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Hippocampal expression of synaptic structural proteins and phosphorylated cAMP response element-binding protein in a rat model of vascular dementia induced by chronic cerebral hypoperfusion☆

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Abstract

The present study established a rat model of vascular dementia induced by chronic cerebral hypoperfusion through permanent ligation of bilateral common carotid arteries. At 60 days after modeling, escape latency and swimming path length during hidden-platform acquisition training in Morris water maze significantly increased in the model group. In addition, the number of accurate crossings over the original platform significantly decreased, hippocampal CA1 synaptophysin and growth-associated protein 43 expression significantly decreased, cAMP response element-binding protein expression remained unchanged, and phosphorylated cAMP response element-binding protein expression significantly decreased. Results suggested that abnormal expression of hippocampal synaptic structural protein and cAMP response element-binding protein phosphorylation played a role in cognitive impairment following chronic cerebral hypoperfusion.

Key Words: cAMP response element-binding protein; chronic cerebral hypoperfusion; growth associated protein 43; learning and memory; synaptophysin; vascular dementia

Abbreviations: CCH, chronic cerebral hypoperfusion; GAP-43, growth-associated protein 43; SYN, synaptophysin; CREB, cAMP response element-binding protein

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INTRODUCTION

Synaptic loss is regarded as the main risk factor for cognitive impairment following vascular dementia induced by chronic cerebral hypoperfusion (CCH)[1-2].

Growth-associated protein 43 (GAP-43) and synaptophysin (SYN) are important proteins involved in synaptic plasticity, nerve fiber growth and development, axonal regeneration, and synaptic functional maintenance[3-6].

During acute injury to the nervous system, cAMP response element-binding protein (CREB) phosphorylation regulates transcription and expression of various genes, promote expression of various proteins (c-Fos, Jun-B, and Bcl-2) and neurotrophic factors (nerve growth factor and brain-derived neurotrophic factor), and mediate regeneration and repair of injured neurons, as well as synaptic plasticity, thereby providing neuroprotection[7].

The present study utilized a rat model of CCH induced by permanent ligation of bilateral common carotid arteries. Learning and memory were tested in model and normal rats using the Morris water maze, and expression of hippocampal SYN, GAP-43, CREB total protein, and phosphorylated protein were analyzed to better understand the molecular mechanisms of CCH-induced cognitive impairment.

RESULTS

Quantitative analysis of experimental animals

A total of 32 Sprague-Dawley rats were randomly assigned to sham-surgery (n = 12, common carotid artery was not ligated) and model (n = 20; CCH[1]) groups. Nine model rats died within 60 days following model establishment, and 23 rats were included in the final analysis.

Learning and memory function decreased in CCH rats

At 60 days after modeling, learning and memory were detected using the Morris water maze, which included spatial reference memory and working memory tests.

On the first day, spatial reference memory testing *via* hidden-platform acquisition training resulted in no significant difference in time spent locating the platform or path length between sham-surgery and model groups. However, after 2–4 days, escape latency and swimming path length increased in the model group compared with the sham-surgery group ($P < 0.01$ or $P < 0.05$, respectively; Figure 1). Spatial probe testing showed that after training, the ratio of swimming time to platform in quadrant to total swimming time (tP/tT), as well as the ratio of swimming path length to total swimming distance (dP/dT), were similar between model and sham-surgery groups ($P > 0.05$). However, the frequency with which the rats accurately crossed the original platform location significantly decreased in the model group compared with the sham-surgery group ($P < 0.01$; Table 1).

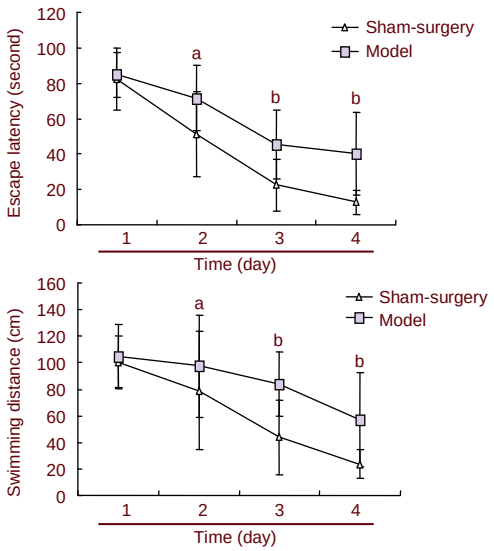

Figure 1 Comparison of escape latency of hidden platform and swimming path length between sham-surgery and model groups.

