MiR-124 protects against cognitive dysfunction induced by sevoflurane anesthesia in vivo and in vitro through targeting calpain small subunit 1 via NF-κB signaling pathway

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Conflict of interest
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Abstract

Background. Postoperative cognitive dysfunction (POCD) is an impairment of cognition that affects post-surgery patients. Sevoflurane anesthesia is linked to cognitive dysfunction correlated to the expression of miRNA levels.

Objectives. In the current study, we investigated if miR-124 can offer protection against cognitive deficits induced by sevoflurane in a spatial learning paradigm, and examined the molecular mechanisms through cell cultures.

Materials and methods. Escape latency, platform crossings in probe trials and swimming speed in the Morris water maze in sevoflurane-treated mice were utilized as a measure of cognitive function. The relative miR-124 expression, and mRNA expressions of Bax, caspase-3 and Bcl-2 in sevoflurane-treated hippocampal cultures were measured using real-time quantitative polymerase chain reaction (RT-qPCR). Moreover, the changes in interleukin (IL)-1β, tumor necrosis factor alpha (TNF-α) and IL-6 were determined using enzyme-linked immunosorbent assay (ELISA). The binding between miR-124 and calpain small subunit 1 (Capn4) was verified with site-directed mutagenesis. The involvement of the nuclear factor kappa B (NF-κB) signaling pathway was examined using western blot analysis.

Results. Our findings indicated that the miR-124 expression was inhibited by sevoflurane treatment in live rats and mouse hippocampal neurons to prevent apoptosis and inflammatory responses. We confirmed Capn4 as a target of miR-124. Treatment with sevoflurane enhanced the expression of Capn4, while overexpression of miR124 suppressed the enhanced expression of Capn4. Also, miR-124 inhibited apoptosis in murine hippocampal neurons induced by sevoflurane via the NF-κB signaling pathway.

Conclusions. Our findings demonstrated that miR-124 exerted its neuroprotective role against sevoflurane via targeting Capn4 and NF-κB signaling pathways. Our work may provide a novel and efficacious treatment for sevoflurane anesthesia-related cognitive dysfunction.

Key words: sevoflurane, NF-κB signaling pathway, miR-124, postoperative cognitive dysfunction, Capn4

Background

Postoperative cognitive dysfunction (POCD) is a syndrome characterized by a reduction in cognitive abilities tested before and after surgery, and is associated with high mortality and morbidity.[1,2] The morbidity caused by POCD is more severe in people above 60 years of age.[3,4] Evidence showed that neuroinflammation exerts a vital role in the prognosis of POCD via facilitating the infiltration of macrophages into the brain, compromising the blood–brain barrier, and damaging neurons and synapses.[5] Another study revealed that sevoflurane anesthesia reduces neurogenesis and neuronal survival in the hippocampus related to cognitive dysfunction.[6] A recent study showed that sevoflurane treatment leads to the reduction of cell survival and apoptosis as well as inflammation in cultured hippocampal neurons.[7] Therefore, sevoflurane anesthesia may serve as a good model for the development of POCD by elevating the apoptosis and inflammatory responses.[8] Given the impact of POCD on patients' quality of life and its demonstrated neurotoxic effects in vitro, there is an unmet need for an efficient neuroprotective agent for the prevention of POCD. MicroRNAs (miRNAs) are a group of small RNAs consisting of 18–22 nucleotides, involved in regulating gene transcription through binding to 3'-UTR of their targets.[9] Previous research suggests that miRNA regulates cognition, plasticity and synaptic transmission. For example, miR-134 overexpression decreases the size of dendritic spines in the hippocampus.[10–12] Similarly, miR-124 plays a vital role in the regulation of synaptic facilitation induced by serotonin at the sensory-motor synapse, and the overexpression of miR-124 reduced the expression of *CREB*, which is a gene related to synaptic plasticity.[13] Emerging evidence suggests that miR-9, miR-124 and miR-29a/b-1 are dysregulated and enhanced the production of Aβ in the brains of patients with Alzheimer's disease (AD).[14,15] MiR-124 is also preferentially expressed in the central nervous system (CNS), up to 100-fold in neurons compared to other organs in the rat.[16] It was also found to regulate cholinergic anti-inflammatory action via decreasing the release of inflammatory mediators such as tumor necrosis factor alpha (TNF-α) and interleukin (IL)-6.[17] It acted as an anti-apoptotic regulator and exerted its neuroprotective effect towards ischemic injury by suppressing the apoptosis of neurons in the cerebral ischemic stroke.[18] However, the role of miR-124 on sevoflurane anesthesia-induced cognitive dysfunction has not been elucidated.

