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Original Article

SNAP25 ameliorates cognitive impairment after subarachnoid hemorrhage in rats

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Abstract: Cognitive impairment can be a long-term complication after subarachnoid hemorrhage (SAH). The synaptosomal-associated protein of 25 kDa gene (SNAP25) is essential for the triggering of vesicular fusion and neurotransmitter release. The study aims to evaluate the effects and the possible mechanism of SNAP25 on cognitive impairment after SAH. Rats (N=60) were randomly assigned into five groups: Normal, Sham, SAH, SAH+NS, and SAH+SNAP25 group. SAH was induced by endovascular perforation. Neurological deficits, SAH severity, brain water content (BWC) and blood-brain barrier (BBB) permeability was determined. Spatial learning and memory abilities were tested by Morris water maze test. SNAP25 expression and the apoptosis of brain neurons were evaluated by immunohistochemical staining. The cytokines (IL-1β, IL-6, TNF-α) in brain tissues were detected by ELISA. The protein levels of apoptosis-related proteins (cleaved caspase-3, Bax) and synaptic plasticity-associated proteins (SYN, Arc and MAP-2) were analyzed by Western blot. This study demonstrated that intraperitoneal injection of SNAP25 accelerated the recovery of neurological dysfunction, effectively relieved subarachnoid hemorrhage, brain edema, and BBB disruption, and improved learning deficits as well as attenuated neuronal apoptosis in SAH rats. In addition, SNAP25 blocked the upregulation of pro-inflammatory factors and apoptosis-related genes, and promoted the expression of synaptic plasticity-associated proteins in rats after SAH. These results indicate that SNAP25 ameliorates cognitive impairment in rats after SAH, suggesting that SNAP25 may represent a novel effective target for the therapy for SAH.

Keywords: Subarachnoid hemorrhage, SNAP25, cognitive impairment, neuronal apoptosis, anti-inflammation

Introduction

Subarachnoid hemorrhage (SAH) is an intractable illness, with a mortality rate of 30% to 50% [1, 2]. Approximately 50% SAH survivors suffer from cognitive impairments, including deficits in attention, learning, memory, language, executive and motor functions [3]. Recent studies have reported that rats reliably developed cognitive dysfunction including spatial learning deficits and visual spatial memory impairments after SAH induced by either prechiasmatic injection of autologous blood or intraluminal perforation [4-6]. So far, several clinical findings have manifested the pathogenesis of cognitive impairment after SAH, and much work has focused on cerebral vasospasm which can cause brain ischemia and contribute to poor outcomes. However, no effective therapies to date can ameliorate cognitive dysfunction. It is noteworthy that abnormal synaptic protein expression was associated with cognitive dysfunction [4]. Nevertheless, little literature concerning the underlying synaptic functions and the molecular mechanisms relating protein changes to synaptic plasticity has been published.

The synaptosomal-associated protein of 25 kDa gene (SNAP25) is a presynaptic plasma membrane protein essential for the triggering of vesicular fusion and neurotransmitter release [7]. It is a characteristic component of synapse and is abundantly expressed by neurons in the hippocampus and other brain regions [8-12]. SNAP25, directly interacting with both syntaxin-1 and vesicle-associated membrane protein 2 (VAMP-2, also known as synaptobrevin-2), forms an integral component of neural soluble *N*-ethylmaleimide-sensitive

factor attachment protein receptor (SNARE) protein complex, which is crucial for the fusion of synaptic vesicles, exocytosis and neurotransmitter release [13-17]. SNAP25 can complex with other proteins such as soluble synaptosomal-associated proteins and the inner plasma [4]. Chen *et al* [4] stated that the expression of SNAP25 in the temporal lobe, hippocampus, and cerebellum of rats was significantly downregulated at days 1 and 3 after SAH, indicating SNAP25 expression and signaling may involve in the pathogenesis of cognitive dysfunction induced by SAH. Besides, SNAP-25 is also known to be implicated in the process of transmitting nerve spikes in the central nervous system and in the memory consolidation in the hippocampus [18, 19]. Thus, we hypothesized that overexpression of SNAP25 may ameliorate the cognitive impairment induced by SAH.