Each rat was trained four times each day, and mean escape latency (A) and swimming path length (B) in each group were calculated each day.

Results were expressed as mean ± SD from 12 rats in the sham-surgery group and 11 rats in the model group, respectively. [a]$P < 0.05$, [b]$P < 0.01$, *vs*. sham-surgery group (Student-Newman-Keuls test).

Table 1 Comparison of spatial search ability between sham-surgery and model groups

Group	tP/tT (%)	dP/dT (%)	Times to cross the platform (times/30 seconds)
Sham-surgery	0.29±0.06	0.28±0.09	1.33±0.49
Model	0.23±0.08	0.22±1.00	0.45±0.52[a]

Results are expressed as mean ± SD from 12 rats in the sham-surgery group and 11 rats in the model group, respectively.

[a]$P < 0.01$, *vs*. sham-surgery group (Student-Newman-Keuls test). tP/tT: Ratio of swimming time to platform in quadrant to total swimming time; dP/dT: ratio of swimming path length to total swimming distance.

In the spatial working memory test, when the platform was moved to the first quadrant, the time spent locating the platform (escape latency), as well as swimming path length, significantly increased in the model group compared with the sham-surgery group ($P < 0.01$). However, when the platform was moved to the second quadrant, the time spent locating the platform significantly increased in the model group compared with the sham-surgery group ($P < 0.05$). In addition, when the platform was placed in the fourth quadrant, the swimming path length significantly increased in the model group compared with the sham-surgery group ($P < 0.05$; Figure 2).

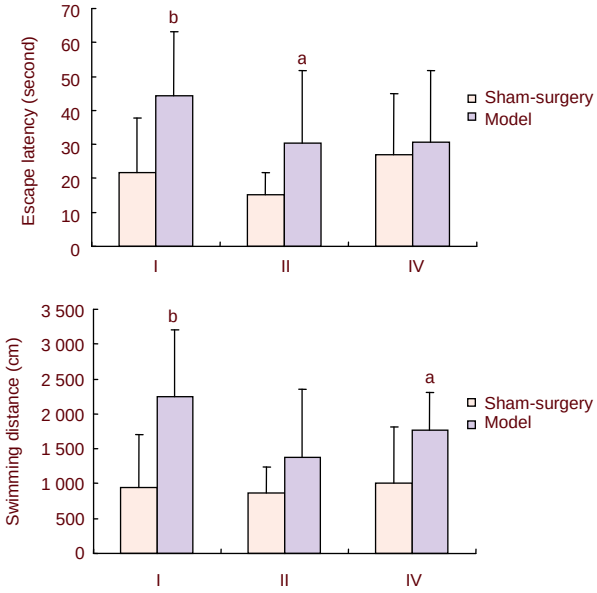

Figure 2 Comparison of escape latency of platform and swimming path length between sham-surgery and model groups.

Following spatial probe tests, the Morris water maze platform was placed in the I, II, IV quadrants, respectively, and the rat was placed into the water at the opposite or adjacent quadrant. Escape latency (A) and swimming path length (B) were recorded.

Results are expressed as mean ± SD from 12 rats in the sham-surgery group and 11 rats in the model group, respectively. [a]$P < 0.05$, [b]$P < 0.01$, *vs*. sham-surgery group (Student-Newman-Keuls test).

Hippocampal SYN expression significantly decreased in CCH rats

Immunohistochemical staining revealed SYN-positive products, which appeared as a stained particle-shaped substance, in the sham-surgery group. The positive cells were densely distributed, and some particles surrounded and outlined the cells. In the model group, SYN expression significantly decreased. Imaging analysis revealed significantly decreased number of hippocampal CA1 SYN-positive cells in the model group compared with the sham-surgery group ($P < 0.01$; Figure 3, Table 2).