Nuclear factor kappa B (NF-κB) is a nuclear transcription factor that contributes to various cellular processes, and its activation is closely related to the stimulation of cytokines.[19] It can be activated by several neurotrophic factors and induces the activation of genes associated with differentiation, growth, activation of immune cells, and survival involving apoptotic factor Bax and inhibition of apoptosis factor Bcl-2.[20] Another study showed that NF-κB activation is responsible for modulating inflammatory mediators, including IL-6, TNF-α and IL-1β, in inflammatory lung injury.[21] Recent research revealed that inflammation is the key factor in the regulation of brain injury pathology. Therefore, NF-κB expressions might be critical during the regulation of cognitive dysfunction.[22] However, the exact mechanism by which NF-κB exerts its effect in the progression of cognitive dysfunction induced by sevoflurane anesthesia is unknown.

Objectives

In this study, in vitro and in vivo models of POCD was implemented with sevoflurane treatment. The impact of miR-124 was investigated in its role as a neuroprotectant to POCD.

Materials and methods

Animals

Sixteen male Sprague Dawley rats (18-month-old) were obtained from the Experimental Animal Center of Hebei Medical University (Shijiazhuang, China) and separated into 2 groups (8 rats in control group and 8 in sevoflurane group). The control group rats were exposed to air inhalation for 6 h. In comparison, the rats in the sevoflurane groups were exposed to 2.5% sevoflurane (600 μg/kg/min) for 6 h in 100% oxygen. The oxygen and sevoflurane levels were monitored using a gas monitor. The rats were intracerebroventricularly injected with lentivirus-miR-124 agomiR (10 μL, n = 4) or agomiR-negative control (n = 4) in the left lateral cerebral ventricles after sodium pentobarbital anesthesia (35 mg/kg). These rats were given a five-day recovery period before start of the sevoflurane inhalation.

All experiments were approved by the Animal Care and Use Committee of Hebei Medical University and were done in line with the instructions provided by the Institutional Animal Care and Use Committee of Hebei Medical University. We took all possible actions to reduce the number of rats used and their suffering.

Morris water maze

The hidden platform was positioned and concealed 1 cm below the surface of the water in the Morris water maze. Starting positions for each trial was pseudo-randomized. Each trial lasted for 60 s, or until the rats were able to locate the hidden platform. If rats were not able to find the hidden platform in 60 s, they were gently guided to the location of the platform. Rats were left on the platform for 15 s before their removal from the pool. We recorded escape latency and platform crossing manually with a stopwatch.

Cell culture

Primary mouse hippocampal neurons were obtained from Thermo Fisher Scientific (Waltham, USA), and cultured in RPMI 1640 (VWR International, Mississauga, Canada) medium with 10% fetal bovine serum (FBS), streptomycin (100 μg/mL) and penicillin (100 U/mL) at 5% CO_2 and 37°C. These neurons were treated with sevoflurane (20 μM) for 48 h and transfected with vectors described below.

Cell transfection

MiR-124 agomiR, pcDNA-Capn4, and their negative controls were obtained from Genomeditech (Shanghai, China). These vectors were transfected into hippocampal neuronal culture using Lipofectamine 2000 (Bioz, Inc., Los Altos, USA) using protocol supplied by the manufacturer.

RT-qPCR

M-MLV Reverse Transcriptase (BioChain, Newark, USA) was used to extract total RNA, and 1 μg of total RNA was used to synthesize cDNA using Prime Script RT reagent kit (TaKaRa Bio Inc., Gothenburg, Sweden) using protocol supplied by the manufacturer. SYBR Premix Ex Taq II (Bioz, Inc.) was used to measure the expression level of miR-124, calpain small subunit 1 (Capn4), Bax, caspase-3, and Bcl-2. A $2^{-\Delta\Delta Ct}$ method was used to determine the fold changes. β-actin was used as an internal control for Capn4, Bax, caspase-3, and Bcl-2, while U6 served as an internal control for miR-124. The primers used are given in Table 1.

Western blot analysis

Total protein content was assessed using a bicinchoninic acid (BCA) kit (Bosterbio, Pleasanton, USA). An equal amount of protein was used in SDS-PAGE. Proteins were transferred onto the polyvinylidene fluoride (PVDF) membranes (Biocompare, San Francisco, USA) after electrophoresis, followed by the blocking with 5% skim milk powder in Tween and Tris-buffered saline (BioLegend, San Diego, USA) for 50 min. These membranes were incubated with primary antibodies including anti-Capn4 (ab92333; 1:2000; Abcam, Cambridge, USA), anti-NF-κB (#8242; 1:1000; Cell Signaling, Beijing, China), anti-phospho-NF-κB (ab183559; 1:1000; Abcam), anti-IKK-β (ab124957; 1:1000; Abcam), anti-p-IKK-β (ab194519; 1:1000; Abcam), and β-actin (ab8227; 1:2000; Abcam) at 4°C overnight followed by the incubation with Goat anti-Rabbit horseradish peroxidase (HRP) (ab7090; 1:2000; Abcam) at 37°C for 1 h. β-actin was the internal control. The chemiluminescence reagent (Thermo Fisher Scientific) was used to visualize the bands and detected under a Las-3000 imaging system (Biocompare).