Endovascular perforation is one of the most widely used methods for the induction of SAH and investigation of its acute sequelae [2]. In this study, we used endovascular perforation to induce a SAH rat model. Then we examined the effects and the possible mechanism of SNAP25 on cognitive dysfunction induced by SAH, providing a novel method for SAH therapy.

Materials and methods

Animals

Sprague-Dawley male rats weighing 250-300 g were purchased from Laboratory Animal Center of Shanghai Institutes for Biological Science. All rats had free access to food and water. This study was approved by the Ethics Committee of the First Affiliated Hospital of Zhengzhou University. All animal procedures were in compliance with the National Institutes of Health (NIH) Guidelines for the Care and Use of Laboratory Animals. Rats (N=60) were randomly divided into five groups with 12 for each: Normal group, Sham group, SAH group, SAH+NS group (received intraperitoneal injection of normal saline for 7 days before SAH), and SAH+SNAP25 group (received intraperitoneal injection of 2 mg/kg SNAP25 for 7 days before SAH).

Establishment of SHA models

SHA was induced by endovascular perforation of internal carotid artery (ICA) bifurcation with a sharpened 3-0 nylon suture as previously described by Bederson et al [20], with some modifications. Briefly, rats were received intraperitoneal anesthesia with 1000 mg/kg urethane, and fixed in a stereotactic frame. The right common carotid artery (CCA), the external carotid artery (ECA), and ICA were exposed. ECA was then ligated and fashioned into a stump. The sharpened monofilament suture was advanced centripetally into ICA from ECA through the common carotid bifurcation. The suture was further advanced distally into the intracranial ICA until resistance was felt (at 18 to 20 mm) and then was pushed 3 mm further to perforate the ICA near its intracranial bifurcation. The suture was then withdrawn into ECA, reperfusing the ICA and producing SAH. Sham-operated rats underwent an identical procedure except that the suture was removed once resistance was felt without puncture. An automatic heating pad was used to maintain body temperature at 37.5 ± 0.5°C throughout the whole experiment. All rats were allowed to recover after incision closure before euthanization.

Neurological evaluation

Neurological scores were evaluated at 48 hours with an 18-point scoring system as Garcia et al [21] described in a blinded fashion. Spontaneous activity (in cage for 5 min), symmetry of movements (four limbs), symmetry of forelimbs (outstretching while held by tail), climbing wall of wire cage, reaction to touch on either side of trunk, and response to vibrissae touch were evaluated with scores (0-3) respectively. The scores at the completion of the evaluation is the summation of all the six individual test scores. Lower scores indicate more severe neurological impairment.

Grading system for SAH

48 hours after SAH, rats were sacrificed under anesthesia and the brain samples were removed rapidly. Then brain samples underwent evaluation of SAH severity according to procedures as Sugawara *et al* [22] previously described. High resolution pictures of the base of the brain depicting the circle of Willis and basilar arteries were taken. The basal cistern can be divided into six segments. According to the amount of subarachnoid blood clots, each segment was allotted scores from 0-3 as follows:

0, no subarachnoid hemorrhage; 1, minimal subarachnoid hemorrhage; 2, moderate blood clot with recognized arteries; and 3, blood clots covering all arteries. Only the arteries within the basal cistern were included for the grading system to maintain reproducibility: basilar artery (BA), anterior cerebral artery (ACA), ICA, proximal posterior cerebral artery (pPCA), and posterior communicating artery (PcomA). The total scores ranging from 0 to 18 indicating the degree of SAH severity is the summation of the scores from all six segments. All SAH animals were divided into three subgroups: 0 to 7 (mild SAH), 8 to 12 (moderate SAH), and 13 to 18 (severe SAH).

Determination of brain water content (BWC)

48 h after SHA experiment, all rats were decapitated under anesthesia. Brain sample around blood clots was isolated and the wet weight was recorded. The dry weight was determined after 48 h of desiccation at 100°C in an oven. The percentage of BWC was calculated as (wet weight-dry weight)/wet weight × 100%.

