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Original Research Article

Up-regulation of miR-20a ameliorates sevoflurane anesthesia-induced cognitive impairment in rats by targeting EphA4

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Abstract

Purpose: *To evaluate the role of miR-20a in sevoflurane (SEV)-induced cognitive impairment in rats and to elucidate the mechanism of action.*
Methods: *A SEV-induced cognitive impairment rat model was developed. The Morris water maze test and fear assay were carried out to assess impaired learning and memory. A cellular SEV-impaired model was developed and the miR-20a level was measured in the animal and cellular models. TUNEL staining and immunoblot assay were performed to determine the SEV effect on apoptosis. Bioinformatic analysis and luciferase assay were conducted to identify the target of miR-20a action. A rescue assay involving miR-20a overexpression in cellular and animal models was developed and used to evaluate function of miR-20a in cognitive defects.*
Results: *The rats showed significant cognitive impairment upon SEV treatment, which inhibited the expression of miR-20a and promoted neuronal apoptosis. Further findings identified EphA4 as a target of miR-20a, which regulates its expression. Overexpression of miR-20a in rats effectively reduced cognitive dysfunction and apoptosis of hippocampus somatic cells caused by SEV treatment.*
Conclusion: *Evidently, miR-20a ameliorates SEV anesthesia-induced cognitive impairment in rats and thus has the potential to serve as a therapeutic target for the treatment of post-operative cognitive dysfunction.*

Keywords: *Postoperative cognitive dysfunction (POCD), miR-20a, Sevoflurane, EphA4, Apoptosis*

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INTRODUCTION

Postoperative cognitive dysfunction (POCD) is a common complication after major surgery that is mainly characterized by mental disorder, anxiety, personality change, and impaired memory [1]. These symptoms can last for days or weeks, but in severe cases, they can last for several months [2]. The occurrence of POCD is closely related to the anesthesia used during the operation [3]. POCD was first proposed by Bedford in 1955 and was defined as "the brain damage caused by anesthesia in the elderly" [4]. The precise pathogenesis of POCD is still unknown today.

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Sevoflurane (SEV) inhalation anesthesia has been used widely in recent years [5]. Increased attention has been paid to studying the effects of SEV inhalation on learning and memory impairment [6]. In recent years, it has been shown that SEV causes obvious memory damage in animals [7]. Although SEV has a significant effect on embryonic brain development, the mechanism is still unclear. Studies have shown that exposure to SEV during pregnancy can lead to impaired learning and memory in offspring and that this effect may be related to the concentration and duration of inhalation of SEV [8].

MicroRNAs (miRNAs) are a class of small non-coding RNAs that can bind to the 3'-untranslated region (3'-UTR) of the transcript of their homologous target genes, thereby regulating their expression [9]. Extensive studies have confirmed a broad association between miRNAs and anesthesia-related cognitive impairment [9-11]; miRNAs, such as miR-665, miR-572, and miR-181, have been reported to affect POCD progression, suggesting that these miRNAs could serve as promising therapeutic targets for treating POCD [12].

EXPERIMENTAL

SEV-treated rat model

All animal experiments in this study were approved by the Ethics Committee of Affiliated Hospital of North Sichuan Medical College (Approval no.2019096) and conducted in accordance with the National Institutes of Health Laboratory Animal Care and Use Guidelines [13]. Male Sprague Dawley rats (250 ± 10 g, 7 weeks old, purchased from Vital River) were housed with free access to water and food. Then the rats were randomly divided into four groups: the sham group with normal air for 6 h (n = 10), the SEV group with 2.5% SEV at 600 μg/kg/min in 100% O_2 for 6 h (n = 10), the negative control (NC) agomir group with caudal vein injection of 1 nmol miR-20a NC packed in liposomes, and the miR-20a agomir group with caudal vein injection of 1 nmol miR-20a agomir (synthesized by GenePharma, China). The miRNA agomir was packed into liposomes and injected every 3 weeks for 15 weeks. Then the rats were allowed to recover for 7 days before further experimentation.

Morris water maze test

To perform the Morris water maze (MWM) test, rats were forced to swim and locate a hidden platform in a rectangular channel. Rats were initially placed on the platform to become familiar with its location in the channel. One day later, the rectangular maze was changed into a circular water maze that was 1.0 cm beneath the surface of the water. During the experiment, rats were placed in different locations in the water. All rats were allowed to remain in the water for 70 s at most. If the rats failed, they were allowed to adjust to the surroundings by being placed in the quadrant opposite the target quadrant. Thereafter, the rats were trained to find a visible platform. Rats were trained four times each day. Each trial had a different platform and starting location. The escape latency times for each rat was recorded.