Table 1. Primer sequences

Primer	Sequence
Capn4 ACCCACTCCGTAACCTC	Forward
Capn4 GGGTAGCAACCGTGAA	Reverse
miR-124 TCGTTAAGGCACGCGGTG	Forward
miR-124 GTGCAGGGTCCGAGGT	Reverse
U6 CTCGCTTCGCAGCACA	Forward
U6 AACGCTTCACGAATTTGCGT	Reverse
Bax TCCAAGAAGCCCTAACGTGT	Forward
Bax ATGATCGTCTGGCTGCTGTA	Reverse
Bcl-2 GTGGTGGCAGATGTGCTTAG	Forward
Bcl-2 TTCAGAGCCACAAACAAGGC	Reverse
Caspase-3 TCGGTCTGGTACAGATGTCG	Forward
Caspase-3 CTTCACCATGGCTCAGAAGC	Reverse
β-actin ACCCAGAAGACTGTGGATGG	Forward
β-actin TCAGCTCAGGGATGACCTTG	Reverse

Luciferase assay

Online bioinformatic prediction tools including the starBase website (http://starbase.sysu.edu.cn/starbase2/index.php) and the TargetScan website (http://www.targetscan.org) were employed to predict the binding sites between Capn4 and miR-124. The primers were designed to target the 3'-UTR of the *Capn4* gene. The target and the mutated *Capn4* sequence were cloned into luciferase reporters to create mutant type (Capn4-MUT) or wild-type (Capn4-WT) plasmids. Neurons were co-transfected with miR-124 mimics or its negative control vector and the Capn4 luciferase reporter plasmids. After 48 h, a dual-luciferase reporter assay (BioVision, Milpitas, USA) was used to determine miR-124 and Capn4 binding.

ELISA assay

Enzyme-linked immunosorbent assay (ELISA; Abcam) was employed to determine the protein levels of IL-1β, IL-6 and TNF-α following the manufacturer's protocol. Supernatants from cell cultures were collected, and a microplate reader (BMG LabTech, Ortenberg, Germany) was used to detect absorbance at 450 nm. The protein levels of cytokines were referenced against a standard curve.

Statistical analyses

IBM SPSS v. 19.0 (IBM Corp., Armonk, USA) and GraphPad Prism v. 7.0 (GraphPad Software, San Diego, USA) software was used to analyze the data. Student's t-test and one-way analysis of variance (ANOVA) were used for the comparison between groups. Post hoc analysis was done using Tukey's correction method. All the experiments were performed in triplicate, and a p-value less than 0.05 was considered statistically significant. Data are presented as the mean ± standard deviation (SD).

Results

The expression of miR-124 was reduced in rats treated with sevoflurane anesthesia

We show that sevoflurane treatment increased escape latency and reduced hidden platform crossings in the Morris water maze, indicating deficits in spatial cognition (Fig. 1A,B). Real-time quantitative polymerase chain reaction (RT-qPCR) showed that miR-124 expression was significantly reduced in sevoflurane treated rats compared to the control group (Fig. 1C). Our findings demonstrated that the reduction in miR-124 expression was associated with cognitive deficits in sevoflurane-treated rats.

MiR-124 suppressed apoptosis induced by sevoflurane treatment in hippocampal neuron culture

The influence of miR-124 on sevoflurane-induced apoptosis in cultured hippocampal neurons was examined. Our results indicate that the expression of miR-124 was markedly reduced in sevoflurane-treated hippocampal neurons compared to that of the control group (**p < 0.001 compared to the control group) (Fig. 2A). Figure 2B shows that miR-124 overexpression in hippocampal neurons in the miR-124 agomiR group (##p < 0.001