Determination of the blood-brain barrier (BBB) permeability

The rats were anesthetized at 48 h after SAH and injected with Evans blue (EB) in normal saline (2%, 2 mL/kg) from femoral vein. 1 h later, rats were transcardially perfused with normal saline, until the perfusate from the right atrium flowed clear. After that, brain samples were quickly stripped, weighed, and then placed into a test tube with 4 mL formamide. Following incubation in a 54°C water bath for 24 h, the samples were centrifuged. The EB in the supernatants of each sample were subsequently measured at 632 nm. The formamide method was used to measure Evans blue content in brain tissue to evaluate BBB damage severity. Evans blue content in brain tissue (μg/g wet brain) was calculated as A × formamide (mL)/wet weight (g), where A refers to the Evans blue content of the sample (μg/mL) given by the standard curve.

Morris water maze

Morris water maze (MWM) experiment was performed one week after SAH as described previously [23, 24], with some modifications. The apparatus consists of a circular pool (180 cm in diameter, 45 cm in depth) filled with water (30 cm in depth) and plexiglass platform (10 cm in diameter). Animal behaviors were monitored by the EthoVision system. Rats able to find the platform 1 cm beneath the surface of the water within 20-40 s in the training session were selected for MWM experiment. Spatial navigation (invisible platform) and probe trials were conducted. The pool is virtually divided into four equal quadrants and the platform was placed at the center of a fixed quadrant. The time the rats spent reaching the underwater platform from the opposite side of the platform is the escape latency. After that, the platform was removed and the rats were placed in the water at the opposite site of the original platform. The swimming tracks within 2 min were recorded. And the percentage time rats spent finding the target zone and the times of crossing the original platform were calculated.

Immunohistochemistry

48 h after SHA experiment, rats in each group were sacrificed under anesthesia. Brain tissues from blood clots were isolated and paraffin sections were prepared. To detect SNAP25 expression, the sections were deparaffinized and hydrated. 3% H_2O_2 was added to block endogenous peroxidase activity. Following a rinse in distilled water and PBS successively, sections were placed in citrate buffer (10 mM, pH=6.0) and heated in a microwave oven at 95°C for 30 min. After being cooled at room temperature for 20 min and washed in PBS, 5% horse serum was added to block non-specific protein binding. Then sections were incubated with a primary rabbit anti-rat antibody for 1 h and then incubated with goat anti-rabbit IgG for 30 min. The sections were stained with diaminobenzidine (DAB). The percentage of SNAP25 positive cells was calculated as the percentage of positive nuclei (brown) in sections (× 400). To evaluate the apoptosis of brain neurons, the paraffin sections were dewaxed, hydrated and washed. Then proteinase K (20 μL/mL) and Triton X-100 in 0.1% sodium citrate were added successively. Following incubation in 0.3% H_2O_2/methanol, the sections were incubated with 50 g TUNEL at 37°C in a humidified chamber and then stained with DAB. Then the sections were sealed and images were acquired by an Olympus microscope. Apoptosis index (AI)

