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Neuroprotective effect of CTRP3 overexpression against sevoflurane anesthesia-induced cognitive dysfunction in aged rats through activating AMPK/SIRT1 and PI3K/AKT signaling pathways

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Abstract. – OBJECTIVE: To investigate the effects of C1q/tumor necrosis factor-related protein-3 (CTRP3) on postoperative cognitive dysfunction (POCD) and elucidate the potential regulatory mechanism in sevoflurane anesthesia-induced aged rats.

MATERIALS AND METHODS: A sevoflurane anesthesia-induced POCD aged rat model was established and hematoxylin and eosin (H&E) staining was used to detect pathological changes of hippocampal neurons. Morris water maze task test was performed to determine the learning and memory ability of rats. Immunofluorescence, quantitative Real Time-Polymerase Chain Reaction (qRT-PCR) and Western blot were used to detect CTRP3 expression. Enzyme-linked immunosorbent assay (ELISA) or qRT-PCR assays were used to evaluate the changes of markers of brain damage and inflammatory cytokines. Terminal deoxynucleotidyltransferase-mediated dUTP nick-end labeling (TUNEL) assay was used to assess the apoptosis of nerve cells in hippocampus. Western blot assay were used to measure the expression levels of apoptosis-related protein, and AMP-activated protein kinase (AMPK)/SIRT1 and PI3K/AKT pathway.

RESULTS: Sevoflurane exposure led to brain injury, cognitive dysfunction in aged rats and decreased the expression of CTRP3. Overexpression of CTRP3 could suppress nerve cell apoptosis, inhibit neuronal inflammation, reduce brain tissue damage and improve cognitive dysfunction of aged rats after sevoflurane anesthesia. Further studies showed that CTRP3 may play a role in POCD by regulating AMPK/SIRT1 and PI3K/AKT signaling pathways.

CONCLUSIONS: CTRP3 may effectively protect against sevoflurane-induced cognitive dysfunction and served as a potential predictive indicator and therapy target for POCD.

Key Words:
CTRP3, AMP-activated protein kinase (AMPK), Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR), Hematoxylin and eosin (H&E), Sevoflurane.

Introduction

Postoperative cognitive dysfunction (POCD) is a central nervous system complication that occurs after anesthesia[1,2]. It is common in elderly patients and can last for months to years, with symptoms including anxiety, confusion, personality changes and memory impairment[3]. POCD can inhibit recovery, increase postoperative complications, prolong length of hospital stay, and negatively affect quality of life after discharge[4]. Neuroinflammation induced by general anesthesia (GA) is increasingly recognized as a key factor in the pathogenesis of POCD[5]. Sevoflurane, a commonly used alkane inhaled anesthetic, has been found to induce increased inflammation and apoptosis of hippocampal neurons in elderly rats, leading to cognitive dysfunction[6,7]. At present, the methods of preventing and treating POCD are relatively limited, and the treatment effect is not ideal. Therefore, exploring the pathogenesis and mechanism of POCD caused by sevoflurane inhalation anesthesia, and looking for effective targets to inhibit neurotoxicity and cognitive dysfunction, is very helpful to improve the quality of life of patients after surgery.

C1q/tumor necrosis factor-related protein-3 (CTRP3) is a new type of adipokine, which belongs to the C1q/TNF protein superfamily and

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is a homologue of adiponectin[8,9]. CTRP3 is a protein consisting of 246 amino acid sequences, with a molecular weight of 26k Da and consists of a secretion signal peptide, a collagen domain, and a C-terminal globular domain[10]. Expression of CTRP3 was firstly found in differentiated 3T3-L1 cell lines, and also in cartilage, kidney, colon, lung and brain[11-16]. CTRP3 has been reported to have multiple biological functions and involved in regulating the physiological and pathological processes of cancer, glucose and lipid metabolism, hypertension, obesity, atherosclerosis and other diseases[9,17-22]. In addition, Wang et al[16] found that CTRP3 exerts neuroprotective effects through AMP-activated protein kinase (AMPK)/HIF-1α/VEGF-dependent pathways in rat models of cerebral hemorrhage, which can reduce brain edema, prevent destruction of the blood-brain barrier (BBB), improve neural function, and promote angiogenesis. However, there is still a paucity of data regarding the role and mechanism of CTRP3 in POCD.

In this study, we aimed to investigate the neuroprotective effect of CTRP3 on sevoflurane-induced neurotoxicity and cognitive deficits and reveal the potential molecular mechanisms.

Materials and Methods

Animals

Sprague-Dawley rats (n=36; male; 15-month-old; weight, 260-280 g) were obtained from Animal Laboratory Center of The First Affiliated Hospital of Zhengzhou University (Zhengzhou, Henan Province, China). The rats were housed under a 12 h light-12 h dark cycle with a constant room temperature of 24-26°C and 60% indoor relative humidity in standard breeding cages, and were allowed *ad libitum* access to water and food. The rats were allowed to adapt to these conditions for at least a week. All procedures were approved by the Ethics Committee of the First Affiliated Hospital of Zhengzhou University and were carried out in accordance with the NIH Guidelines for Care and Use of the Laboratory Animals.

