Amyloid beta-peptide worsens cognitive impairment following cerebral ischemia-reperfusion injury

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Research Highlights
(1) This study combined intracerebroventricular injection of amyloid β-peptide with cerebral ischemia and reperfusion to establish a new animal model of Alzheimer’s disease. We found that a single injection of amyloid β-peptide alone could not impair learning and memory abilities in rats or cause death of hippocampal neurons. However, intracerebroventricular injection of amyloid β-peptide lowering cerebral ischemia and reperfusion could aggravate learning and memory in rats and cause nerve cell death.
(2) In this study, the synergistic effect of amyloid β-peptide and cerebral ischemia-reperfusion injury exacerbated nerve damage by inducing glycogen synthase kinase 3β and protein phosphatase 2A activity, which resulted in the phosphorylation of tau protein.

Abstract
Amyloid β-peptide, a major component of senile plaques in Alzheimer’s disease, has been implicated in neuronal cell death and cognitive impairment. Recently, studies have shown that the pathogenesis of cerebral ischemia is closely linked with Alzheimer’s disease. In this study, a rat model of global cerebral ischemia-reperfusion injury was established via occlusion of four arteries; meanwhile, fibrillar amyloid β-peptide was injected into the rat lateral ventricle. The Morris water maze test and histological staining revealed that administration of amyloid β-peptide could further aggravate impairments to learning and memory and neuronal cell death in the hippocampus of rats subjected to cerebral ischemia-reperfusion injury. Western blot showed that phosphorylation of tau protein and the activity of glycogen synthase kinase 3β were significantly stronger in cerebral ischemia-reperfusion injury rats subjected to amyloid β-peptide administration than those undergoing cerebral ischemia-reperfusion or amyloid β-peptide administration alone. Conversely, the activity of protein phosphatase 2A was remarkably reduced in rats with cerebral ischemia-reperfusion injury following amyloid β-peptide administration. These findings suggest that amyloid β-peptide can potentiate tau phosphorylation induced by cerebral ischemia-reperfusion and thereby aggravate cognitive impairment.

Key Words
neural regeneration; brain injury; cerebral ischemia-reperfusion; Alzheimer’s disease; amyloid β-peptides; tau proteins; glycogen synthase kinase 3β; protein phosphatase 2A; phosphorylation; grants-supported paper; neuroregeneration
INTRODUCTION

Alzheimer’s disease is characterized by two hallmark brain lesions—extracellular deposition of amyloid β-peptide (Aβ) and intracellular neurofibrillary tangles\(^1\). Aβ, the major constituent of senile plaques in the brain of Alzheimer’s disease patients, has been implicated in the pathogenesis of the disease. Yankner et al\(^2\) reported that Aβ was neurotoxic to cultured hippocampus neurons, while Iwasaki et al\(^3,4\) found that fibrillar Aβ could induce prominent neuronal cell death and the expression of cell death-related genes in primary cortical neuronal cultures. These dying neurons exhibited morphological features of apoptosis\(^5\). Neurofibrillary tangles are composed of bundles of paired helical filaments and straight filaments, the major protein component of which is abnormally hyperphosphorylated tau protein\(^1\). The biological function of tau is regulated by its degree of phosphorylation. Hyperphosphorylation of tau depresses its microtubule assembly activity and its binding to microtubules, thus leading to microtubule destabilization, the appearance of neurofibrillary tangles and neurodegeneration in the brain of Alzheimer’s disease patients\(^6\).

Phosphorylation of tau can be regulated by many protein kinases and phosphatases. Glycogen synthase kinase 3 is a serine/threonine kinase that was first isolated and purified as an enzyme capable of phosphorylating and inactivating glycogen synthase\(^7,8\). Glycogen synthase kinase 3 is inhibited when phosphorylated at a serine residue (Ser\(^1\)) for glycogen synthase kinase 3β and Ser\(^21\) for glycogen synthase kinase 3α located in the N-terminal domain\(^7,8\). Glycogen synthase kinase 3β is shown to phosphorylate tau protein both in vitro and in vivo at multiple sites, some of which are abnormally hyperphosphorylated in the brains of Alzheimer’s disease patients\(^9\). Currently, glycogen synthase kinase 3β is recognized as a multifunctional kinase involved in many kinds of biological processes, such as embryonic development, tumorigenesis, neurodegeneration and cell death\(^9\). A growing number of studies have shown that glycogen synthase kinase 3β is the most likely candidate for the protein kinase responsible for the abnormal phosphorylation of tau in the brain of Alzheimer's disease patients\(^10-11\). Immunohistochemical studies have found that glycogen synthase kinase 3β is located in neurofibrillary tangles\(^10\). Moreover, protein levels of glycogen synthase kinase 3β are elevated in the brain of Alzheimer’s disease patients\(^11\).