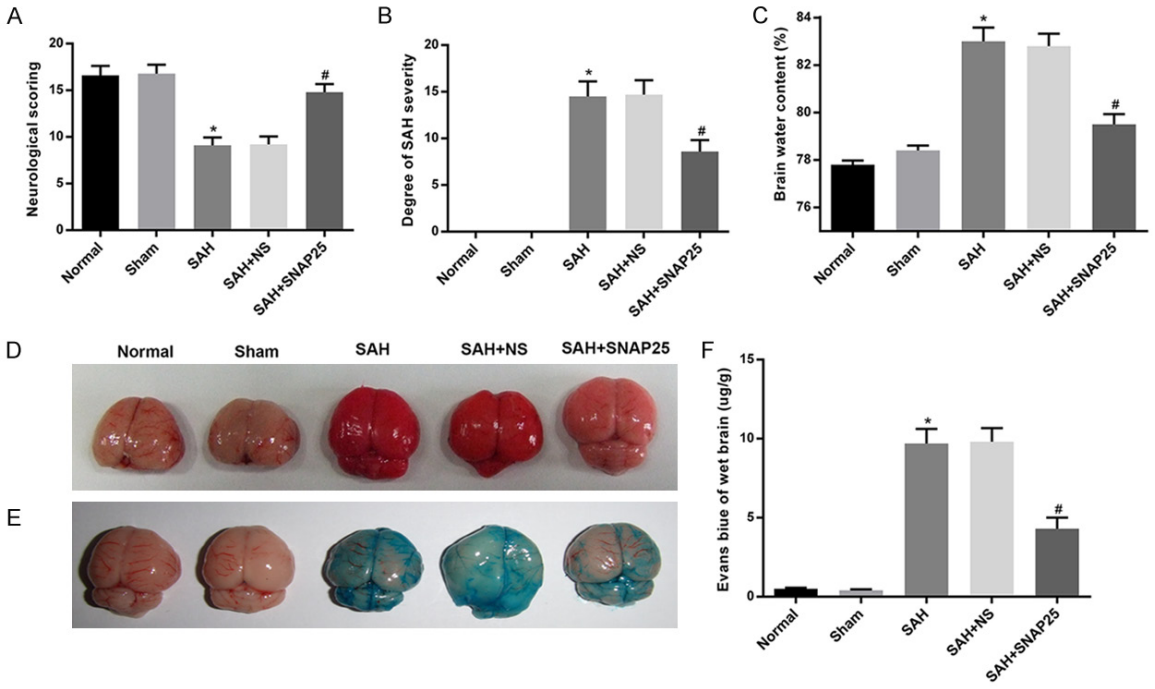

Figure 1. SNAP25 injection ameliorated neurological deficits, subarachnoid hemorrhage, cerebral edema, and BBB disruption in rats after SAH. SD rats (N=60) were randomly divided into five groups with 12 for each: Normal, Sham, SAH, SAH+NS, and SAH+SNAP25 group. SHA was induced by endovascular perforation. 48 h after SHA, (A) the extent of neurological impairment was assessed with an 18-point scoring system, (B) and brain samples underwent evaluation of SAH severity. (C) then BWC in each rat was determined and (D) brain tissues in each group were photographed to assess brain edema. Furthermore, (E) images of brain tissues in each group after EB injection were shown and (F) EB contents in brain tissues (μg/g wet brain) were calculated to evaluate the degree of BBB disruption. $^{*}P<0.05$: vs. Normal, $^{\#}P<0.05$: vs. SAH.

was calculated as the percentage of positive nuclei (brown) in sections (× 400) stained by TUNEL.

Enzyme-linked immunosorbent assay (ELISA)

The levels of various cytokines including interleukin 1-beta (IL-1β), interleukin-6 (IL-6), and tumor necrosis factor-alpha (TNF-α) in brain tissues of rats in each group were measured with an ELISA kit (R&D Systems, USA) according to the manufacturer's protocol.

Western blot

Rats were sacrificed under anesthesia and brain tissues were isolated. In brief, tissues were lysed in in 200 μL lysis buffer. Then equal protein from cell lysates was resolved by 10% SDS-PAGE gels and transferred to PVDF membrane (Millipore, USA). After that, primary antibodies against cleaved caspase-3, Bax, synaptophysin (SYN), activity-regulated cytoskeletal protein (Arc) or microtubule-associated protein 2 (MAP-2) (Abcam, UK) were added, followed by secondary antibodies horseradish peroxidase-conjugated goat anti-rabbit IgG. β-actin was used as an internal control. The protein analysis was visualized by Quantity One software (Bio-Rad Laboratories, USA).

Statistical analysis

All statistical analyses were performed using SPSS 18.0. Differences between two groups were evaluated by Student's t-test. Multiple comparison between the groups was performed using S-N-K method. Data are presented as the mean ± SD from three independent experiments. $P<0.05$ was considered statistically significant.