Sevoflurane Exposure Treatment and Animal Grouping

The rats were put into anesthesia box respectively, one end of the box is the air inlet, which is connected to the anesthesia machine (EZ-SA800 Single Animal System, EZ Anesthesia, Palmer, PA, USA), the other end is the air outlet, which is connected to the exhaust gas absorption device. Sevoflurane anesthesia treatment was using 2.5 % sevoflurane (Flow 1 L/min) in the oxygen for 6 h *via* anesthesia machine, labeled as sevoflurane (Sev) group, and the rats of control group were received regular air inhalation (Flow 1 L/min) for 6 h. Experiment 1, the rats were randomly divided into 2 groups: (1) control (Ctrl) and (2) sevoflurane (Sev). Experiment 2, the rats were randomly divided into four groups: (1) Ctrl, (2) Sev, (3) Sev+AAV9-NC and (4) Sev+AAV9-CTRP3. For transduction of rats with overexpressed-CTRP3 or respective controls, AAV9 vectors carrying either the CTRP3 or green fluorescent protein (GFP) gene (produced by Hanbio Biotechnology Co., Shanghai, China) were intracerebrally injected into rats as previously described[23]. After transfection for 5 days, the following experiments were performed.

Morris Water Maze Task

The Morris water maze task was performed to evaluate the effects of sevoflurane and regular air exposure on learning and memory ability in rats, as previously mentioned[23,24]. Briefly, the maze was made of the water tank with a diameter of 200 cm and 60 cm high walls, and was filled with water at 25°C. The maze was divided into four quadrants with a hidden platform (10 cm in diameter), which was located 1 cm below the water surface. Place navigation test lasted 5 days, rats in each group were trained to swim 2 hours a day. We placed the platform in the center of any quadrant, and at the same time every day, let the rats face the pool wall from any of the other three quadrants into the water, swim and find the platform, observed and recorded the time that the rats found and climbed the platform within 60 seconds, as the escape latency time. When the rats could not find the hidden platform within a limited time, then, we gently guided the rats to stay on the platform for 20 seconds and the latency was recorded as 60 seconds. The spatial probe test was conducted to evaluate the spatial memory of rats withdraw the underwater platform on the second day after the completion of the place navigation test (i.e., the sixth day of the experiment). The rats were lowered into the water from the opposite side of the original target quadrant for twice, allowed them explore for 120 s freely, recorded the number of platform crossings, the time spent and distance covered in the target quadrant, and tracked the trajectory using

ANY-maze Video Tracking System (version 5.1, Stoelting Co., Wood Dale, IL, USA).

Hematoxylin-Eosin (H&E) Staining

The brain tissues were harvested, embedded with paraffin and sectioned. The slices were then stained with HE staining kit (Beyotime, Beijing, China) according to the manufacturer's protocol. The slices were observed pathological changes under a microscope.

Enzyme Linked Immunosorbent Assay (ELISA)

The supernatants from the rat brain tissues were obtained, and ELISA kit (USCN Life Science & Technology Company, Missouri, TX, USA) was used to measure the levels of S-100β, NSE, IL-1β, TNF-α and IL-6 based on the manufacturer's directions. Optical density (OD) values were measured at 450 nm using a microplate reader (EXL808; BioTek instruments, Inc., Winooski, VT, USA) and the corresponding concentration of the sample was obtained by using the standard curve.

Immunofluorescence Analysis

The hippocampus of rats was sliced in 20 μm thick slices for immunofluorescence staining. The slices were washed with phosphate buffer solution (PBS), permeated with 0.3% Triton X, and blocked with 10 % normal goat serum (NGS). Then, the slices were incubated with different primary antibodies overnight, after which the secondary antibody labeled with red fluorescence was added. Finally, the samples were stained by 4′,6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich, St. Louis, MO, USA) for 10 min, and then, pictures were captured by a confocal microscope.

Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR)

The PCR-based analysis was performed as previously described[25]. In brief, the total RNA was extracted from the hippocampus of rats using TRIzol reagent (Invitrogen, Carlsbad, CA, USA), and reverse-transcribed by PrimeScript RT reagent kit (TaKaRa Bio, Inc., Otsu, Shiga, Japan) according to manufacturers' instructions. Transcript levels were measured on ABI PRISMR 7300 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). Relative quantification of gene expression based on the threshold cycle (CT) values and normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) using $2^{-\Delta\Delta CT}$ method. The primers were as follows: CTRP3 forward 5′-ATGGAGGTGAGCAGAAGAGC-3′ and reverse 5′-CACAGTCCCCGTTTTAGCAT-3′; IL-1β forward 5′-CAGTGAGGAGAATGACCTGTTC-3′; and reverse 5′-CGAGATGCTGCTGTGAGATT-3′; TNF-α forward 5′-AGCATGATCCGAGATGTGGAA-3′, and reverse 5′-TAGACAGAAGAGCGTGGTGGC-3′; IL-6 forward 5′-GTTGCCTTCTTGGGACTGATG-3′ and reverse 5′-ATACTGGTCTGTTGTGGGTGGT-3′; GAPDH forward 5′-GGTGAAGGTCGGAGTCAACG-3′ and reverse 5′-TGGGTGGAATCATATTGGAACA-3′.