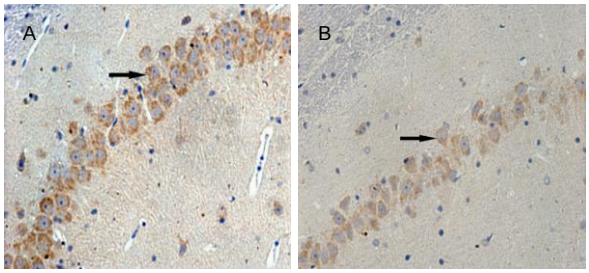

Figure 3 Synaptophysin (SYN) expression in hippocampal CA1 region (immunohistochemical staining, × 200). Arrows represent SYN immunoreactive products.

(A) SYN immunoreactive products are visible as thick, darkly stained particles and dense distribution in the sham-surgery group.

(B) SYN immunoreactive products are lightly stained in the model group.

Table 2 Synaptophysin (SYN) expression in the hippocampus

Group	Immunohistochemistry		Western blot
	SYN-positive cells (n = 6, n/mm)	SYN absorbance (n = 6)	Absorbance of SYN/GAPDH (n = 5)
Sham-surgery	39.58±11.36	24.26±5.68	2.05±1.52
Model	25.83±8.87[b]	12.52±1.69[b]	1.28±0.65[a]

Results are expressed as mean ± SD. [a]P < 0.05, [b]P < 0.01, *vs.* sham-surgery group (Student-Newman-Keuls test).

Western blot results showed that hippocampal SYN protein expression was significantly less in the model group than in the sham-surgery group (P < 0.05; Figure 4, Table 2).

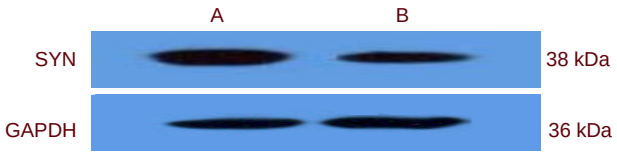

Figure 4 Synaptophysin (SYN) expression in hippocampal CA1 region (western blot).

There were five rats in each group. GAPDH served as the internal reference. A: Sham-surgery group; B: model group.

Hippocampal GAP-43 expression decreased in CCH rats

Immunohistochemical staining revealed GAP-43 immunoreactive products. Compared to the sham-surgery group, the number of hippocampal CA1 GAP-43-positive cells significantly decreased in the model group (P < 0.05), but no difference was observed in absorbance values between the two groups (P > 0.05; Figure 5, Table 3).

Western blot results showed no significant difference in hippocampal GAP-43 protein expression between model and sham-surgery groups (P > 0.05; Figure 6, Table 3).

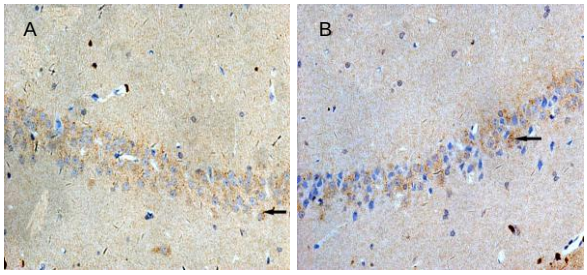

Figure 5 Growth-associated protein 43 (GAP-43) expression in hippocampal CA1 region (immunohistochemical staining, × 200). Arrows represent GAP-43-positive cells.

(A) In the sham-surgery group, GAP-43 immunoreactive products are visible as thick particles that are densely distributed.

(B) In the model group, GAP-43-positive cells are lightly stained with no typical neuronal structures.

Table 3 Growth-associated protein 43 (GAP-43) expression in the hippocampus

Group	Immunohistochemistry		Western blot
	GAP-43-positive cells (n = 6, n/mm)	GAP-43 absorbance (n = 6)	Absorbance of GAP-43/GAPDH (n = 5)
Sham-surgery	29.75±9.16	28.53±7.15	1.24±0.56
Model	18.75±8.54[a]	23.28±5.19	1.23±0.31

Results are expressed as mean ± SD. [a]P < 0.01, *vs.* sham-surgery group (Student-Newman-Keuls test).