Results

SNAP25 injection ameliorated neurological deficits, subarachnoid hemorrhage, cerebral edema, and BBB disruption in rats after SAH

Mean neurological scores of the SAH and SAH+NS rats were significantly lower than tho-

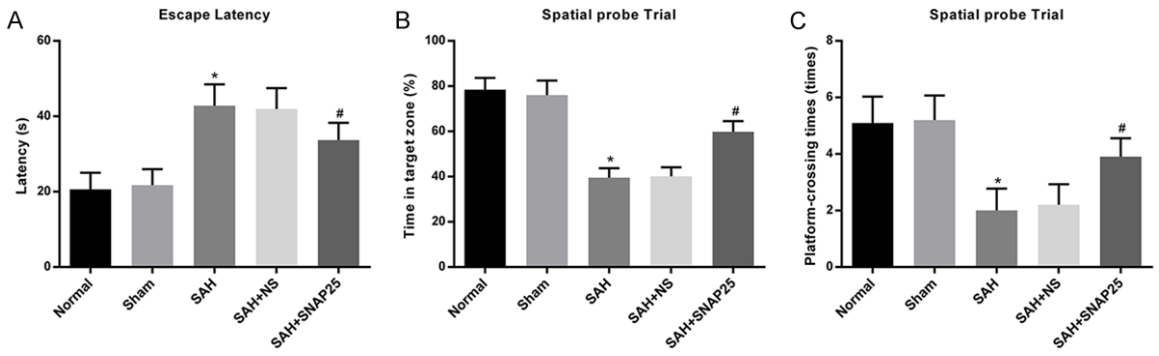

Figure 2. Spatial navigation and probe trials in Morris water maze. A. The escape latency to find the submerged platform, B. The percentage time spent finding the target zone, and C. The times of crossing the original platform were calculated. $^{*}P<0.05$: vs. Normal, $^{\#}P<0.05$: vs. SAH.

se of Normal and Sham groups, and SNAP25 treatment improved neurological scores after SAH (**Figure 1A**). Besides, compared with the two control groups, a markedly increase in the degree of SAH severity was detected in SAH and SAH+NS groups, and SNAP25 moderately reduced the SHA severity (**Figure 1B**). Moreover, for BWC and cerebral edema, SAH and SAH+NS rats showed a drastically increase in brain water in comparison with Normal and Sham rats, and interestingly, SNAP25 decreased BWC and cerebral edema to a level similar to that of Normal or Sham group (**Figure 1C** and **1D**). For BBB permeability, marked extravasation of EB dye into all brain regions was observed and high levels of EB of wet brains were also detected in SAH and SAH+NS groups, when compared with Normal or Sham group. Treatment of SNAP25 greatly ameliorated BBB permeability, as shown by the decreased amount of EB extravasation (**Figure 1E** and **1F**). These results demonstrated that induction of SAH led to more severe neurological dysfunction, subarachnoid hemorrhage, accompanied by the enhancement of cerebral edema formation and an increase in BBB permeability. Fortunately, SNAP25 injection ameliorated such lesion after SAH.

SNAP25 injection ameliorated the impairment of learning and memory in rats after SAH

To evaluate the effect of SNAP25 on spatial learning and memory in rats, Morris water maze experiment was performed one week after SAH. Results of spatial navigation trial demonstrated that, compared with Normal or Sham group, SAH and SAH+NS rats exhibited a significantly reduced learning with longer escape latency. And SNAP25-injected rats showed improved performance with shorter latency than SAH and SAH+NS rats (**Figure 2A**). For spatial probe trial, we found that Normal and Sham rats reached their maximum performances, with the highest level in time in target zone and times of crossing the original platform. Expectedly, compared with the two control groups, SAH and SAH+NS groups showed a markedly reduction in them. However, compared with SAH and SAH+NS groups, injection of SNAP25 induced the improvement of learning and memory, with an increase in the target zone time and platform-crossing times throughout the trial. (**Figure 2B** and **2C**). Our results indicated that the impairment of learning and memory in rats due to SAH could be attenuated by SNAP25 injection.