Western Blot

Total protein extraction from hippocampus tissues were homogenized in radio-immunoprecipitation assay (RIPA) buffer and the protein concentration was calculated by a BCA Protein Assay Kit (Thermo Fisher Scientific, San Jose, CA, USA). An equal amount of proteins was electrophoresed in 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore Co., Billerica, MA, USA). The membranes were blocked with 5% non-fat milk-TBST solution for 1 h, followed by incubation with primary antibodies, including rabbit polyclonal anti-CTRP3 (1:1000, ab36870, Abcam, Cambridge, UK), rabbit monoclonal to anti-Bax (1:1000, ab32503, Abcam, Cambridge, UK), rabbit monoclonal to anti-Bcl-2 (1:1000, ab32124, Abcam, Cambridge, UK), rabbit polyclonal to anti-AMPK (1:1000, ab131512, Abcam, Cambridge, UK), rabbit polyclonal to anti-p-AMPK (1:1000, ab23875, Abcam, Cambridge, UK), Mouse monoclonal to anti-Sirt1 (1:1000, ab110304, Abcam, Cambridge, UK), rabbit polyclonal anti-Akt (1:500, ab8805, Abcam, Cambridge, UK), rabbit polyclonal to anti-p-Akt (ab38449, Abcam, Cambridge, UK), rabbit monoclonal anti-GAPDH (1:3000, ab181602, Abcam, Cambridge, UK). Then, the membranes were incubated with horseradish peroxidase (HRP)-labeled corresponding secondary antibodies. Finally, Gel imaging system (Thermo Fisher Scientific, Waltham, MA, USA) was used to detect and analyze the protein band intensities of membranes. GAPDH was used as an internal control and Image-pro Plus 6.0 (Media Cybernetics Inc., Rockville, MD, USA) was used for quantitative analysis.

Terminal Deoxynucleotidyl Transferase-Mediated dUTP Nick-End Labeling (TUNEL) Assay

TUNEL staining was performed to detect the apoptosis of hippocampus tissues according to the instructions of TUNEL Detection Kit (Roche Diagnostics, Shanghai, China). The numbers of TUNEL-positive cells in the hippocampal were observed and captured under a light microscope (Olympus DX51 fluorescence microscope; Olympus Corp., Tokyo, Japan).

Statistical Analysis

The Statistical Product and Service Solution (SPSS) 21.0 software (IBM Corp. Armonk, NY, USA) was used for data analysis. Measurement data were expressed as mean ± standard deviation (SD). Comparisons between two groups were analyzed by Student's *t*-test, while comparisons among multiple groups were analyzed using one-way analysis of variance (ANOVA), followed by Tukey's post-hoc test. A value of $p<0.05$ was considered to be statistically significant.

Results

CTRP3 Expression Was Downregulated in Sevoflurane Anesthesia-Induced Rats

HE staining was performed to observe the pathological changes of hippocampal neurons in sevoflurane anesthesia-induced rats. Sevoflurane (Sev) group showed nuclear concentration and vacuolization, disordered and loose arrangement and smaller cells in hippocampal neutrons (Figure 1A). ELISA assay demonstrated that serum S-100β and NSE level were significantly higher in the Sev group compared with the Ctrl group (Figure 1B). Morris water maze test indicated that sevoflurane anesthesia prolonged the latency time, but reduced the number of platform