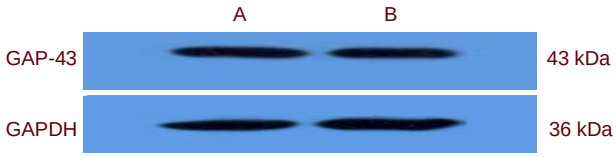

Figure 6 Growth-associated protein 43 (GAP-43) expression in the hippocampus (western blot).

There were five rats in each group. GAPDH served as internal reference. A: Sham-surgery group; B: model group.

Influence of CCH on hippocampal CREB and phosphorylated protein expression

Western blot results showed slightly less hippocampal CREB total protein expression in the model group than in the sham-surgery group (P > 0.05), but hippocampal phosphorylated CREB expression was significantly less in the model group than in the sham-surgery group (P < 0.05; Figure 7).

DISCUSSION

Ligation of bilateral common carotid arteries has been shown to induce acute brain ischemia during the early

stage. Subsequent CCH, as a result of basilar artery and Willis' circle blood regulation, gradually forms the collateral circulation. Ligation of bilateral common carotid arteries results in brain ischemia/hypoxia *via* CCH, which results in damage to the hippocampus, cortex, white matter, and visual system, further leading to neurological functional loss and learning and memory deficits[8]. Ligation of bilateral common carotid arteries has been frequently used to study animal models of CCH.

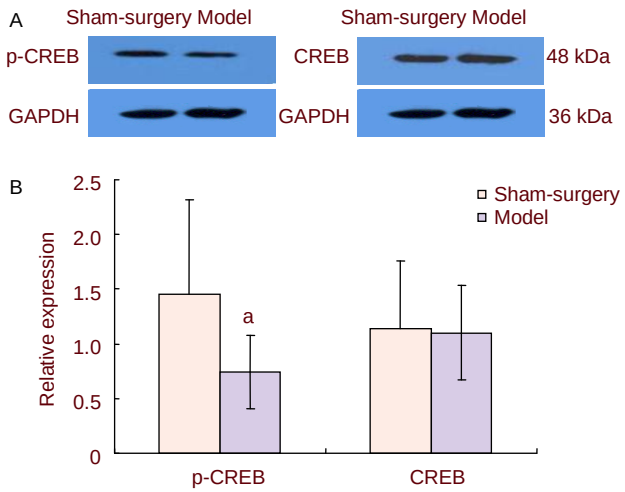

Figure 7 Hippocampal cAMP response element-binding protein (CREB) and phosphorylated CREB (p-CREB) expression.

There were five rats in each group. (A) Western blot electrophoresis; (B) quantitative results from image analysis software (mean ± SD). $^{a}P < 0.05$, *vs.* sham-surgery group (Student-Newman-Keuls test). p-CREB or CREB expression was expressed as absorbance value ratio of target protein to GAPDH.

GAP-43, a specific protein associated with growth (43 kDa), is distributed only in differentiated neurons with axonal growth. Because it is a calmodulin-binding protein and is located in growth cones and synaptic preterminals, GAP-43 connects plasma membranes and binds membrane skeleton proteins, such as actin and fodrin, to influence synaptic growth. Therefore, GAP-43 is regarded as a neuronal determinant during development and a marker of nerve regeneration[9]. GAP-43 expression reaches a peak in mammals at postnatal 1 week and gradually decreases with age, which suggests that increased GAP-43 protein was time-limited, because endogenous repair did not completely compensate for function. Following injury, neurons at the injury site exhibit functional compensation *via* collateral sprouting and reactive axonal regeneration, which further stimulates GAP-43 expression. Notably, GAP-43 expression significantly increases during early injury and gradually decreases with injury repair[10]. In the present study, the number GAP-43-positive cells in the hippocampal CA1 region decreased in the CCH-induced vascular dementia group compared with the sham-surgery group, and western blot assays showed similar GAP-43 protein expression changes between CCH-induced vascular dementia and sham-surgery groups.