SNAP25 injection attenuated the reduction of SNAP25 and the increase of neuronal apoptosis in brain tissues of rats after SAH

48 h after SAH experiment, rats in each group were sacrificed under anesthesia. Brain tissues were isolated and paraffin sections were prepared. To evaluate the expression of SNAP25 and the apoptosis of brain neurons, the sections were deparaffinized, hydrated, and washed. Data revealed that, compared to Normal and Sham group, decreased SNAP25 expression and increased TUNEL-positive cells were observed in SAH and SAH+NS rats. However, rats treated with SNAP25 injection showed increased SNAP25 expression and reduced TUNEL-positive cells when compared with SAH and SAH+NS rats (**Figure 3**).

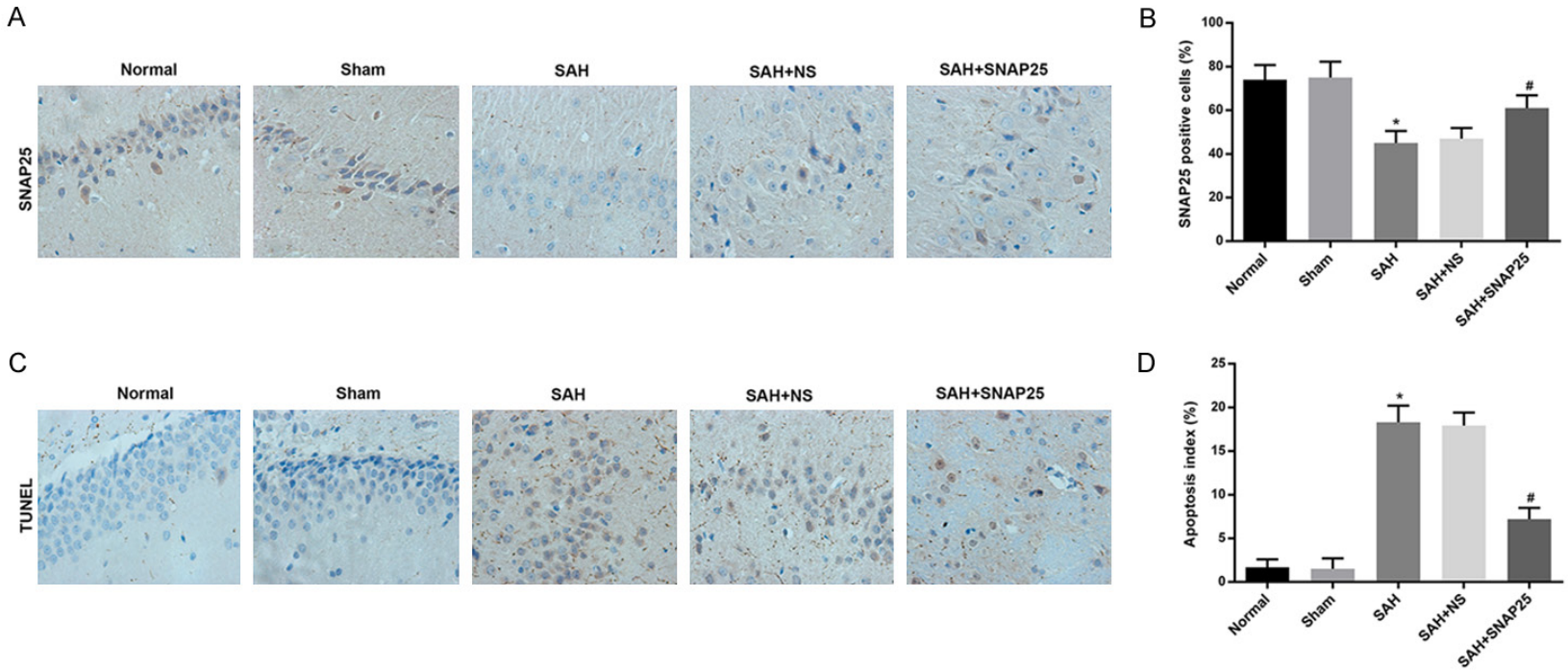

Figure 3. SNAP25 injection attenuated the reduction of SNAP25 and the increase of neuronal apoptosis in brain tissues of rats after SAH. A, B. SNAP25 immunoreactivity in brain tissues of SAH rats and the percentage of SNAP25-positive cells (brown) were shown. C, D. TUNEL staining was performed to evaluate the apoptosis of brain neurons and AI was calculated. $^{*}P<0.05$: vs. Normal, $^{\#}P<0.05$: vs. SAH.

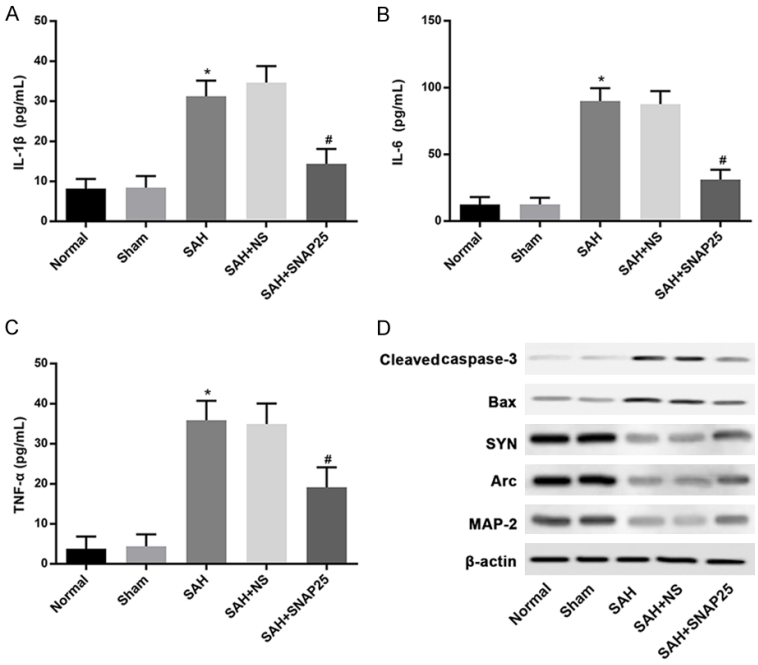