Fear conditioning

Each animal was placed in a chamber that smelled of 70% alcohol and subjected to three tone-foot shock pairings (tone: 2000 Hz, 85 db, 30 s; foot shock: 1 mA, 2 s) with an inter-trial time of 1 min in a slightly darkened room. At 30 s after the conditioning training, the animal was removed from this test chamber. Then 24 h later, the rats were returned to the chamber for 8 min. The amount of time the animal exhibited freezing behavior was recorded in 8 s intervals. Then the animal was placed in a second chamber that was wiped with 1% acetic acid to establish a different context and smell from the first test chamber. Freezing behavior was noted for 3 min. The auditory stimulus was applied for three cycles, each of which consisted of 30 sec of stimulus followed by a 1 min interval. Freezing behavior was recorded for 5 min.

Quantitative PCR assay

This experiment was performed as described previously [14]. Briefly, total RNA was extracted from rat hippocampus neuronal cells with the miRNeasy Mini Kit (Qiagen, Dusseldorf, Germany). Then, RNA was reverse-transcribed into cDNA using M-MLV reverse transcriptase with specific reverse transcription (RT) primers. Then, SYBR Green PCR Master Mix (Applied Biosystems, Thermo Fisher Scientific, USA) was used to measure the expression levels of miR-20a. U6 was used as an internal control for miR-20a. The RT primers used are listed in Table 1.

Cell culture and cell transfection

The HT22 mouse hippocampal neuronal cell line (ATCC, Rockville, MD, USA) was incubated in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 15% fetal bovine serum (Invitrogen, Shanghai, China).

Table 1: Sequences of RT primers used in quantitative PCR assay

Gene	Forward primers (5'-3')	Reverse primers (5'-3')
miR-20a	GCCCGCTAAAGTGCTTATAGTG	CCAGTGCAGGGTCCGAGGT
U6	TGCGGGTGCTCGCTTCGGCAGC	CCAGTGCAGGGTCCGAGGT

HT22 cell exposure to SEV was performed in a gas-tight chamber at 37°C, and 1% 2T and 5% SEV were administered respectively by a SEV-specific vaporizer (Yu Yan, China).

The miR-20a inhibitor (miR-20a inhibitor) and its negative control (miR inhibitor-NC), as well as the miR-20a mimic (miR mimic) and its control (miR mimic-NC), were synthesized by GenePharma (Shanghai, China). HT22 cells were transfected with the oligonucleotides above using Lipofectamine 2000 reagent (Thermo Fisher Scientific).

TUNEL staining

Cells were fixed with 4% paraformaldehyde. After washing three times with PBS, the cells were stained with the Cell Death Detection Kit (Sigma-Aldrich, St. Louis, MO, USA). Nuclei were stained with DAPI. The TUNEL-stained images were captured using a Nikon Labophot 2 microscope.

Immunoblot analysis

Cell lysate was harvested using lysis buffer containing 50 mM Tris (pH 7.4), 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, and 0.1% SDS. Protein samples were resolved by SDS-PAGE and transferred onto polyvinylidene fluoride membranes. Then the membranes were blocked with 5% BSA for 1 h. Subsequently, primary antibodies against Bax (1:1000, 2774; Cell Signaling Technology [CST], Danvers, MA, USA), Bcl-2 (1:1000, 3498; CST), cleaved-caspase-3 (1:1000, 9661; CST), EphA4 (1:1000, 8793; CST), and β-actin (1:1000, 4970; CST) were incubated with the membrane overnight at 4°C. Then the membranes were incubated with secondary antibody conjugated with horseradish peroxidase (1:1000, 7074; CST). Protein bands were detected using a chemical luminescence reagent (Pierce, Rockford, IL, USA).

Luciferase reporter assay

The 3'-UTR region of the *EphA4* gene was amplified using PCR and cloned into the pMIR-REPORT Luciferase vector (Ambion, Thermo Fisher Scientific). The EphA4-MUT reporter was constructed using a Site-Directed Mutagenesis Kit. Then, HEK293 cells were transfected with the pMIR-REPORT vector, EphA4-WT or EphA4-MUT reporter, and the miR-20a mimic, mimic-NC, miR inhibitor-NC or miR-20a inhibitor. After transfection, luciferase activity was measured.