As a reliable marker of nerve synapses, SYN expression accurately reflects synaptic distribution and density, and has been extensively used *in vivo* or *in vitro* to trace synaptogenesis[11]. Results from the present study showed that at 60 days after CCH, cognition was impaired and hippocampal SYN expression was significantly reduced. It is likely that after brain ischemia, hippocampal neurons degenerated or died, which led to axonal terminal degeneration or loss. Ischemia has been shown to result in decreased neuronal substance metabolism and protein synthesis, axoplasmic transport difficulties, and reduced axonal terminal SYN expression[12].

A previous study demonstrated that the cAMP messenger system plays a key role in long-term memory[13]. In addition, CREB is a constructive expression transcription factor that links the cAMP response element promoter site, allowing it to become activated by phosphorylated Ser-133. Phosphorylated-CREB promotes cAMP response element sequence transcription and regulates a large number of downstream genes in response to external stimulations. This gene transcription and expression results in structural changes or formation of synaptic connections, allowing long-term storage of acquired information[14]. In a rat model of ischemia/reperfusion injury, phosphorylated CREB expression has been previously shown to significantly attenuate neuronal loss in the hippocampal CA1 region[15], although these protective effects were limited. CREB phosphorylation in the hippocampal CA1 region highly correlates with the degree of ischemia, *i.e.*, mild ischemia benefits CREB phosphorylation, while severe ischemia inhibits CREB phosphorylation[16-17]. In the present study, hippocampal phosphorylated CREB protein expression significantly decreased in the model group at 60 days following CCH. In summary, following CCH, the amount of GAP-43 and SYN-positive cells in the hippocampal CA1 region significantly decreased, and hippocampal SYN and phosphorylated CREB protein expression significantly decreased. Results demonstrated that abnormal expression of hippocampal synaptic structural protein and CREB protein phosphorylation could be a molecular mechanism of CCH-induced cognitive impairment in rats.

MATERIALS AND METHODS

Design

A randomized, controlled, animal experiment.

Time and setting

The experiments were performed in the Laboratory Animal Center of Capital Medical University, Beijing, China from December 2010 to June 2011.

Materials

A total of 32 healthy, specific-pathogen free, male,

Sprague-Dawley rats, weighing 240 ± 20 g, were purchased from Vital River, Beijing, China (No. SCXK (Jing) 2006-0009). The rats were housed for 3 days to acclimate the environment. Experimental procedures were performed in accordance with the *Guidance Suggestions for the Care and Use of Laboratory Animals*, issued by the Ministry of Science and Technology of China[18].

Methods

Establishment of CCH model

The CCH model was established using ligation of bilateral common carotid arteries, as previously described[1]. Briefly, the rats were anesthetized and placed in a supine position. A median incision was made at the neck, and bilateral common carotid arteries were isolated and ligated, and the incision was sutured. The sham-surgery group was subjected only to dissociation of bilateral common carotid arteries. Both groups were housed under identical conditions.

Morris water maze test for learning and memory

At 60 days after injury, the Morris water maze (Institute of Materia Medica, Chinese Academy of Medical Sciences, Beijing, China) was used to detect learning and memory. The water maze comprised a circular pool (98 cm in diameter, 60 cm in height, and a black inner wall) filled with water (21 ± 1°C) to a depth of 30 cm. A platform, 8 cm in diameter, was submerged to 1 cm below the water surface. Swimming traces, escape latencies, swimming speed. and distance traveled were monitored by an automatic tracking system (Institute of Materia Medica, Chinese Academy of Medical Sciences, China). Spatial reference memory test: the platform was placed in the southwest quadrant (quadrant III), 22 cm from the center of the pool. The surroundings of the water maze were fixed and free of interference from noise or light. (1) Hidden-platform acquisition training: the rat was respectively placed in the water from opposite and adjacent quadrants, and the time spent locating the platform (locating the platform and remaining there for 5 seconds) within 90 seconds, *i.e.*, escape latency and swimming traces were recorded. If the rat failed to climb onto the platform within 90 seconds, the escape latency was recorded as 90 seconds, and the rat was guided to the platform and allowed to remain on the platform for 5 seconds. Each rat was trained four times per day, and the mean value of escape latency and swimming distance of each group per day was calculated. (2) Spatial probe test: on the fifth day after hidden-platform acquisition training, the platform was removed. The rats were placed in the quadrant adjacent to the original platform, facing the wall. Measurements were as follows: the quadrant (quadrant III) where the platform was previously located was regarded as the target area, and the ratio of swimming time for platform in quadrant to total swimming time (tP/tT), as well as the ratio of swimming path length to the total swimming distance (dP/dT), were measured. In addition, the frequency that rats crossed the location where the platform was previously located was recorded.