Figure 4. SNAP25 injection attenuated the upregulation of pro-inflammatory factors and apoptosis-related genes, promoted the expression of synaptic plasticity-associated protein in rats after SAH. A-C. The levels of various cytokines (IL-1β, IL-6, TNF-α) in brain tissues of rats in each group were measured with an ELISA kit. D. The protein expression of apoptosis-related proteins (cleaved caspase-3, Bax) and synaptic plasticity-associated proteins (SYN, Arc and MAP-2) was analyzed by Western blot. $^{*}P<0.05$: vs. Normal, $^{\#}P<0.05$: vs. SAH.

SNAP25 injection attenuated the upregulation of pro-inflammatory factors and apoptosis-related genes, promoted the expression of synaptic plasticity-associated protein in rats after SAH

Data demonstrated that, concentrations of pro-inflammatory mediators IL-1β, IL-6, and TNF-α were low in the rat brains in Normal and Sham groups. When compared with the two control groups, levels of the three inflammatory cytokines in SAH and SAH+NS groups were markedly induced after SAH. Interestingly, SNAP25 administration after SAH could greatly suppress SAH-induced elevation of IL-1β, IL-6, and TNF-α (**Figure 4A-C**). In addition, Western blot analysis revealed that, compared with Normal and Sham rats, SAH and SAH+NS rats displayed the upregulation of apoptosis-related genes (cleaved caspase-3 and Bax), accompanied by the downregulation of synaptic plasticity-associated proteins (SYN, Arc, and MAP-2). Meanwhile, SNAP25 injection attenuated such effects induced by SAH (**Figure 4D**).