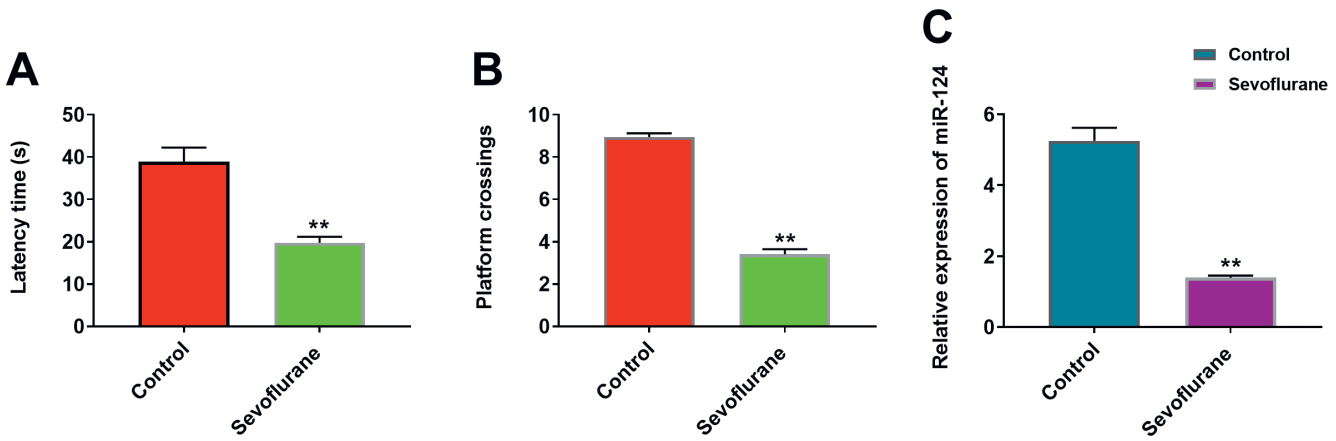

Fig. 1. MiR-124 was inhibited in rats treated with sevoflurane

A and B. The escape latency and platform crossing were assessed in the Morris water maze; C. MiR-124 expression was determined in the left lateral cerebral ventricle of the rats using RT-qPCR (**p < 0.001).

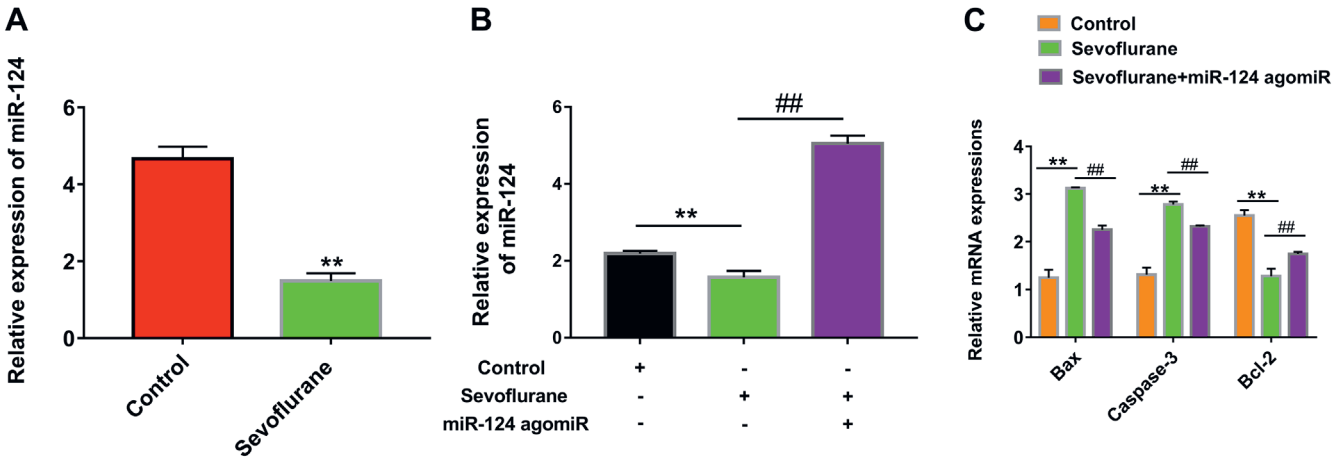

Fig. 2. MiR-124 decreased sevoflurane-induced apoptosis in hippocampal neurons

A and B. RT-qPCR was performed to determine the relative expression of miR-124 in hippocampal neurons; C. Bax, caspase-3 and Bcl-2 mRNA expression levels were determined in sevoflurane-treated hippocampal neurons using RT-qPCR (**p < 0.001 compared to the control group, ##p < 0.001 compared to the sevoflurane group).

