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Expression of synaptosomal-associated protein-25 in the rat brain after subarachnoid hemorrhage

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Research Highlights

(1) Synaptosomal-associated protein is an important factor for extracellular secretion and transmitter release, and it is involved in learning consolidation in the hippocampus. Therefore, in this study, we aimed to observe changes in synaptosomal-associated protein-25 expression in the temporal lobe, hippocampus, and cerebellum of rats following subarachnoid hemorrhage.
(2) This study was the first to determine expression of synaptosomal-associated protein-25 in arachnoid hemorrhage models. Synaptosomal-associated protein-25 expression was significantly lower following subarachnoid hemorrhage, which may be related to cognitive disorders after subarachnoid hemorrhage.

Abstract

Synaptosomal-associated protein-25 is an important factor for synaptic functions and cognition. In this study, subarachnoid hemorrhage models with spatial learning disorder were established through a blood injection into the chiasmatic cistern. Immunohistochemical staining and western blot analysis results showed that synaptosomal-associated protein-25 expression in the temporal lobe, hippocampus, and cerebellum significantly lower at days 1 and 3 following subarachnoid morrhage. Our findings indicate that synaptosomal-associated protein-25 expression was down-regulated in the rat brain after subarachnoid hemorrhage.

Key Words

neural regeneration; brain injury; synaptosomal-associated protein-25; subarachnoid hemorrhage; cognition; cortex; hippocampus; cerebellum; grants-supported paper; neuroregeneration

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INTRODUCTION

A substantial portion of people that survive subarachnoid hemorrhage have cognitive impairments, rather than focal neurological deficits[1]. These include deficits in attention, memory, learning, language, executive and motor functions, among which learning and executive functions are the most commonly affected by subarachnoid hemorrhage[2]. In those patients that return to the community, cognitive dysfunction is present in 44% of them. With such dismal consequences, multiple trials are ongoing with the aim of improving the functional outcomes following subarachnoid hemorrhage[2]. The first experimental study concerning cognitive dysfunction after subarachnoid hemorrhage in animal models was reported by Takata and colleagues[3]. These authors demonstrated that rats reliably developed long-term vestibu-

Conflicts of interest: None declared.

Ethical approval: Experimental protocols were permitted by the Animal Ethics Committee of the Soochow University in China.

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lomotor dysfunctions and visual spatial memory impairments following subarachnoid hemorrhage and that cognitive dysfunctions were correlated with selective neuronal loss in the hippocampus and cortex. Afterwards, another team assessed long-term cognitive behaviors in the intraluminal perforation model of subarachnoid hemorrhage in rats[4]. They found that rats had a mild, but statistically significant, cognitive impairments at 5 weeks post-lesion in the intraluminal perforation model of subarachnoid hemorrhage. A recent study using the same model as in the present study was published in 2010, which demonstrated that subarachnoid hemorrhage induced by prechiasmatic injection of autologous blood in rats was associated with spatial learning deficits. Much work on subarachnoid hemorrhage has focused on cerebral vasospasm. This is based on the assumption that severe vasospasm can reduce cerebral blood flow, cause brain ischemia and infarction, and contribute to poor outcomes. It is notable that neuronal death seems to progress over months after cerebral vasospasm. However, the neuronal injury is minimal, suggesting that other functional abnormalities account for the learning deficits observed[5]. Thus, until now, no study of cognitive dysfunction after subarachnoid hemorrhage has focused on the underlying synaptic functions related to cognitive behaviors, and the molecular mechanisms relating protein changes to synaptic plasticity in subarachnoid hemorrhage model remain unclear. Abnormal synaptic protein expression may lead to cognitive dysfunction.

Synaptosomal-associated protein of 25 kDa is a characteristic component of synapses and is highly expressed by neurons in the central nervous system[6-7]. Synaptosomal-associated protein of 25 kDa is an integral component of the synaptic vesicle-docking/fusion core complex and plays an essential role in exocytosis/neurotransmitter release. Synaptosomal-associated protein of 25 kDa is a t-SNARE[7-8] that has several binding regions, allowing it to complex with other proteins such as soluble synaptosomal-associated proteins[9-13] and the inner plasma membrane[14-18]. These unique binding domains are thought to help stabilize the synaptic vesicle fusion complex[19-23]. Although the absence of synaptosomal-associated protein of 25 kDa may not stop neurotransmission, it could dramatically alter vesicle release. Synaptosomal-associated protein of 25 kDa is associated with several physiological functions, including synaptic vesicle release, synaptic vesicle recycling[24], and neurite extension, as seen in neuron repair and synaptogenesis[25]. Synaptosomal-associated protein of 25 kDa is also developmentally regulated with embryonic and adult forms[26].

