However, the expression levels of PSD95 and p-CREB/CREB ratio in miR-181-I + sevoflurane group were obvious increased compare with sevoflurane group ($P < 0.05$), indicating that miR-181 inhibition could increase the expression levels of PSD95 and p-CREB/CREB ratio in hippocampus.

Discussion

General anesthetics are always considered to be effectively safe, but increasing studies confirm that these anesthetics can induce neurotoxicity or neurodegeneration in the developing brains. In our study, we found that miR-181 was significantly upregulated in hippocampus following general anesthesia by sevoflrane. In addition, sevoflrane could induce hippocampal memory impairment and apoptosis, and miR-181 inhibition could alleviate these negative effects induced by sevoflrane to some extent, indicating a protective role of miR-181 inhibitor in brain development against apoptosis or neurodegeneration.

Previous study has shown that miR-181 is involved in neuroinflammatory responses of astrocytes [11]. A evidence also demonstrated that miR181 could target multiple Bcl-2 family members and subsequently influence apoptosis in astrocytes [15]. Moreover, miR-181 can function as tumor-suppressor that commonly down-regulated and induced apoptosis in tumor cells [16, 17]. In addition, inhibition of miR-181 is proved to reduce forebrain ischemia-induced neuronal loss [18]. miR-181 is identified as a brain-specific miRNA associated with neural plasticity and memory and may contribute to the impaired cognitive function [19]. Thus, the results of our experiment are in line with previous findings and suggest that miR-181 inhibition could protect against hippocampal apoptosis or memory impairment after sevoflrane anesthesia.

To further explore the potential regulatory mechanism of miR-181 in hippocampus after sevoflrane anesthesia, we also determined the effect of miR-181 inhibitor on the expression of neurodevelopmental proteins in hippocampus by western blot. The results showed miR-181 inhibition could increase the expression of PSD95 and p-CREB/CREB that were significant decreased after sevoflrane exposure in hippocampus, suggesting that miR-181 inhibitor may play a neuroprotective role in hippocampus via regulating the levels of PSD95 and p-CREB/CREB ratio after sevoflrane anesthesia.

PSD-95, a membrane-associated guanylate kinase (MAGUK), is shown to be concentrated at glutamatergic synapses [20, 21]. PSD-95 as a synaptic protein can play important roles in regulating glutamate receptor anchoring, synaptic stability and certain types of memory [22]. Wang et al. confirmed that decreased PSD-95 in the hippocampus might be responsible for sevoflurane anesthesia induced long-term memory impairment and hippocampal apoptosis in the developing brain [23]. PSD-95 is also reported to be involved in the Iodine deficiency induced hippocampal apoptosis [24]. In addition, PSD-95 can control N-methyl-D-aspartate glutamate (NMDA) receptor clustering, thus to play a key role in synapse stabilization and plasticity [25, 26]. Moreover, expression profiling following induction of neuronal activity demonstrates that miR-181 expression is altered after NMDA receptor-dependent synapse plasticity [27], implying the potential link between PSD-95 and miR-181 in synapse plasticity. In our study, we found that miR-181 inhibition obvious increased hippocampal PSD-95 expression, which were generally in accordance with previous findings and indicated that inhibition of miR-181 might improve sevoflrane-induced hippocampal apoptosis and memory impairment via regulating PSD-95 expression.

CREB and p-CREB are implicated in the formation of hippocampal long-term potentiation (LTP), synaptic plasticity, and memory [28]. It has been suggested that NMDAR overactivation-mediated ERK/CREB dysregulation may be an important mechanism involved in chronic intermittent hypoxia (CIH)-induced neurocognitive impairments and hippocampal apoptosis [29] Shaerzadeh et al. also found that p-CREB/CREB ratio was observed to significantly decrease in hippocampus neurons, implying the crucial roles of p-CREB/CREB in neuronal loss [30]. Furthermore, miR-181a is shown to involved in insulin-like growth factor-1 (IGF-1)-mediated CREB1 regulation [31]. In our study, increased p-CREB/CREB ratio was also observed in miR-181-I + sevoflurane group compare with sevoflurane group. It is thus intriguing to speculate that inhibition of miR-181 might improve sevoflrane-induced hippocampal apoptosis and memory impairment via regulating p-CREB/CREB ratio.

Taken together, our study implies a potential mechanism of miR-181 negatively regulating sevoflrane-induced hippocampal apoptosis and memory impairment, and its roles in hippocampus is likely to be played through regulating the levels of PSD95 and p-CREB/CREB

ratio. MiR-181 inhibitor may effectively protect against sevoflurane-induced hippocampal impairments.

Disclosure of conflict of interest

None.

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