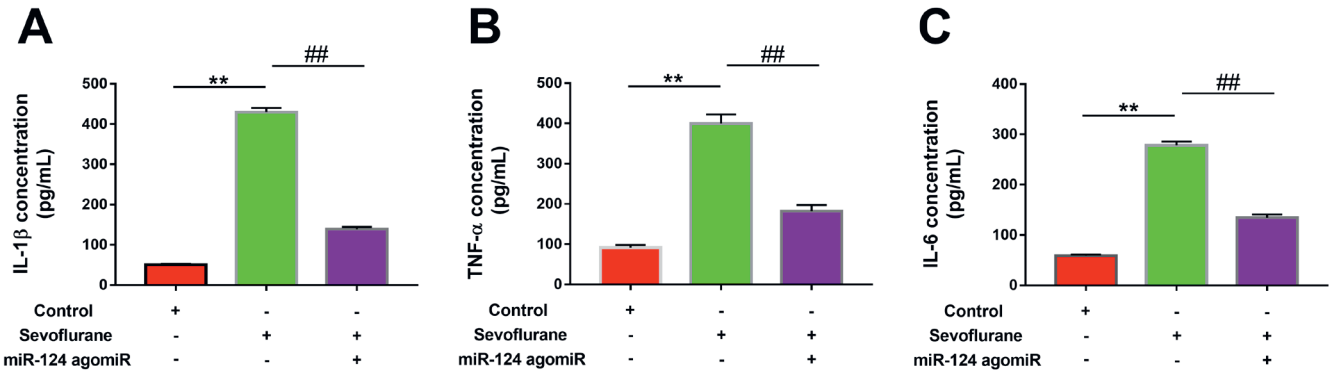

Fig. 3. MiR-124 suppressed the inflammation induced by sevoflurane in hippocampal neurons

A–C. The protein concentrations of IL-1β, IL-6 and TNF-α in sevoflurane-treated hippocampal neurons were detected using ELISA (**p < 0.001 compared to the control group, ##p < 0.001 compared to the sevoflurane group).

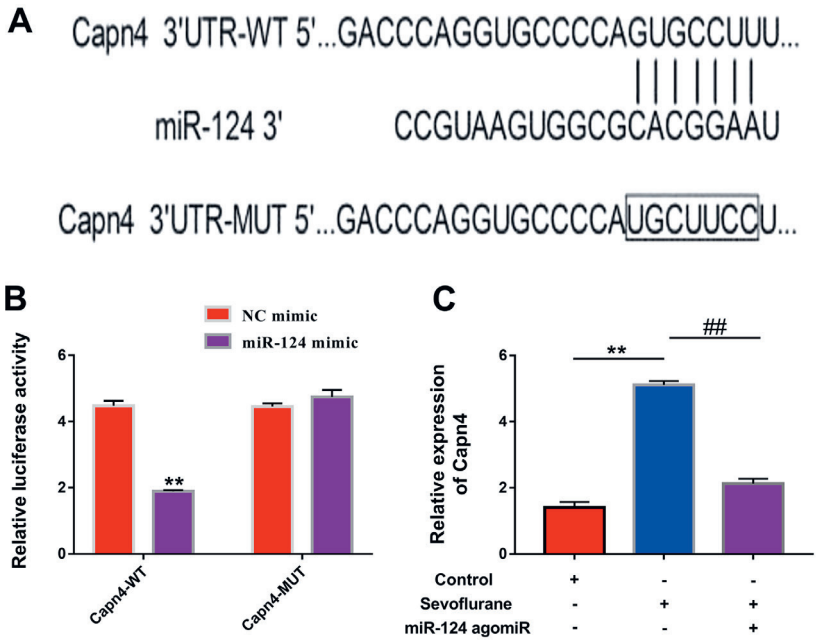

Fig. 4. Capn4 is a direct target of miR-124

A and B. The starBase software and luciferase assay were employed to predict and verify the relationship between miR-124 and Capn4; C. RT-qPCR was performed to measure the mRNA expression of Capn4 in sevoflurane-treated hippocampal neurons (**p < 0.001 compared to the control group, ##p < 0.001 compared to the sevoflurane group).

compared to the sevoflurane group). Also, Bax, caspase-3 and Bcl-2 RNA levels from RT-qPCR revealed that sevoflurane treatment increased the mRNA level of Bax and caspase-3 and decreased the level of Bcl-2. On the other hand, miR-124 agomiR reversed the impact of sevoflurane on the mRNA expression of these pro-apoptotic mediators (Fig. 2C). These data showed that miR-124 suppressed apoptosis induced by sevoflurane in hippocampal neurons.

Impact of miR-124 on sevoflurane--induced neuroinflammation in hippocampal neurons

Our ELISA data show that the level of IL-1β, IL-6 and TNF-α was increased by sevoflurane treatment compared to control. At the same time, miR-124 agomiR inhibited the level of these inflammatory mediators (**p < 0.001 compared to the control group, ##p < 0.001 compared to the sevoflurane group) (Fig. 3A–C). These data support that miR-124 inhibits sevoflurane-induced neuroinflammation in hippocampal neurons.