Synaptosomal-associated protein of 25 kDa in the hippocampus, as a synaptosomal associated protein with a critical role in exocytosis and neurotransmitter release, is involved in memory consolidation[27]. However, no studies have yet focused on the potential contribution of synaptosomal-associated protein of 25 kDa to dysfunctional behavioral processes following subarachnoid hemorrhage.

The aim of the current study was to evaluate changes in synaptosomal-associated protein of 25 kDa expression in the cortex, hippocampus, and cerebellum of rats following subarachnoid hemorrhage.

RESULTS

Quantitative analysis of experimental animals

Forty-two rats were assigned randomly to a normal group (normal feeding), control group (physiological saline injected into the prechiasmatic cistern), and subarachnoid hemorrhage group (non-heparinized fresh autologous arterial blood from the tail was injected into the prechiasmatic cistern to establish the subarachnoid hemorrhage model). According to different time points, animals in the subarachnoid hemorrhage group were assigned into five subgroups, namely Day 1, Day 3, Day 5, Day 7, and Day 14 groups. There were six rats in each group. All 42 rats were involved in the final analysis.

General observations of brain tissue from subarachnoid hemorrhage rats

No significant changes in body weight, mean arterial blood pressure, temperature, or injected arterial blood gas data were detected in any of the experimental groups (data not shown). The cerebral blood flow and mean arterial blood pressure in the control and subarachnoid hemorrhage groups are shown in Figure 1. Blood pressure began to increase and cerebral blood flow began to decrease at 15 minutes following subarachnoid hemorrhage and then returned to baseline levels at 30 minutes. As shown in Figure 2, the rats in the Day 1 and Day 3 groups exhibited blood clots over the basal surface of the brainstem and Circle of Willis but the blood staining at the base of the Circle of Willis was not observed in the Day 5, Day 7, and Day 14 groups.

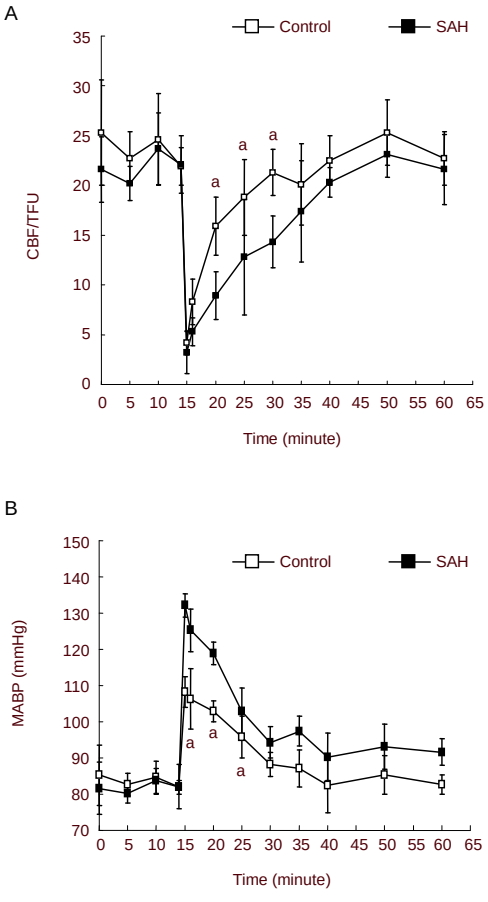

Figure 1 The time course of cerebral blood flow (CBF) and mean arterial blood pressure (MABP) in the control (n = 6) and subarachnoid hemorrhage (SAH) groups (n = 30).

The data are expressed as mean ± SD. [a]P < 0.05, *vs*. SAH group. One-way analysis of variance and Fisher's least significant difference *post-hoc* test were used to compare the intergroup differences. 1 mmHg = 0.133 kPa.