Statistical analysis

Data were analyzed using SPSS 13.0 (SPSS Inc., Chicago, Illinois, USA). All data are presented as the mean ± standard deviation (SD) (n = 3). The Student's t-test and analysis of variance (ANOVA) were performed to assess statistical significance, which was defined as $p < 0.05$.

RESULTS

SEV-induced memory impairment inhibits miR-20a level in rat hippocampus

To determine the memory and spatial learning ability of rats following SEV administration, the Barnes maze test and fear conditioning assay were conducted. As shown in Figure 1 A, rats in the SEV group exhibited impaired cognitive ability. Simultaneously, SEV-exposed rats displayed reduced freezing behavior when compared with control rats in the contextual fear conditioning test. Freezing behavior between the SEV-treated and control rats differed minimally during the tone fear conditioning test (Figure 1 B). The miRNA-20 level was also monitored in the two groups. Interestingly, we noticed a reduced miR-20a level in SEV-treated rats (Figure 1 C), suggesting that SEV treatment might suppress miR-20a level in the hippocampus of rats.

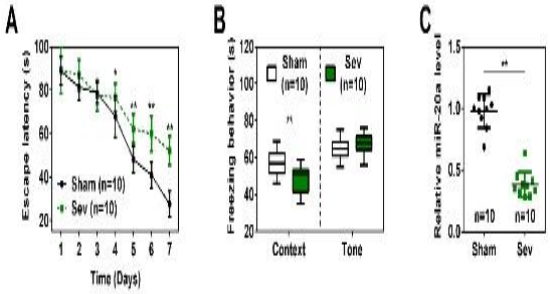

Figure 1: SEV-induced memory impairment inhibits miR-20a level in hippocampus of rats. (A) The escape latency in sham or SEV-induced rats. (B) The freezing behavior in sham or SEV-induced rats. *$P < 0.05$, **$p < 0.01$ compared with the control group

SEV promotes neuronal apoptosis and inhibits miR-20a *in vitro*

HT22 cells exposed to varying concentrations of SEV were subjected to TUNEL staining to assess apoptosis. SEV increased the TUNEL-positive cell number in a dose-dependent manner (Figure 2 A). Meanwhile, SEV treatment upregulated the protein level of Bax-2 and cleaved Caspase-3 and inhibited Bcl-2 in a dose-dependent manner (Figure 2 B). Similarly, miR-20a was decreased by SEV exposure (Figure 2 C). These data suggest that SEV induces neuronal apoptosis *in vitro*.

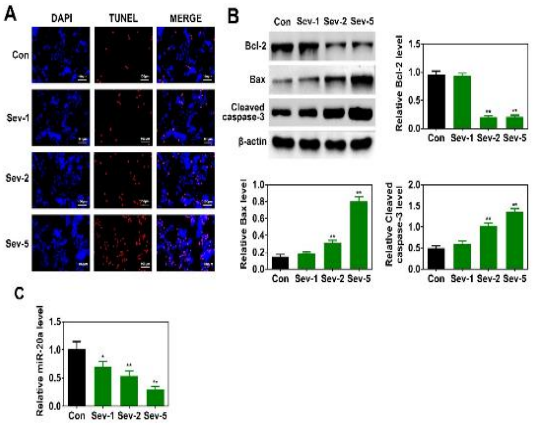

Figure 2: SEV promotes neuronal apoptosis and inhibits miR-20a *in vitro*. (A) TUNEL staining of HT22 cells increased with SEV concentration. (B) The level of apoptosis-related proteins was examined in SEV-treated cells by immunoblot. (C) qPCR assay measured the relative miR-20a level in HT22 cells. **P* < 0.05, ***p* < 0.01 compared with the control group

MiR-20a overexpression suppresses SEV-induced neuronal apoptosis

To unveil the function of miR-20a in SEV-induced cell apoptosis, we examined the effect of miR-20a overexpression on HT22 cells. First, the efficacy of transfection of the miR-20a mimic was confirmed by qPCR. Results revealed that miR-20a levels increased after transfection (Figure 3 A). Then apoptosis was examined in cells exposed to 5 % SEV. The data demonstrate that increased apoptosis in SEV-treated cells was suppressed by miR-20a transfection, suggesting the protective effect of miR-20a in neurons (Figure 3 B). Moreover, we assessed the level of apoptosis-related proteins in the different groups. The elevated expression level of Bax and cleaved Caspase-3 induced by SEV were inhibited by miR-20a transfection (Figure 3 C), further confirming that miR-20a suppressed SEV-induced apoptosis.