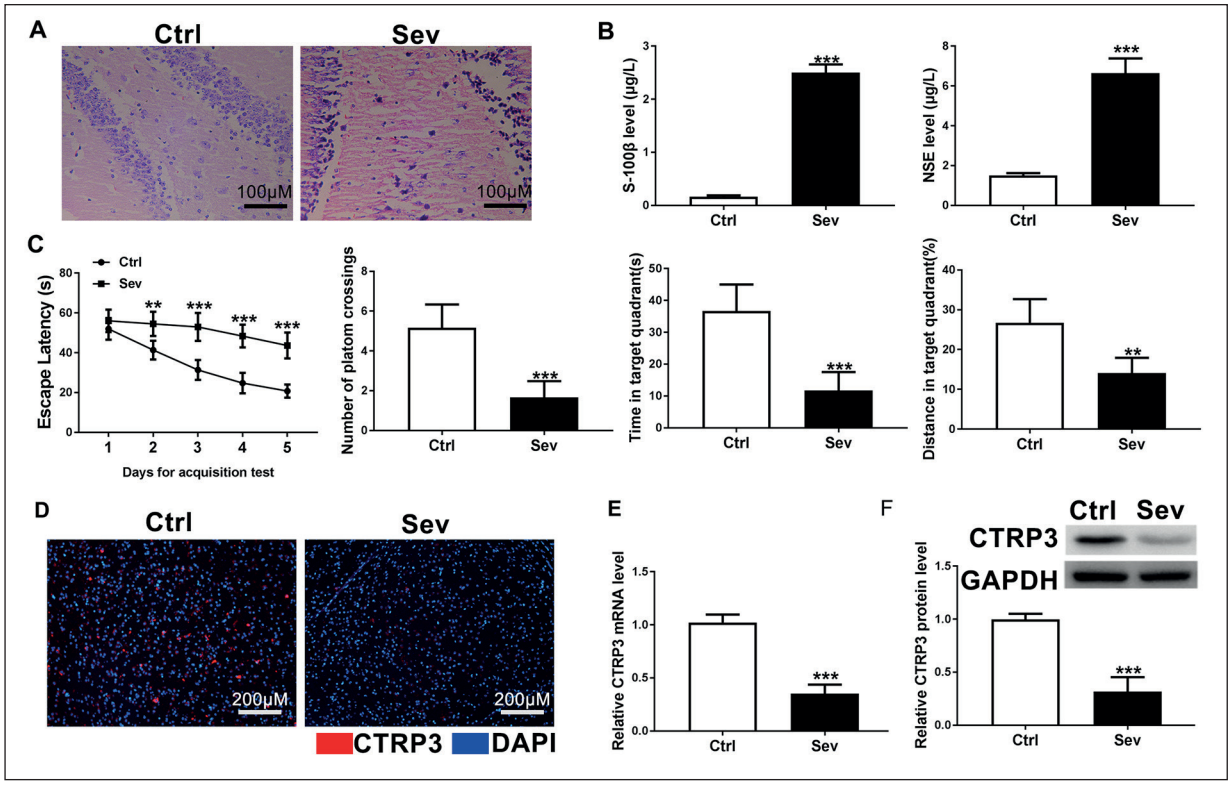

Figure 1. CTRP3 expression was down-regulated in sevoflurane anesthesia-induced rats. **A,** Hematoxylin and eosin (H&E) staining was performed to observe the pathological changes of hippocampal neurons in rats. **B,** Enzyme-linked immunosorbent assay (ELISA) was performed to detect the expression levels of S-100β and NSE. **C,** Morris water maze test was used to measure the latency time, the number of platform crossing, the percentage of time spent and the distance covered in the target quadrant. **D-F,** Immunofluorescence (**D**), qRT-PCR (**E**) and Western blot (**F**) were used to determine the levels of CTRP3 in the hippocampi of rats. **$p<0.01$, ***$p<0.001$ *vs.* the Ctrl group. Sev, sevoflurane; Ctrl, Control.

crossing, the percentage of time spent and the distance covered in the target quadrant (Figure 1C). These results revealed that sevoflurane anesthesia induced brain injury and learning and memory impairments in aged rats. In addition, CTRP3 expression was significantly decreased in sevoflurane anesthesia-induced rats using immunofluorescence, qRT-PCR and Western blot (Figure 1D-F).

Overexpression of CTRP3 Attenuated Sevoflurane-Induced Neuronal Apoptosis in Aged Rats

In order to investigate the effect of CTRP3 on sevoflurane anesthesia-induced neuronal apoptosis, CTRP3 was successfully overexpressed in sevoflurane anesthesia-induced aged rats (Figure 2A). TUNEL assay showed that the amount of TUNEL positive cells in the rat hippocampus were remarkably increased by sevoflurane treatment, whereas the overexpression of CTRP3 attenuated the effects of sevoflurane treatment on cell apoptosis in aged rats (Figure 2B). Furthermore, Western blot assay indicated that Bcl-2 level was significantly decreased, but Bax level was significantly increased by sevoflurane treatment, whereas the overexpression of CTRP3 reversed the above effects (Figure 2C). These results suggested that CTRP3 could supress sevoflurane anesthesia-induced neuronal apoptosis in aged rats.

Overexpression of CTRP3 Reduced S evoflurane Anesthesia-Induced Neuronal Inflammation in Aged Rats

To explore whether CTRP3 could affect sevoflurane anesthesia-induced neuronal inflammation, the concentration of inflammatory cytokines were dectected by ELISA and qRT-PCR. As illustrated in Figure 3A and 3B, sevoflurane anesthesia caused the elevation of IL-1β, TNF-α and IL-6, whereas the overexpression of CTRP3