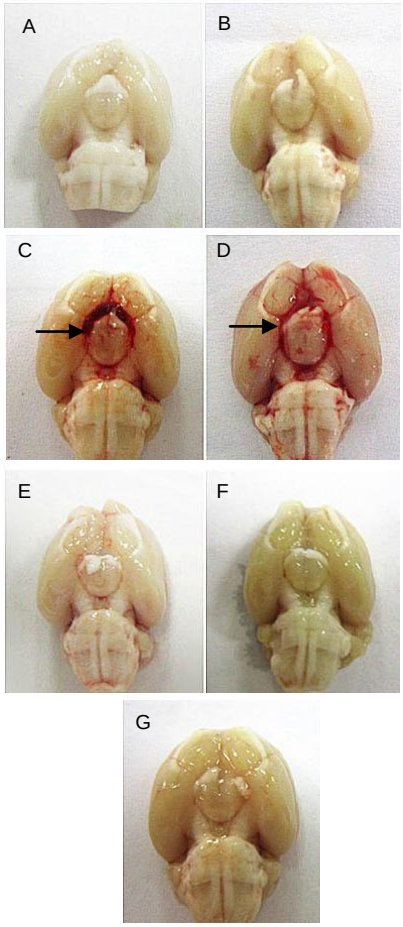

Figure 2 Brain specimens of subarachnoid hemorrhage (SAH) rats.

(A–G) Ventral view of typical brains from Normal (A), Control (B), SAH Day 1 (C), SAH Day 3 (D), SAH Day 5 (E), SAH Day 7 (F), and SAH Day 14 (G) groups.

The images in C and D show blood clots (arrows) at the base of the Circle of Willis and brainstem.

Western blot analysis of synaptosomal-associated protein of 25 kDa protein expression in the temporal lobe, hippocampus, and cerebellum of subarachnoid hemorrhage rats

Synaptosomal-associated protein of 25 kDa protein expression was detected by western blot analysis. In the cortex, high levels of synaptosomal-associated protein of 25 kDa protein expression were found in the normal and control groups (Figure 3). Synaptosomal-associated protein of 25 kDa protein expression was significantly lower after subarachnoid hemorrhage and peaked on day 1. There were statistically significant differences between the control group and subarachnoid hemorrhage Day 1, 3, and 5 groups (P < 0.01 or P < 0.05). In the hippocampus and cerebellum, high levels of synaptosomal-associated protein of 25 kDa protein expression were also found in the normal and control groups (Figure

3). Synaptosomal-associated protein of 25 kDa protein expression was significantly lower in the hippocampus and cerebellum following subarachnoid hemorrhage on days 1–7 ($P < 0.01$).

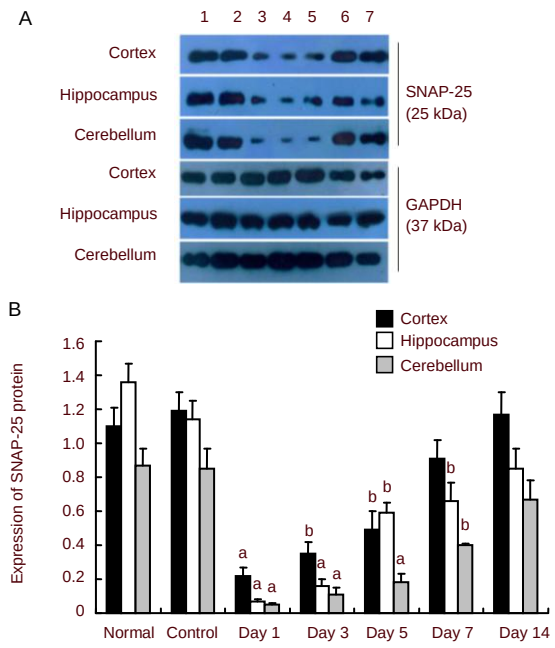

Figure 3 Synaptosomal-associated protein-25 (SNAP-25) protein expression in brain tissue of subarachnoid hemorrhage (SAH) rats.

(A) Western blot bands of autoradiograms of SNAP-25 protein in rat brain tissue. These bands show that expression of SNAP-25 protein was lower in the SAH groups and the lowest time-point was approximately on days 1 and 3. Lane 1: Normal; lane 2: control; lanes 3–7: Days 1, 3, 5, 7 and 14 groups.

(B) Quantitative analysis of SNAP-25 protein expression in rat brain tissue. SNAP-25 protein expression (SNAP-25/GAPDH absorbance) in SAH groups was significantly lower than in the control group. The data are expressed as mean ± SD ($n = 6$ in each group). $^{a}P < 0.01$, $^{b}P < 0.05$, *vs*. control group. One-way analysis of variance and Fisher's least significant difference *post-hoc* test were used to compare the intergroup differences. GAPDH: Glyceraldehyde phosphate dehydrogenase.