Capn4 was target of miR-124

Bioinformatic prediction tools – the starBase website and the TargetScan website – indicated that miR-124 may bind to Capn4. Our luciferase assay confirmed the binding between miR-124 and Capn4. Our results show that miR-124 mimics significantly reduced the luciferase activity in the Capn4-WT group compared to the Capn4-MUT group (Fig. 4A,B). Moreover, the expression of Capn4 was measured by using RT-qPCR, and our findings revealed an increased expression of Capn4 in response to sevoflurane treatment in hippocampal neurons compared to the controls. At the same time, miR-124 inhibited the mRNA level of Capn4 in hippocampal neurons (Fig. 4C). These results demonstrated that Capn4 is a direct target of miR-124.

MiR-124 inhibited sevoflurane-induced apoptosis in hippocampal neurons via the NF-κB signaling pathway

To investigate the downstream effectors of miR-124, we applied LY294002 to hippocampal neurons to test the involvement of the NF-κB pathway. The RT-qPCR results show that the miR-124 mimics reduced caspase-3 and Bax mRNA levels and enhanced the level of Bcl-2. On the other hand, treatment with NF-κB inhibitor and Capn4 overexpression reversed the effects of miR-124 mimics on sevoflurane-induced apoptosis in hippocampal neurons ($^{\#}p < 0.001$ compared to the sevoflurane group, $^{\$}p < 0.001$ compared to the miR-124 mimic group, nonsignificant compared to pcDNA-Capn4; Fig. 5A). Our western blot data indicate that miR-124 mimics suppressed the Capn4 protein level and increased the phosphorylation of NF-κB and IKK-β in sevoflurane-treated hippocampal neurons. However, the overexpression of Capn4 and treatment with NF-κB inhibitor showed the opposite effect on Capn4, p-NF-κB and p-IKK-β protein levels ($^{\#}p < 0.001$ compared to the sevoflurane group, $^{\$}p < 0.001$ compared to the miR-124 mimic group, nonsignificant compared to pcDNA-Capn4; Fig. 5B). These data demonstrated that miR-124 attenuated the apoptosis induced by sevoflurane treatment in hippocampal neurons via the NF-κB signaling pathway.

MiR-124 rescued cognitive function in rats treated with sevoflurane anesthesia

Next, we explored the impact of miR-124 on cognitive deficits induced by sevoflurane exposure in rats. Our results indicate that miR-124 decreased the escape latency and increased platform crossing in rats treated with sevoflurane compared to controls ($^{**}p < 0.001$ compared to the control group, $^{\#\#}p < 0.001$ compared to the sevoflurane group, Fig. 6A–C). Moreover, western blot analysis results revealed that miR-124 agomiR reversed sevoflurane-induced increases in protein levels of Capn4, p-NF-κB and p-IKK-β ($^{**}p < 0.001$ compared to the control group, $^{\#\#}p < 0.001$ compared to the sevoflurane group; Fig. 6D). These findings revealed that miR-124 improved cognitive function in rats treated with sevoflurane.

Discussion

In the current study, we showed that miR-124 expression was decreased in the freely moving rats and cultured mouse hippocampal neurons by sevoflurane. This decrease was associated with a general increase in the expression of apoptotic factors and neuroinflammatory markers in cultured hippocampal neurons. We were also able to show that Capn4 binds to miR-124 and triggers downstream effects through the NF-κB signaling pathway. All these molecular changes may be related to the amelioration of spatial cognition in sevoflurane-treated rats by miR-124.

The miRNAs play a critical role in sevoflurane-induced apoptosis. For instance, miR-96 enhanced the apoptosis in hippocampal neurons after treatment with sevoflurane, exacerbating the impact of sevoflurane on hippocampal neurons and cognitive function.[23] Another study revealed that miR-34a was involved in the modulation of hippocampal apoptosis induced by sevoflurane which was inhibited by the knockdown of miR-34a in hippocampal neurons.[24] Furthermore, miR-665 has been shown to play a neuroprotective role by reducing sevoflurane-induced apoptosis in hippocampal neurons.[25] Previous studies showed that