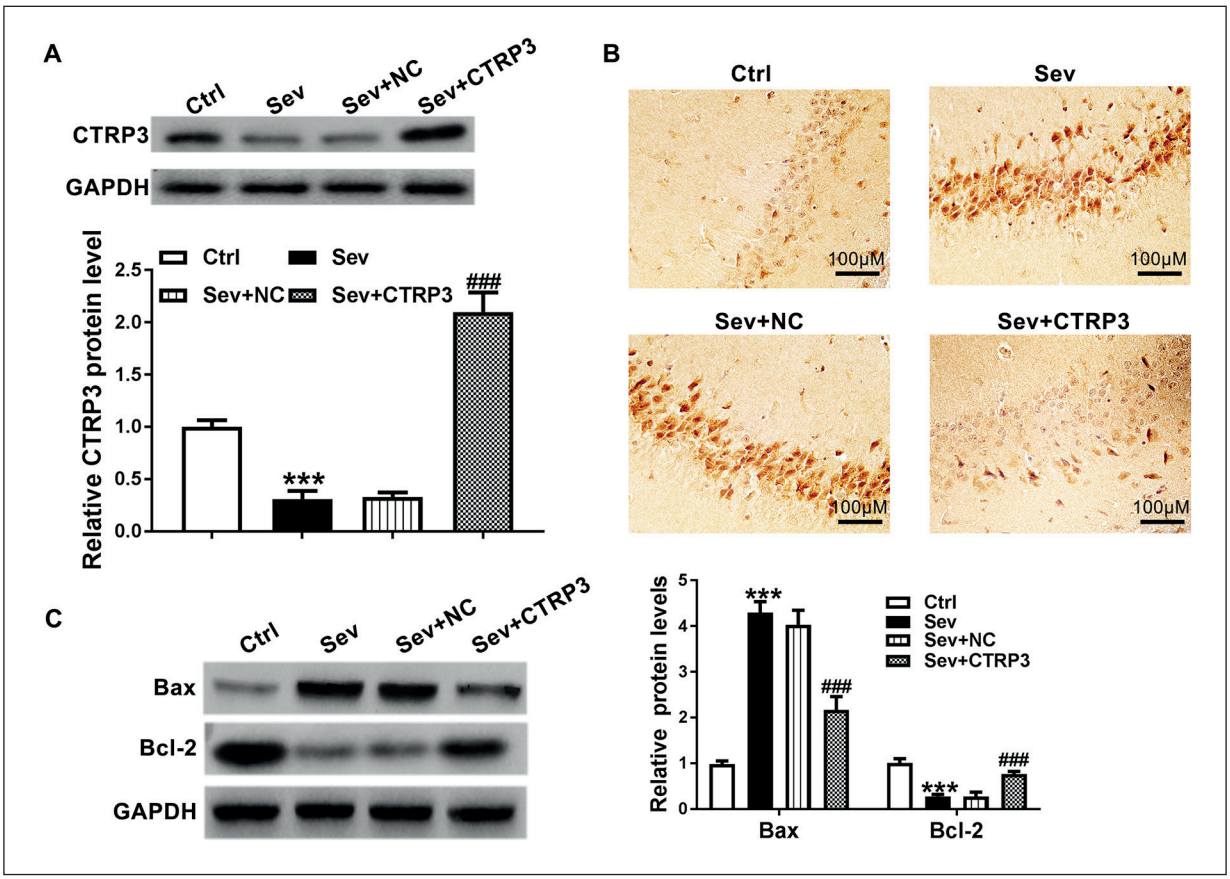

Figure 2. Overexpression of CTRP3 attenuated sevoflurane-induced neuronal apoptosis in aged rats. **A,** Representative Western blot bands and quantitative evaluation of CTRP3 in the hippocampi of rats. **B,** Apoptosis-positive cells were measured by TUNEL assays. **C,** Representative Western blot bands and quantitative evaluation of Bax and Bcl-2. ***p<0.001 *vs.* the Ctrl group; $^{###}p$<0.001 *vs.* the Sev + NC group.