Immunohistochemical detection of synaptosomal-associated protein of 25 kDa expression in the temporal lobe, hippocampus, and cerebellum of subarachnoid hemorrhage rats

In temporal lobe

To assess the localization and expression of synaptosomal-associated protein of 25 kDa in the cortex after subarachnoid hemorrhage, we performed an immunohistochemical study. Synaptosomal-associated protein of 25 kDa-positive cells were observed in the normal and control groups (Figure 4A, B). Fewer synaptosomal-associated protein of 25 kDa-positive cells in the subarachnoid hemorrhage groups were found in the cortex (Figure 4C–F). Synaptosomal-associated protein of 25 kDa-positive cells were present mainly in neurons (Figure 4G). Compared with the control group, synaptosomal-associated protein of 25 kDa expression was significantly lower in the cortex on days 1, 3, 5, and 7 following subarachnoid hemorrhage ($P < 0.01$, $P < 0.01$, $P < 0.01$, and $P < 0.05$ for Day 1, Day 3, Day 5, and Day 7 groups *vs*. control group, respectively; Figure 4H). In the subarachnoid hemorrhage Day 14 group, synaptosomal-associated protein of 25 kDa expression was not significantly different compared with the control group (Figure 4H).

In the hippocampus

Synaptosomal-associated protein of 25 kDa-positive cells were observed in the hippocampus of the normal and control groups, which indicates rich expression of synaptosomal-associated protein of 25 kDa in the normal hippocampus of rats (Figure 5A, B). Fewer synaptosomal-associated protein of 25 kDa-positive cells in the subarachnoid hemorrhage groups were found on days 1 and 3 (Figure 5C, D). Expression of synaptosomal- associated protein of 25 kDa was mainly localized to neurons (Figure 5). On days 1 and 3 following subarachnoid hemorrhage, synaptosomal-associated protein of 25 kDa expression was significantly lower compared with the control group (Figure 5H). There were no statistically significant differences between Day 7 or Day 14 groups and the control group ($P > 0.05$; Figure 5H).

In the cerebellum

Synaptosomal-associated protein of 25 kDa was expressed at a high level in the normal and control groups. In contrast, in the subarachnoid hemorrhage groups, synaptosomal-associated protein of 25 kDa was expressed at a low level in a time-independent manner (Figure 6). There was a statistically significant difference between the control group and the Day 1, 3, 5, and 7 groups ($P < 0.01$ or $P < 0.05$). Expression of synaptosomal-associated protein of 25 kDa was mainly localized to neurons and was significantly lower in the subarachnoid hemorrhage groups. Synaptosomal-ass- ociated protein of 25 kDa expression was not significantly different between the control group and Day 14 group ($P > 0.05$).

DISCUSSION

To our knowledge, this study is the first to report that synaptosomal-associated protein of 25 kDa expression is lower in cortex, hippocampus, and cerebellum early after subarachnoid hemorrhage in rats.

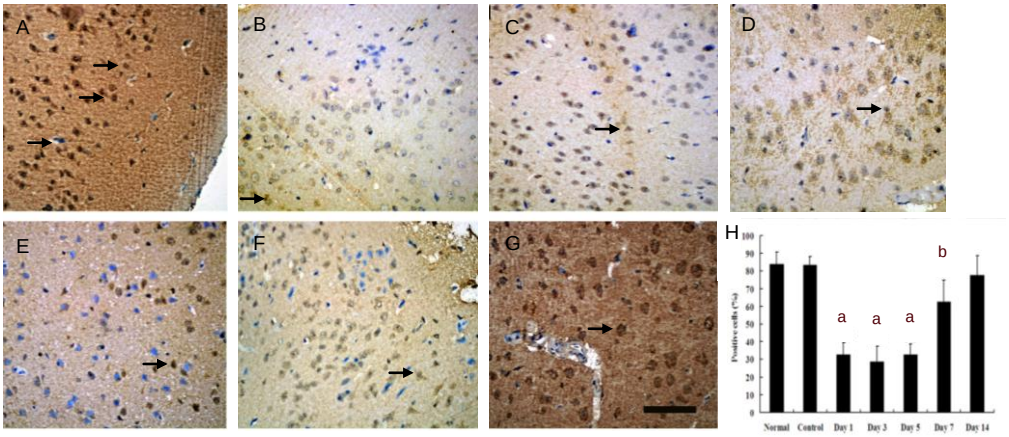

Figure 4 Synaptosomal-associated protein-25 (SNAP-25) immunoreactivity in the temporal lobe of subarachnoid hemorrhage rats (scale bars: 50 μm, light microscopy, diaminobenzidine staining, × 200).