Discussion

Cognitive impairment can be a long-term complication after SAH [5]. Approximately 50% of SAH patients die from early brain injury (EBI), and many survivors suffer from lasting cognitive deficits [25]. The treatment of cognitive impairment has been regarded as a major target in the management of SAH patients. In the present study, we determined the effects of intraperitoneal administration of SNAP25 on neural and cognitive function, as well as neuron apoptosis in SAH rats. In addition, we investigated the effect of SNAP25 on the expression of pro-inflammatory mediators, apoptosis-related genes, and the synaptic plasticity-associated proteins.

In this study, SHA rats developed EBI including neurological deficits, subarachnoid hemorrhage, increased cerebral edema, and BBB disruption, as well as medium-term cognitive dysfunction, which was in agreement with other studies [22, 26-29]. Thus, reducing the EBI and secondary cognitive impairment is important for the improving poor prognosis of SAH. Hou *et al* [18, 30] stated that, SNAP25, in the hippocampal CA1 and CA3 regions, was involved in the consolidation of contextual fear memory and water-maze spatial learning and memory. Our study found that SNAP25 accelerated the recovery of neurological dysfunction, effectively relieved subarachnoid hemorrhage, brain edema, and BBB disruption, and improved learning deficits in SAH rats, indicating SNAP25 may alleviate the EBI and medium-term cognitive dysfunction by some mechanisms.

Apoptosis plays an important role in neuronal injury after SAH, and the reduction of neuronal apoptosis after SAH can be one of the important strategies for neuroprotection [31]. To find the mechanism of the protective effect of SNAP25 in SAH, we performed TUNEL staining

to evaluate the neural apoptosis. Our results demonstrated that SNAP25 could attenuate SAH-induced neuronal apoptosis in brains of SHA rats. Besides, Western blot analysis revealed that the elevation of apoptosis-related genes (cleaved caspase-3 and Bax) induced by SAH was blocked by SNAP25. This may be the possible mechanism of neuroprotection of SNAP25 in SAH rats.

The decrease of inflammation may also be involved in the regulation of cognitive function after SAH [31]. Toll-like receptor 4 (TLR4) is one of cerebral inflammatory pathways and plays an important role in the secondary brain damage of SAH [32]. Our study found that SNAP25 inhibited TLR4 pathway-related mediators such as IL-1β, IL-6, and TNF-α in SAH rats. This indicated that SNAP25 may exert its inhibitory effects of SHA-induced cognitive impairment by its anti-inflammatory effects.

SNAP25 is a key component of synaptic vesicle-docking/fusion machinery and is likely to play multiple roles in synapses, including synaptic vesicle release, synaptic vesicle recycling [33], and neurite extension [34]. Synapse formation is required for the development of the nervous system and dynamic changes of synapses in the mature brain correlated with cognitive functions. In view of this, our study examined the changes of synaptic plasticity-associated proteins including SYN, Arc, and MAP-2. SYN is a presynaptic vesicle protein located in axons and responsible for formation of synapses [35]. MAP-2 is a dendritic protein implicated in the assembly of the microtubules in the dendrites and in dendritic plasticity [36]. SYN and MAP-2 affect synaptic activity and have integral roles in cognition [37]. Arc is an immediate early gene widely involved in hippocampal-dependent learning and memory and is believed to play an integral role in synapse-specific plasticity [38]. Our study found that SNAP25 promoted the expression of the three synaptic plasticity-associated proteins in rats after SAH, providing another possible mechanism of alleviating cognitive dysfunction in SAH.

In summary, our study suggests for the first time that SNAP25 may attenuate the SAH-induced EBI, medium-term cognitive dysfunction, neuronal apoptosis and activation of inflammation, possibly by its anti-apoptotic and anti-inflammatory properties as well as its ability of promoting expression of synaptic plasticity-associated proteins. Our study provided relatively complete elucidation of the amelioration of SNAP25 on cognitive impairment in SHA models.

Disclosure of conflict of interest

None.

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