Spatial working memory test: the platform was respectively placed in quadrant I, II, and IV. The rat was respectively placed in the water from opposite and adjacent quadrants, and the time spent locating the platform (escape latency), as well as length of swimming path, were measured.

Immunohistochemical staining for hippocampal SYN and GAP-43 expression

Following learning and memory testing, six rats from each group were selected and endocardially perfused with 4% paraformaldehyde and 0.1 M phosphate buffered solution (pH 7.4). The entire brain was harvested, and coronal sections of tissues, 4 mm caudal to optic chiasma, were fixed with 4% paraformaldehyde, paraffin-embedded, and coronally sectioned (5 μm) for immunohistochemical staining. The sections were incubated with 0.01% citric acid (pH 6.0), followed by exposure to microwave twice for 10 minutes each, to retrieve the antigens. The sections were incubated with rabbit anti-rat SYN (1:100; Epitomics, Burlingame, CA, USA) or GAP-43 (1:200; Epitomics) monoclonal antibodies at 4°C for 24 hours, followed by goat anti-rabbit IgG (1:10 000; Epitomics) at room temperature for 1 hour. The stainings were colorized with diaminobenzidine for 10 minutes, dehydrated, cleared, and mounted. Negative controls were incubated in phosphate buffered solution rather than primary antibodies. Sections were observed by microscopy (Leica, Solms, Germany). Positive cells from three fields of view (× 200) in the hippocampal CA1 region were collected from each group using a Leica digital microscope camera. The number of cells per millimeter (*n*/mm), as well as absorbance of positive particles in each field of view, were quantified using NIS-Elements Basic Research image analysis system.

Western blot analysis of SYN, GAP-43, CREB, and phosphorylated-CREB hippocampal expression

Five rats were selected from each group and anesthetized. Hippocampal tissues were harvested and homogenized. Tissue protein was extracted using Tis-tissue protein extraction reagent (Beijing Comwin Biotech, Beijing, China), and the protein was quantified using bicinchoninic acid (kit purchased from Beijing Comwin Biotech). The protein samples were mixed with 5 × sodium dodecyl sulfate polyacrylamide gel electrophoresis sample buffer and boiled for 5 minutes (3 μg/μL). The samples were separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis, electrotransferred to nitrocellulose filters at 350 mA and 4°C for 80 minutes, blocked with 5% defatted milk at room temperature for 1 hour, and incubated in rabbit anti-rat SYN (1:20 000), GAP-43 (1:20 000), phosphorylated-CREB (1:2 000; Epitomics), CREB (1:2 000; Epitomics), or GAPDH (1:5 000; Epitomics) monoclonal antibodies overnight at 4°C. The membranes were washed three times with Tris-buffered saline/Tween-20, incubated with secondary antibody

goat anti-rabbit IgG-horseradish peroxidase (Epitomics) for 60 minutes, washed three times with Tris-buffered saline/Tween-20, and visualized by enhanced chemiluminescence. Protein blots were analyzed using Image Quant TL software and band absorbance was quantified. GAPDH expression was used as the internal reference.

Statistical analysis

Data were expressed as mean ± SD and analyzed using SPSS 10.0 for Windows (SPSS, Chicago, IL, USA). Differences between groups were compared using the Student-Newman-Keuls test. $P < 0.05$ was considered statistically significant.

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Author contributions: Hui Zhao designed the study and revised the manuscript. Zhiyong Li contributed to animal experiments and statistical analysis. Yali Wang and Qiuxia Zhang conducted immunohistochemistry and molecular biology experiments. Hui Zhao, Zhiyong Li, and Qiuxia Zhang were responsible for funding.

Conflicts of interest: None declared.

Ethical approval: This study received permission from the Animal Ethics Committee of Capital Medical University, China.

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