The SNAP-25-positive cells were abundant in the normal and control groups (A, B), but fewer SNAP-25-positive cells were detected in the subarachnoid hemorrhage Day 1–7 groups (C–F). The SNAP-25-positive cells in the subarachnoid hemorrhage groups were present mainly in neurons (shown as brown and arrows).

(H) The percentage of SNAP-25-positive cells (Y axis) in each group. The data are expressed as mean ± SD (n = 6 in each group). Ordinates represent the percentage of cells positive for SNAP-25. $^{a}P < 0.01$, $^{b}P < 0.05$, *vs*. control and normal groups. One-way analysis of variance followed by Fisher's least significant difference *post-hoc* test was used to compare the intergroup differences.

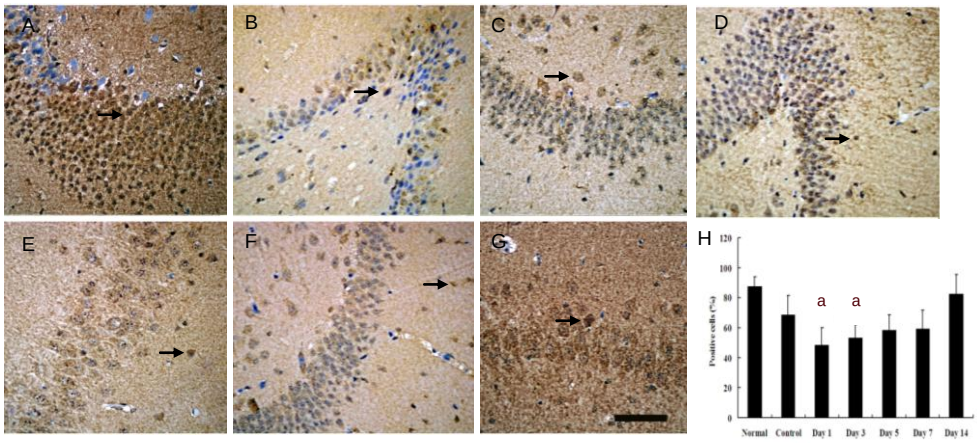

Figure 5 Synaptosomal-associated protein-25 (SNAP-25) immunoreactivity in the hippocampus of subarachnoid hemorrhage (SAH) rats (scale bars: 50 μm, light microscopy, diaminobenzidine staining, × 200).

Relatively levels of SNAP-25-positive cells were observed in the normal (A), control group (B), SAH Day 5 (E), SAH Day 7 (F), and SAH Day 14 groups (G), which indicates that there is abundant expression of SNAP-25 in the normal hippocampus of rats or at the late stages of SAH. SNAP-25-positive cells were present mainly in neurons (shown as brown and arrows). In the SAH Day 1 and 3 groups (C, D), relatively few SNAP-25-positive cells were observed.

(H) The percentage of SNAP-25-positive cells (Y axis) in each group. The data are expressed as mean ± SD (n = 6 in each group). Ordinates represent the percentage of cells positive for SNAP-25. $^{a}P < 0.01$, *vs*. control and normal groups. One-way analysis of variance followed Fisher's least significant difference *post-hoc* test was used to compare the intergroup differences.

Lower levels of synaptosomal-associated protein of 25 kDa expression were apparent on days 1 and 3 and synaptosomal-associated protein of 25 kDa expression recovered by day 14. This leads to the hypothesis that decreased synaptosomal-associated protein of 25 kDa expression in the brain may be important in modulating the function of synapses, and we tentatively put forward that this phenomenon may be critically involved in neurobehavioral dysfunction after subarachnoid hemorrhage.