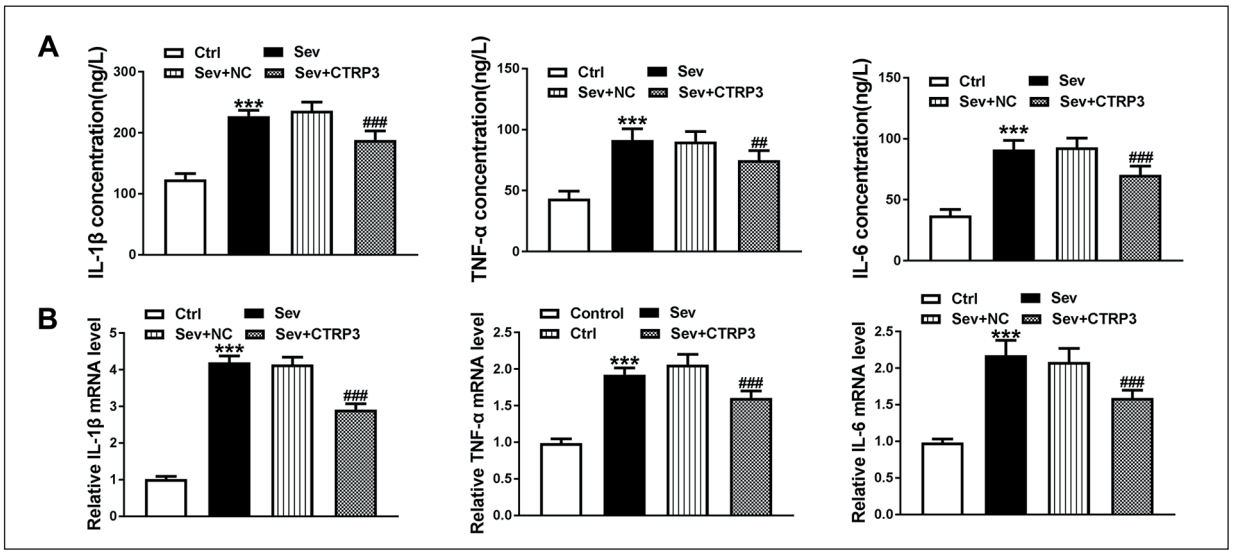

Figure 3. Overexpression of CTRP3 reduced sevoflurane anesthesia-induced neuronal inflammation in aged rats. **A, B,** ELISA and qRT-PCR were performed to detect the expression levels of IL-1β, TNF-α and IL-6 in the hippocampi of rats. ***$p<0.001$ *vs.* the Ctrl group; $^{##}p<0.01$, $^{###}p<0.001$ *vs.* the Sev + NC group.

inhibited the sevoflurane anesthesia-induced increased levels of IL-1β, TNF-α and IL-6. These findings revealed that CTRP3 reduced sevoflurane anesthesia-induced neuronal inflammation in aged rats.

CTRP3 Improved Cognitive Dysfunction in Aged Rats Induced by Sevoflurane Anesthesia

We further investigated the effect of CTRP3 on the cognitive function in sevoflurane anesthesia-induced aged rats. HE staining indicated that the rats in Sev group dispalyed evident pathological changes in brain tissues with distinctive brain injury comapred to the Ctrl group, whereas overexpression of CTRP3 weakened the above damage (Figure 4A). ELISA assay also demonstrated that overexpression of CTRP3 reduced the enhancement of S-100β and NSE levels in aged rats induced by sevoflurane anesthesia (Figure 4B). Morris water maze test was conducted to evaluate the effects of CTRP3 on learning and memory abilities. The results showed that the overexpression of CTRP3 reduced the latency time and the number of platform crossing, while increased the percentage of time spent and the distance covered in the target quadrant in aged rats induced by sevoflurane anesthesia (Figure 4C). These data semonstrated that CTRP3 improved cognitive dysfunction in sevoflurane anesthesia-induced aged rats.

CTRP3 Activated AMPK/SIRT1 and PI3K/AKT Signaling Pathways in Sevoflurane Anesthesia-Induced Aged Rats

To clarify the mechanism underlying the role of CTRP3 in neurological deficits and cognitive impairment in sevoflurane anesthesia-induced aged rats, Western blot assay (Figure 5A and 5B) were used to analyze the changes in the AMPK/SIRT1 and PI3K/AKT signaling pathways. The expression levels of p-AMPK, Sirt1 and p-Akt in the Sev group were significantly decreased compared with Ctrl group. Compared with the Sev group, overexpression of CTRP3 caused the elevated levels of p-AMPK, Sirt1 and p-Akt. These findings demonstrated that CTRP3 protected sevoflurane-induced brain injury in aged rats possibly by activating the AMPK/SIRT1 and PI3K/AKT signaling pathways.

Discussion

The pathogenesis of POCD is complex, most of which is caused by a combination of anesthesia and surgery on the basis of central nervous system degeneration in elderly patients, and the neurological decline caused by multiple factors, including inflammatory reactions and apoptosis in the central nervous system. Sevoflurane is a commonly used inhaled anesthetic and the mechanism of sevoflurane anesthesia-caused POCD