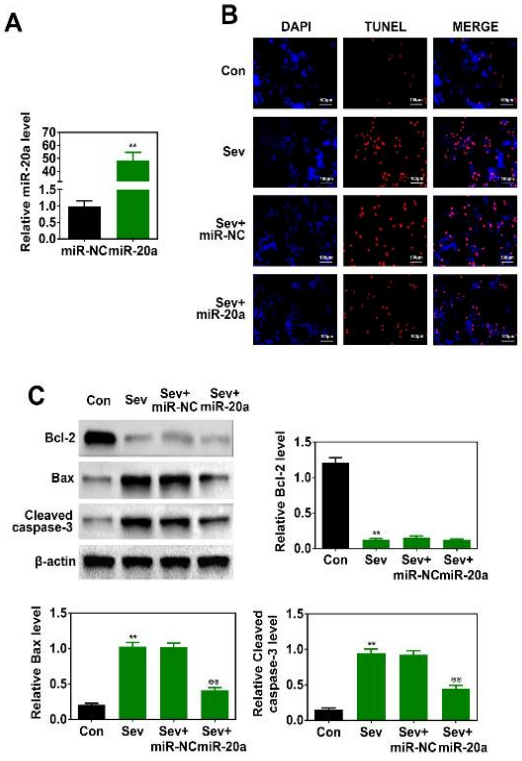

Figure 3: MiR-20a overexpression suppresses SEV-induced neuronal apoptosis. (A) Relative miR-20a levels in control or miR-20a-transfected cells were measured by qPCR. (B) TUNEL assay of SEV-treated HT22 cells in the presence of miR-20a or miR-NC overexpression. (C) Apoptosis-related protein level was assessed in SEV-treated or -untreated cells overexpressing miR-20a or miR-NC

MiR-20a targets *EphA4* to suppress its expression

We screened the Targetscan database (http://www.targetscan.org/) to identify the potential target of miR-20a. A 3'-UTR of *EphA4* was found to be complementary to the miR-20a sequence (Figure 4 A). To verify the regulatory relationship, luciferase assays were performed. The *EphA* luciferase activity was inhibited or elevated in miR-20a mimic- or miR-20a inhibitor-transfected cells, respectively, indicating that miR-20a targets *EphA4* (Figure 4 B). *EphA4* mutant luciferase activity remained unchanged in the miR-20a mimic- or miR-20a inhibitor-transfected groups (Figure 4B). The level of EphA4 protein was elevated or inhibited by miR-20a inhibitor or miR-20a mimic, respectively (Figure 4 C). Moreover, EphA4 protein level was induced in SEV-treated rat hippocampus (Figure 4D). Taken together, these data suggest that miR-20a regulated *EphA4* expression.

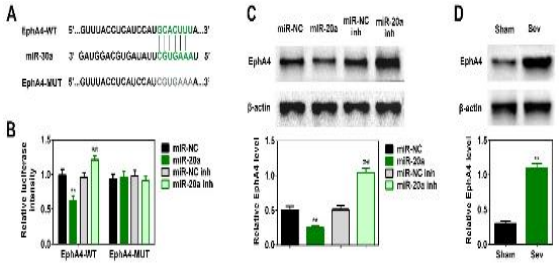

Figure 4: MiR-20a targets *EphA4* to suppress its expression. (A) The predicted binding nucleotides of miR-20a and *EphA4*, along with the mutant sequence of predicted binding nucleotides in *EphA4*. (B) Luciferase assay was performed to measure the EphA4 (WT) or EphA4 (MUT) reporter activity following transfection of the indicated miR-20a mimics or miR-20a inhibitor. (C) EphA4 protein level in cells transfected with the indicated miR-20a mimics or miR-20a inhibitor. (D) EphA4 protein level in sham or SEV-treated rat hippocampus; $^{**}p < 0.01$ compared with the miR-NC group; $^{@@}p < 0.01$ compared with the miR-NC inhibitor group

MiR-20a overexpression reverses SEV-induced cognitive impairment

Since miR-20a overexpression could reverse apoptosis induced by SEV, we investigated whether the cognitive defects induced by SEV could be improved by miR-20a. SEV-treated rats were subjected to miR-20a agomir or NC agomir transfection followed by the Barnes maze test and fear conditioning assay. miR-20a agomir injection reversed memory impairment and learning ability induced by SEV (Figure 5 A). The decreased freezing behavior in SEV-exposed rats was also reversed by miR-20a injection in the contextual fear conditioning test. However, the freezing behavior changed only minimally among the different groups during the tone fear conditioning test (Figure 5 B). In addition, EphA4 was induced by SEV exposure and was decreased by miR-20a (Figure 5 C).