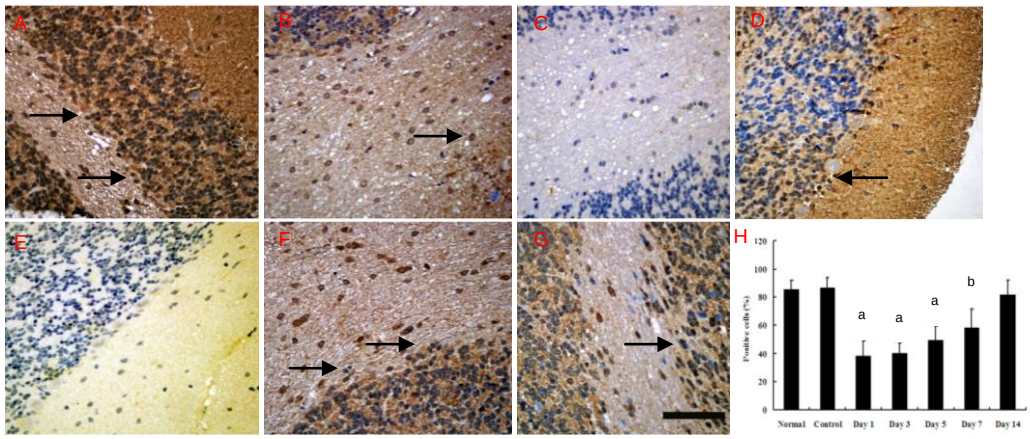

Figure 6 Synaptosomal-associated protein-25 (SNAP-25) immunoreactivity in the cerebellum of subarachnoid hemorrhage (SAH) rats (scale bars: 50 μm, light microscopy, diaminobenzidine staining, × 200).

SNAP-25-positive cells were present mainly in Purkinje cells (shown as brown and arrows). (A) Normal group; (B) control group; (C) SAH Day 1 group; (D) SAH Day 3 group; (E) SAH Day 5 group; (F) SAH Day 7 group; (G) SAH Day 14 group.

(H) The percentage of SNAP-25-positive cells in each group. The SAH groups showed statistically significant down-regulation of SNAP-25 expression compared with control group. Data are expressed as mean ± SD (n = 6 in each group). Ordinates represent the percentage of cells positive for SNAP-25 protein (Y axis). $^{a}P < 0.01$, $^{b}P < 0.05$, *vs*. control and normal groups. One-way analysis of variance followed Fisher's least significant difference *post-hoc* test was used to compare the intergroup differences.

Any factor that causes abnormalities in the function and structure of the cerebral cortex and subcortex may lead to cognitive impairments. Brain abnormalities such as abnormalities of nerve growth factor receptors, neuropeptides, and neurotransmitters including acetylcholine, norepinephrine, and, dopamine are likely to cause cognitive impairments. Patients suffering from subarachnoid hemorrhage always have varying degrees of cognitive impairment. Recently, the protection of cognitive function after subarachnoid hemorrhage has been getting more and more attention. As such, our research is focused in this area.

Synaptosomal-associated protein of 25 kDa is one of the so-called soluble N-ethylmaleimide-sensitive fusion protein receptor proteins that play essential roles in neurotransmitter release through the formation of a core complex with other soluble N-ethylmaleimide-sensitive fusion protein receptor proteins, VAMP-2/synaptobrevin 2, and syntaxin 1[28-33]. In addition to its essential role in neurotransmitter release, the possible involvement of synaptosomal-associated protein of 25 kDa in neurite extension and sprouting[34-38], regulation of ion channel functions[27, 39-41], and neurotransmitter receptor incorporation into the plasma membrane[42-45] has been suggested. Thus, synaptosomal-associated protein of 25 kDa is likely to play multiple roles in synapses, and the elucidation of its regulatory mechanisms will contribute to the understanding of synaptic plasticity. It should be noted that this study has examined synaptosomal-associated protein of 25 kDa expression in the adult rat brains and we tentatively put forward that synaptosomal-associated protein of 25 kDa was somewhat abundant in the neurons of cortex, hippocampus, and cerebellum in the physiological state, and it was down-regulated after blood injection in this subarachnoid hemorrhage model.

Synapse formation is required for the development of the nervous system and dynamic changes of synapses in the mature brain are associated with cognitive functions such as learning and memory. Notably, aberrant synapse structures are present in mental retardation and neurological disorders[46-47]. Elucidating the molecular machinery that organizes synapses is therefore relevant to our understanding both of physiological functions as well as debilitating brain disorders. As mentioned by Hou *et al* [27], synaptosomal-associated protein of 25 kDa in the hippocampal CA3 area is involved in regulating long-term potentiation and formation of long-term memory, consistent with its role in learning and memory functions in the hippocampal CA1 area.