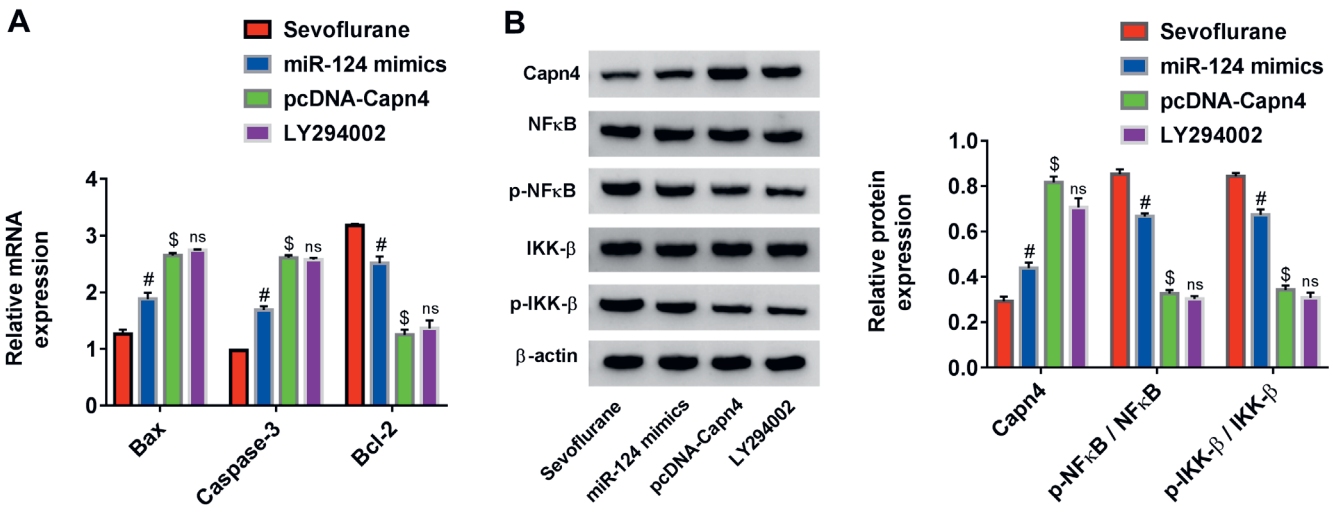

Fig. 5. MiR-124 attenuated sevoflurane-induced apoptosis in hippocampal neurons via the NF-κB signaling pathway

A. The mRNA levels of Bax, caspase-3 and Bcl-2 were determined using RT-qPCR; B. Protein expression levels of Capn4 and NF-κB signaling pathways were assessed using western blot ($^{\#}p < 0.001$ compared to the sevoflurane group, $^{\$}p < 0.001$ compared to the miR-124 mimic group, nonsignificant (ns) compared to pcDNA-Capn4).