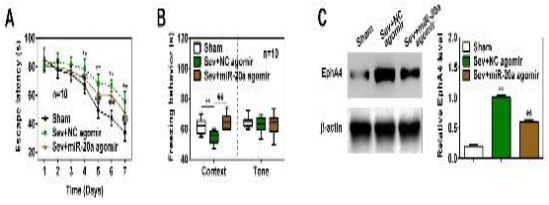

Figure 5: MiR-20a overexpression alleviates SEV-induced cognitive impairment. (A) Escape latency values in sham, SEV-induced, or miR-20a agomir-injected SEV-treated rats. (B) Freezing behavior in sham, SEV-induced, or miR-20a agomir-injected SEV-treated rats. (C) EphA4 protein levels in sham, SEV-induced, or miR-20a agomir-injected SEV-treated rats. $^{**}P < 0.01$ versus sham group; $^{@@}p < 0.01$ compared with the SEV-treated group

DISCUSSION

POCD is a common condition that is more likely to occur in the elderly, with a morbidity of approximately 10 – 60 % [15]. Anesthetic agents affect the central nervous system, and preoperative agents have been associated with postoperative psychiatric disorders [16]. Even though some studies have shown that, after SEV sevoflurane administration, patients have a lower degree of cognitive decline and a faster recovery time, understanding how SEV causes cognitive impairment is still worth further study [17]. In this study, a SEV anesthesia rat model of POCD was developed to simulate cognitive impairment, and our results demonstrate that miR-20a upregulation ameliorates SEV-induced cognitive impairment. These experimental results suggest a potential treatment for POCD.

Using Morris water maze and conditional fear experiments, we found that SEV-treated rats exhibit significant cognitive impairment and miR-20a downregulation. SEV also promoted neuronal apoptosis as evidence by the TUNEL assay. These experiments confirm the effects of SEV on neurons and cognition, as well as demonstrate a correlation between SEV and miR-20a expression.

miR-20a is a critical miRNA involved in multiple cellular functions [18]. miR-20a promotes kidney injury in sepsis rats through autophagy, and regulates fibroblast-like synoviocyte proliferation and apoptosis in rheumatoid arthritis [19]. After SEV treatment, the expression of miR-20a was downregulated in the hippocampus of newborn rats and lungs of adult rats [20]. Importantly, miR-20a has been reported to affect cognitive function [20]. After nerve injury in rats, miR-20a levels were downregulated. Lastly, overexpression of miR-20a reversed sensory function. Therefore, it can be inferred that miR-20a may play a role in SEV-induced cognitive impairment [21]. In this study, miR-20a expression was found to be decreased upon SEV treatment, and overexpression of miR-20a in rats effectively reduced the cognitive dysfunction and apoptosis of hippocampus somatic cells caused by SEV treatment. These findings further confirm the key role of miR-20a in cognitive impairment regulation.

EphA4 plays a very important role in regulating neuronal function [22]. EphA4 has been shown to be required for neural circuits controlling skilled reaching, and inhibition of the EphA4 pathway blocked trigeminal neuropathic pain [23]. EphA4 interacts with vascular endothelial growth factor receptor 2 in the neural stem and affects

progenitor cell differentiation [24]. A previous study demonstrated that EphA4 expression was upregulated in the brain with nerve injury, and that depletion of EphA4 could improve the injury [25]. Here, we found that EphA4 is regulated by miR-20a, and therefore may regulate the progression of POCD. These data provide evidence that EphA4 may serve as a target for POCD treatment.

CONCLUSION

Rats showed cognitive impairment upon SEV treatment, which also decreased miR-20a expression and promoted neuronal apoptosis. miR-20a overexpression inhibited the apoptosis of neurons. miR-20a targets *EphA4* and regulates its expression. Overexpression of miR-20a in rats reduces the cognitive dysfunction and apoptosis of hippocampus somatic cells caused by SEV treatment. Thus, these findings suggest that miR-20a is a potential therapeutic target for POCD treatment.

DECLARATIONS

Conflict of interest

No conflict of interest is associated with this work.

Contribution of authors

We declare that this work was performed by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Duo Qian designed the study and supervised the data collection. Changlin Chen and Biqian Dong analyzed and interpreted the data. Yu Qiu prepared the manuscript for publication and reviewed the draft of the manuscript. All authors have read and approved the manuscript.

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