In this study, synaptosomal-associated protein of 25 kDa was down-regulated in the cortex, hippocampus, and cerebellum, which suggests that long-term memory formation may be impaired after subarachnoid hemorrhage.

However, additional work is needed in this area to fully explore the consequences of these findings. The future of this topic lies in a complete elucidation of cognitive dysfunction in relation to synaptosomal-associated protein of 25 kDa in subarachnoid hemorrhage models.

Our findings show that synaptosomal-associated protein of 25 kDa expression in the rat brain (cortex of temporal lobe, hippocampus, cerebellum) was significantly lower after subarachnoid hemorrhage, and reached its minimum on day 1. The degree of the decrease in synaptosomal-associated protein of 25 kDa varied across different brain tissues. The decline was the most obvious in the hippocampus, but less in cortex of temporal lobe, and finally in the cerebellum.

In summary, we found that synaptosomal-associated protein of 25 kDa expression was down-regulated after subarachnoid hemorrhage. Previous studies showed that synaptosomal-associated protein of 25 kDa is critical for cognitive function, and the present study demonstrated the lower expression of synaptosomal-associated protein of 25 kDa in the brain following subarachnoid hemorrhage, which suggests that synaptosomal-associated protein of 25 kDa expression and signaling could participate in the pathogenesis of cognitive dysfunction induced by subarachnoid hemorrhage. Accordingly, the role of synaptosomal-associated protein of 25 kDa in the pathogenesis of cognitive dysfunction related to synaptic plasticity-related proteins deserve further study.

MATERIALS AND METHODS

Design

A randomized, controlled animal experiment.

Time and setting

This experiment was conducted at the Experimental Center of the First Affiliated Hospital of Soochow University, China from March 2011 to July 2012.

Materials

Sprague-Dawley rats aged 3 months and weighing 300–350 g were purchased from the Animal Center of Chinese Academy of Sciences, Shanghai, China (license No. SYXK 2012-0045). The rats were housed in temperature and humidity controlled animal quarters with a 12-hour light/dark cycle.

Animal use and care protocols, including all operation procedures, were approved by the Animal Care and Use Committee of Soochow University and conformed to the *Guide for the Care and Use of Laboratory Animals* issued by the National Institutes of Health. The protocols were also conducted in accordance with the *Guidance Suggestions for the Care and Use of Laboratory Animals*, formulated by the Ministry of Science and Technology of China[48].

Methods

Establishment of subarachnoid hemorrhage models

Following intraperitoneal anesthesia with urethane (1 000 mg/kg), each rat's head was fixed in a stereotactic frame. Body temperature was maintained at 37.5 ± 0.5°C with an automatic heating pad (LSI Letica Scientific Instruments, Barcelona, Spain). The tail artery was cannulated to measure mean arterial blood pressure and to obtain blood samples. The rats were placed in a stereotaxic frame (David Kopf Instruments, Tujunga, California, USA) with the mouthpiece at 0°. A laser Doppler flowmeter (MBF3D, Moor Instruments, Delaware, USA) was used for continuous monitoring of cerebral blood flow in the cerebral cortex supplied by the middle cerebral artery. To place the laser Doppler flowmeter probes, a bur hole was drilled 5 mm left and 1 mm posterior to the bregma without injury to the dura mater.

The experimental subarachnoid hemorrhage model was produced using stereotaxic insertion of a needle with a rounded tip and a side hole into the prechiasmatic cistern as previously described[49-53]. An infusion of 0.3 mL of non-heparinized fresh autologous arterial blood was slowly injected into the prechiasmatic cistern over 20 seconds with a syringe pump using aseptic techniques. Control animals were injected with 0.3 mL of saline. The animals were allowed to recover for 45 minutes after subarachnoid hemorrhage. After the operation, each rat was then returned to their cages maintained at 23 ± 1°C. 20 mL of 0.9% NaCl was injected subcutaneously after the operation to prevent dehydration. Heart rate and rectal temperature were monitored, and rectal temperature was kept at 37.0 ± 0.5°C, using physical cooling (ice bag) when required throughout the experiments. We observed that the inferior basal temporal lobe was always stained by blood. Tissues from the cortex, hippocampus, and cerebellum were separated on ice, paraffin-embedded sections fixed, and frozen in liquid nitrogen immediately for molecular biological and biochemical experiments.