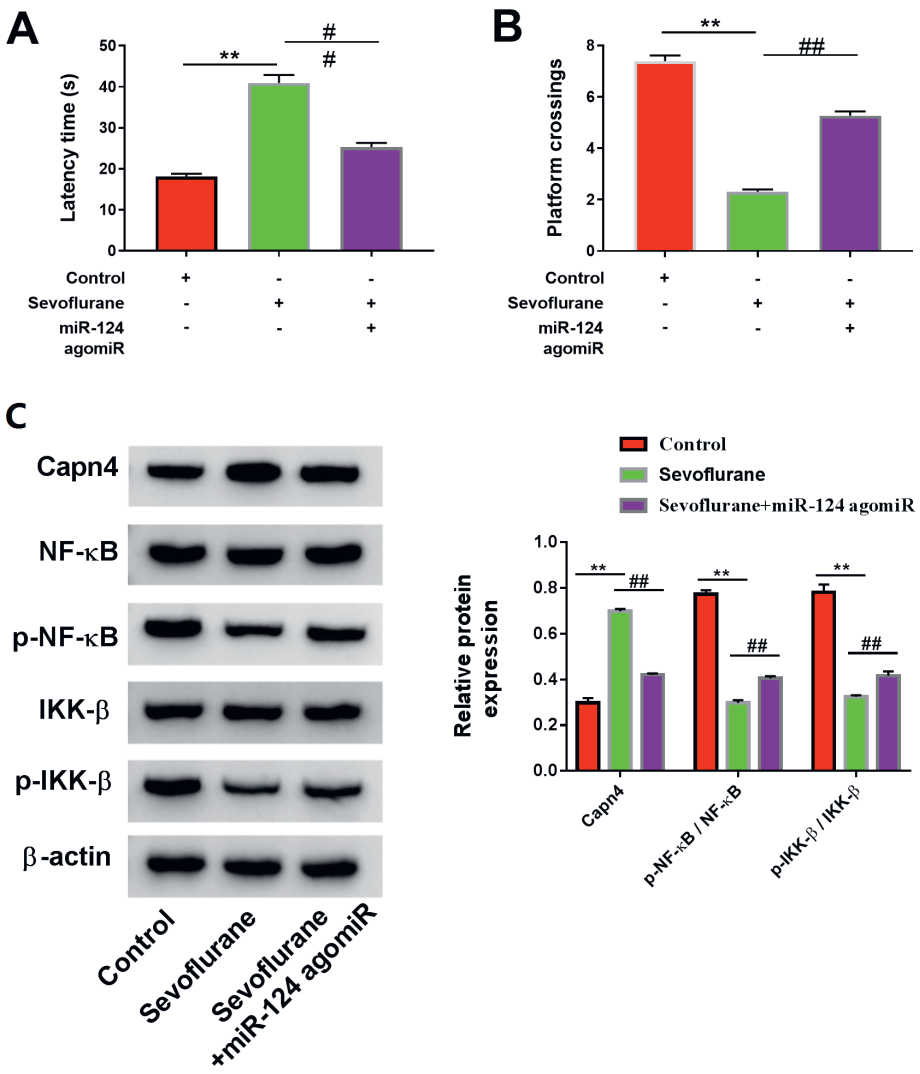

Fig. 6. MiR-124 improved spatial cognition in rats treated with sevoflurane

A and B. The escape latency and hidden platform crossings in the Morris water maze; C. Western blot analysis was used to examine the protein expressions of Capn4, and NF-κB signaling pathways (**p < 0.001 compared to the control group, ##p < 0.001 compared to the sevoflurane group; ns – nonsignificant).

miR-124 was upregulated in the neural precursor cells and during CNS development related to synaptic plasticity.[26,27] There is also evidence that miR-124 exerts its function in modulating inflammatory process by suppressing the release of inflammatory modulators.[17,28] Recent research revealed that miR-124 enhanced the spatial learning and memory in rats, offering resistance to apoptosis, and improved viability in hippocampal neurons.[29] Despite converging evidence showing that miRNAs play a major role in neuroprotection and neural plasticity relating to normal cognition, the impact of miR-124 in the context of sevoflurane exposure has not been described previously. In our research, we elucidated that miR-124 suppressed sevoflurane-induced apoptosis in cultured hippocampal neurons by decreasing the mRNA expressions of caspase-3 and Bax, while enhancing the expressions of Bcl-2. Previous research suggested that the expressions of inflammatory mediators such as IL-1β and TNF-α are elevated in sevoflurane-treated hippocampal neurons, while miR-410-3p suppressed the expressions of IL-1β, IL-6 and TNF-α.[30] Expressions of IL-1β, IL-6 and TNF-α can also be inhibited by the overexpression of miR-640 in sevoflurane-treated hippocampal neurons.[31] Our findings show that also miR-124 inhibited the expression of IL-1β, IL-6 and TNF-α in hippocampal neurons. These data support the role of miR-124 in suppressing inflammation and apoptosis induced by sevoflurane in vitro, subserving the rescue of sevoflurane-induced cognitive deficits.

The calpains belong to a calcium-dependent neutral cysteine proteases family and are involved in various biological activities, including cell cycle progression and cell mobility.[32] Calpain small subunit 1 (Capn4) exerts an important function in maintaining the activity and stability of calpain.[33] It plays a critical role in the modulation of cell differentiation and proliferation in osteoblasts,[34] and also mediates apoptosis in cardiomyocytes.[35] A recent study showed that miR-124 suppressed the invasion and proliferation of neural progenitor cells (NPC) cells by regulating its target gene *Capn4*.[36] Calpain can induce apoptosis, inflammatory response and NF-κB signaling following myocardial infarction, and the targeted deletion of Capn4 leads to improvement of cardiac function and decrease in mortality.[37] A previous study showed that andrographolide ameliorated POCD in aged rats via downregulating

NF-κB/MAPK pathways.[38] Upon the activation of resident microglia and macrophage in the hippocampus, NF-κB signaling is stimulated to promote neuroinflammation and increase transcription, subsequently contributing to cognitive impairment.[39,40] It has been reported that during surgical treatment, sevoflurane suppresses the NF-κB-mediated production of epithelial cell-derived inflammatory mediators, such as IL-8 and IL-6, which are the leading cause of ischemia/reperfusion injury.[41] Furthermore, suppression of NF-κB signaling pathways may inhibit cognitive dysfunction after sevoflurane anesthesia via inhibiting IL-6, IκBα, TNF-α, and IL-1β.[42] Similarly, our results revealed that miR-124 could inhibit the apoptosis induced by sevoflurane in hippocampal neurons by NF-κB signaling. The overexpression of miR-124 rescued apoptosis induced by sevoflurane in hippocampal neurons in the presence of an NF-κB inhibitor. Lastly, our research revealed that miR-124 significantly reduced the escape latency and increased hidden platform crossing, suggesting that miR-124 can improve or mitigate cognitive dysfunction induced by sevoflurane via the NF-κB signaling pathway.

Our data are consistent with the idea that miR-124 exerts its neuroprotective role against sevoflurane-induced cognitive dysfunction through Capn4 binding to modulate the NF-κB signaling pathway. These findings support for the further exploration of miR-124 related manipulations for POCD.

Limitations

This study only related the NF-kB signaling pathway, which is a limitation.

Conclusions

Our findings demonstrated that miR-124 exerted its neuroprotective role against sevoflurane via targeting Capn4 and NF-κB signaling pathways. Our work may provide a direction for sevoflurane anesthesia-related cognitive dysfunction.

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