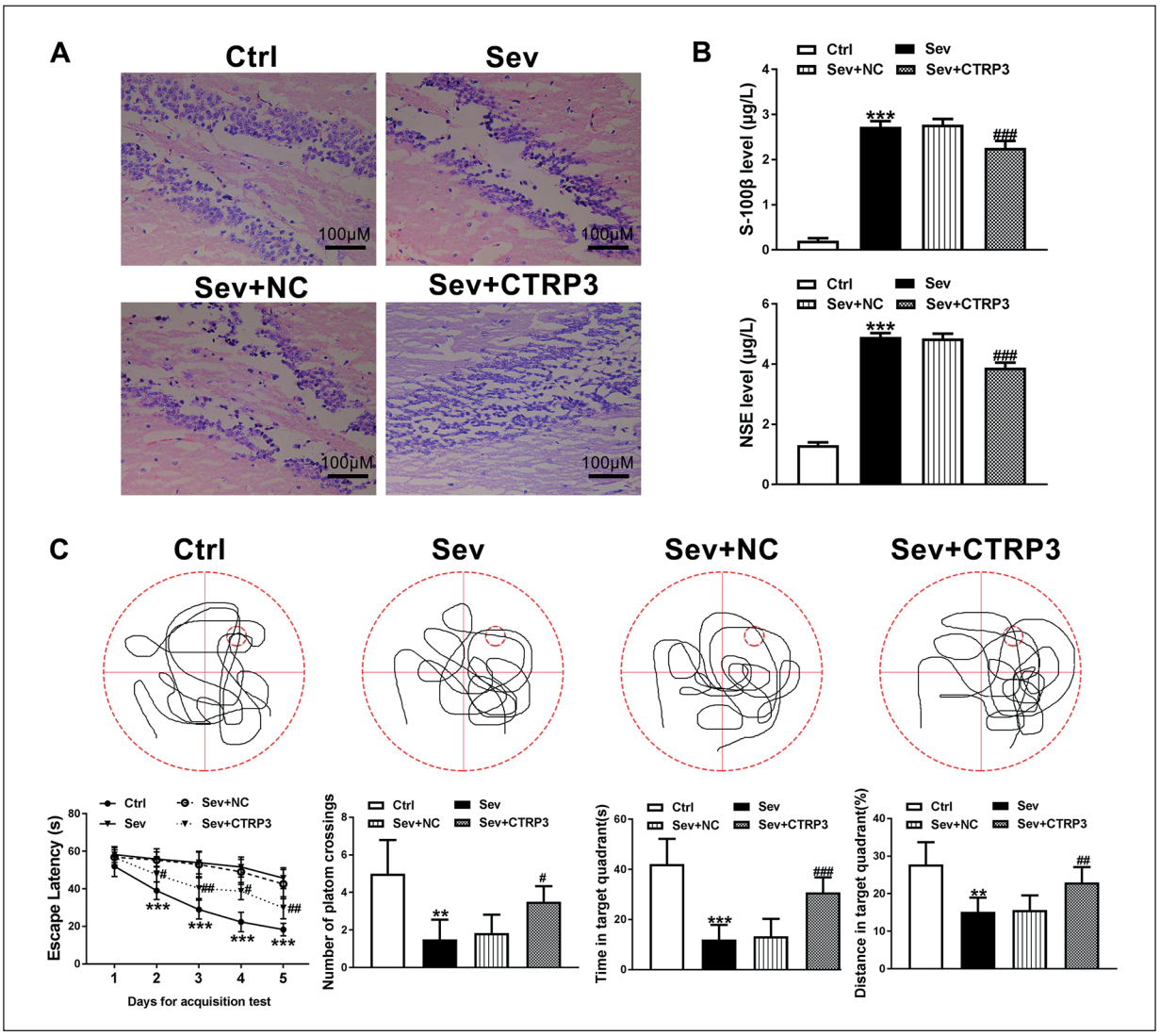

Figure 4. CTRP3 improved cognitive dysfunction in aged rats induced by sevoflurane anesthesia. **A,** HE staining was performed to observe the pathological changes of hippocampal neurons in rats. **B,** ELISA was performed to detect the expression levels of S-100β and NSE. **C,** Morris water maze test was used to measure the latency time, the number of platform crossing, the percentage of time spent and the distance covered in the target quadrant. $^{**}p<0.01$, $^{***}p<0.001$ *vs.* the Ctrl group; $^{\#}p<0.05$, $^{\#\#}p<0.01$, $^{\#\#\#}p<0.001$ *vs.* the Sev + NC group.

has been reported to be closely associated with neuroinflammation and apoptosis[26-28]. Therefore, in-depth understanding of the mechanism of POCD induced by sevoflurane and search for appropriate targets and mechanisms to reduce or even eliminate the occurrence of cognitive dysfunction arethe difficulties of anesthesiological research. Herein, this study provided evidence that CTRP3 upregulation protected the neurological deficits and cognitive dysfunction triggered by sevoflurane exposure in aged rats *via* mediating AMPK/SIRT1 and PI3K/AKT signaling pathways.

Firstly, animal models of sevoflurane exposure were established in this study. The results revealed that sevoflurane exposure led to serious hippocampal neuron injury, caused the elevated levels of serum markers of brain tissue damage (S-100β and NSE)[29], and increased impairments in learning and memory in aged rats. CTRP3 could attenuate brain injury after intracerebral hemorrhage, suggesting the potential role of CTRP3 in regulating brain damage[16,30]. We thus suspected that CTRP3 might be also correlated with sevoflurane anesthesia-caused POCD. Consistently, CTRP3 expression in aged rats was re-