Preparation of brain samples

The rats scheduled for death were anesthetized with an intraperitoneal injection of ketamine (100 mg/kg) and

xylazine (0.5 mg/kg). Perfusion was then performed. The thorax was opened with a cannula placed in the left ventricle, the descending thoracic aorta was clamped, and the right atrium was open. Perfusion was performed with 250 mL of PBS (pH 7.4) at 37°C under a perfusion pressure of 120 cm H_2O. After perfusion, the brain sample was removed and rinsed in 0.9% normal saline (4°C) several times to wash away blood and blood clot. And then the cortex, hippocampus and cerebellum were separated on ice under microscope and frozen in liquid nitrogen immediately for molecular biological and biochemical experiments.

Western blot analysis for synaptosomal-associated protein of 25 kDa protein expression in the cortex, hippocampus, and cerebellum

The frozen brain tissue was mechanically lysed in 20 mmol/L of Tris at pH 7.6, which contained 0.2% sodium dodecyl sulphate, 1% Triton X-100, 1% deoxycholate, 1 mmol/L phenylmethylsulphonyl fluoride, and 0.11 U/mL aprotinin (Sigma-Aldrich Inc., St. Luis, MO, USA). Lysates were centrifuged at 12 000 × *g* for 20 minutes at 4°C. The protein concentration was estimated by the Bradford method using the Nanjing Jiancheng protein assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, Jiangsu Province, China). The samples (60 μg per lane) were separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis and electro-transferred onto a polyvinylidene-difluoride membrane (Bio-Rad Laboratory, Hercules, CA, USA). The membranes from the sample were blocked with 5% skimmed milk for 2 hours at room temperature and incubated overnight at 4°C with a primary rabbit anti-rat monoclonal antibody directed against synaptosomal-associated protein of 25 kDa (1:200; Santa Cruz Biotechnology, Santa Cruz, CA, USA). GAPDH (rabbit anti-rat, 1:6 000, Sigma-Aldrich) was used as a loading control. After the membrane was washed six times for 10 minutes in TBS + Tween-20, it was incubated in a horseradish peroxidase-conjugated secondary goat anti-rabbit monoclonal antibody (1:400 in PBST, Santa Cruz Biotechnology) for 2 hours.

The blotted protein bands were visualized by enhanced chemiluminescence using western blot detection reagents (Amersham, Arlington Heights, IL, USA) and exposed to X-ray film. The developed films were digitized using an Epson Perfection 2480 scanner (Seiko Corp, Nagano, Japan). Optical densities were obtained using Glyko Bandscan software (Glyko, Novato, CA, USA) and the synaptosomal-associated protein of 25 kDa expression levels were normalized to GAPDH.

Immunohistochemical detection for synaptosomal-associated protein of 25 kDa expression in the cortex, hippocampus, and cerebellum

Immunohistochemistry on formalin-fixed paraffin-embedded sections was performed to determine synaptosomal-associated protein of 25 kDa immunoreactivity.

Sections were deparaffinized and rehydrated in graded concentrations of ethanol to distilled water. Endogenous peroxidase activity was blocked with 3% H_2O_2 for 5 minutes, followed by a brief rinse in distilled water and a 15-minute wash in PBS. Sections were placed in 10 mmol/L citrate buffer (pH 6.0), and heated in a microwave oven at 95°C for 30 minutes. Sections were cooled at room temperature for 20 minutes and rinsed in PBS. Non-specific protein binding was blocked by a 40-minute incubation in 5% horse serum. Sections were incubated with a primary rabbit anti-rat antibody (1:200; Santa Cruz Biotechnology) for 1 hour at room temperature, followed by a 15-minute wash in PBS. Sections were incubated with goat anti-rabbit IgG (1:500; Santa Cruz Biotechnology) for 60 minutes at room temperature. 3,3'-Diaminobenzidine was used as the chromogen and counterstained with hematoxylin. Sections incubated in the absence of primary antibody were used as negative controls.

Microscopy of the immunohistochemically stained tissue sections was performed by an experienced pathologist blinded to the experimental condition with a microscopic image system (Carl Zeiss International, Shanghai, China).

Statistical analysis

All data are presented as mean ± SD. SPSS 16.0 software (SPSS, Chicago, IL, USA) was used for statistical analysis of the data. Differences between experimental groups were determined by one-way analysis of variance followed by the Fisher's least significant deference *post-hoc* test. Statistical significance was accepted at $P < 0.05$.

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