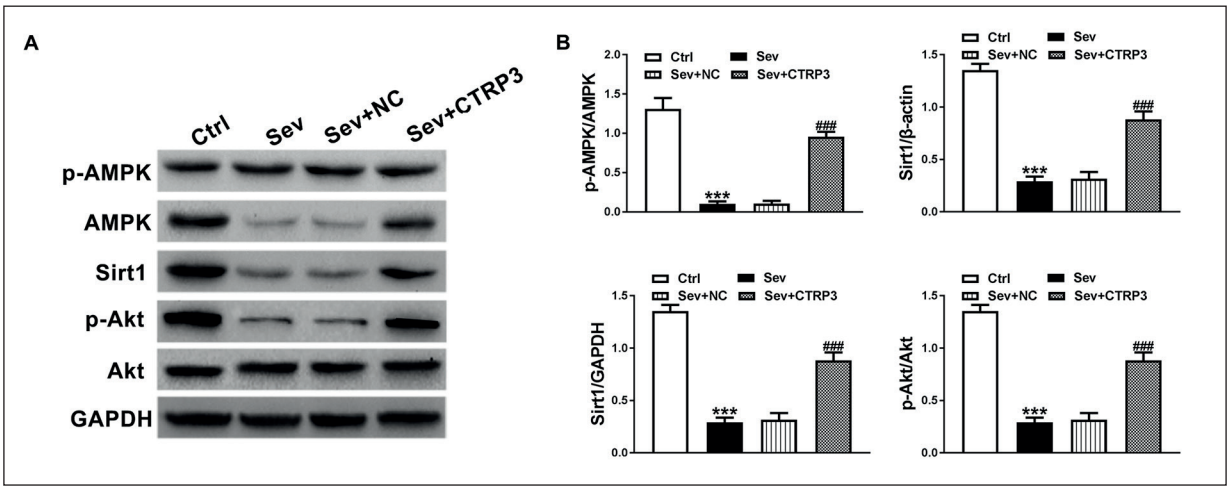

Figure 5. CTRP3 activated AMPK/SIRT1 and PI3K/AKT signaling pathways in sevoflurane anesthesia-induced aged rats. **A, B,** Representative Western blot bands and quantitative evaluation of AMPK, p-AMPK, Sirt1, AKT and p-AKT. ***p<0.001 *vs.* the Ctrl group; ###p<0.001 *vs.* the Sev + NC group.

duced in response to sevoflurane exposure. These findings indicated that CTRP3 might play a role in sevoflurane anesthesia-induced aged rats.

CTRP3 inhibited accumulation of inflammatory factors (such as TNF-α, IL-1β, and MCP-1) and cell apoptosis in high glucose-induced human umbilical vein endothelial cells, simulating the model of diabetic endothelial dysfunction *in vitro*[31]. CTRP3 also ameliorated the expression of inflammatory markers (CRP, TNF-α and IL-6) and reduced cell apoptosis induced by oxidized low-density lipoprotein (ox-LDL) in atherosclerosis[32]. Additionally, CTRP3 inhibited inflammation and apoptosis in depression, diabetic cardiomyopathy and doxorubicin-induced cardiac dysfunction[22,33-35]. Moreover, in critical illness and sepsis patients, the plasma concentration of CTRP3 was negatively correlated with inflammatory cytokines[36]. This study is the first evidence demonstrating that CTRP3 inhibited neuronal apoptosis and the expression of IL-1β, TNF-α and IL-6 proinflammatory cytokines in sevoflurane anesthesia-induced aged rats. Furthermore, overexpression of CTRP3 reduced brain damage and the expression of S-100β and NSE, and decreased learning and memory impairments, suggesting that CTRP3 exerted a neuroprotective role in sevoflurane anesthesia-induced aged rats.

Evidence suggested that CTRP3 could protect cardiomyocytes by ameliorating mitochondrial dysfunction by increasing phosphorylation of AMPK and the expression of Sirt1[37]. CTRP3 also plays an important role in diabetic retinopathy and osteoclastogenesis by regulating AMPK signaling pathway[38,39]. Moreover, CTRP3 was also reported to activate AMPKα and Akt in the diabetic hearts[35]. Recently, CTRP3 has been demonstrated to inhibit inflammation and cell apoptosis through PI3K/Akt pathway[32]. Generally, the activated AMPK/SIRT1 and PI3K/AKT signaling pathways have been shown to play protective role in nerve damage caused by anesthesia[40-42]. Therefore, it was speculated that the neuroprotective effect of CTRP3 might manifest through the activation of AMPK and AKT signaling pathways. Further mechanism studies by Western blot showed that CTRP3 increased the expression of phosphorylated-AMPK, Sirt1 and phosphorylated-AKT after sevoflrane exposure, indicating that CTRP3 may play a neuroprotective role by activating AMPK/SIRT1 and PI3K/AKT signaling pathways in sevoflurane anesthesia-induced aged rats.

Conclusions

Our study implies an underlying mechanism of CTRP3 negatively regulating inflammation, neuronal apoptosis and memory impairment by mediating AMPK/SIRT1 and PI3K/AKT signaling pathways in sevoflurane anesthesia-induced aged rat. Thus, CTRP3 may serve as a new therapeutic biomarker and for neurological deficits caused by sevoflurane exposure. The

clinical value and treatment strategy of CTRP3 for anaesthesia-induced neural dysfunction need further analysis.

Conflict of Interest

The Authors declare that they have no conflict of interests.

Authors' Contribution

WZ conceived and designed the experiments, LHY analyzed and interpreted the results of the experiments, YCX performed the experiments.

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