Protein phosphatase 2A is a member of the family of serine/threonine phosphatases and is ubiquitously expressed in most tissues and cells. Protein phosphatase 2A accounts for as much as 1% of total cellular protein and is responsible for the major portion of serine/threonine phosphatase activity\(^12-13\). The activity of protein phosphatase 2A is decreased in the brains of Alzheimer’s disease patients\(^14\), but its function remains unclear.

Ischemia-induced cell death is one of the most serious effects caused by brain ischemia\(^15-16\). The precise pathogenic mechanism of brain ischemia is not clear. Despite numerous agents that can prevent the cascade of events leading to ischemic neuronal death in animal models, human clinical trials with these agents have proven disappointing\(^15-16\). In the last ten years, increasing evidence has indicated that cerebrovascular diseases have important roles in the pathogenesis and evolution of Alzheimer’s disease. Stroke has been shown to be closely associated with Alzheimer’s disease in many studies\(^17-21\). Wen et al\(^22\) found that tau hyperphosphorylation may contribute to the brain damage induced by transient cerebral ischemia. Many studies have found that brain ischemia can result in Alzheimer’s disease-like neuropathology\(^23-24\), such as increased production of Aβ, abnormal phosphorylation of tau protein and overexpression of presenilin and apolipoprotein E. Clinical studies have shown that Alzheimer’s disease patients with a history of cerebrovascular disease have a more rapid development of dementia\(^25\). Other studies have found that hypoxia can promote...
Aβ-induced apoptosis of hippocampal neurons cultured in vitro [26]. Epidemiological studies suggest that elderly people with silent stroke have an increased risk of Alzheimer’s disease [27]. Despite many studies reporting a close association between brain ischemia and Alzheimer’s disease, the underlying mechanisms remain unclear.

To further disclose the possible association between Alzheimer’s disease and brain ischemia, we developed an in vivo rat model of dementia using cerebral ischemia-reperfusion combined with intracerebroventricular administration of Aβ. As Aβ is metabolized very rapidly in the brain, we administered Aβ daily. Spatial learning and memory of rats was monitored, and the survival of hippocampal pyramidal cells, phosphorylation of tau protein, and the activity of glycogen synthase kinase 3β and protein phosphatase 2A were examined.

The pathogenesis of Alzheimer’s disease is very complicated. Many environmental factors and genetic factors may jointly contribute to the formation of Alzheimer’s disease. To date, there are no ideal in vivo animal models that possess all pathological features, which greatly restrict the development of therapeutic drugs for Alzheimer’s disease. Although many animal models of Alzheimer’s disease are available, these models only contain one or several pathological features of Alzheimer’s disease. In this study, we developed an in vivo rat model of dementia by injecting Aβ following cerebral ischemia-reperfusion; two important factors for inducing Alzheimer’s disease. Our results showed that neuronal damage was aggravated in this model. Therefore, this animal model of dementia may be used for studying the pathogenic mechanism of Alzheimer’s disease and for therapeutic drug development.

RESULTS

Quantitative analysis of experimental animals

Eighty adult male Sprague-Dawley rats were randomly divided into sham surgery, cerebral ischemia-reperfusion, Aβ administration without brain ischemia (Aβ), and cerebral ischemia-reperfusion plus Aβ administration (combination) groups. The numbers of rats in each group were 20. All 80 rats were included in the final results analysis.

Effects of cerebral ischemia-reperfusion and Aβ administration on spatial memory

The Morris water maze test was conducted on rats after 15 minutes brain ischemia followed by 3 days of reperfusion. In the place navigation test, the escape latency in the cerebral ischemia-reperfusion group was significantly longer than that in the sham operation group (Figure 1A). The escape latency in the combination group was significantly longer than that in the cerebral ischemia-reperfusion group (P < 0.05). However, the escape latency in the Aβ administration group was not significantly different from that of the sham operation group.

![Figure 1](image-url)  
**Figure 1** Morris water maze test for spatial learning and memory ability of rats subjected to amyloid β-peptide (Aβ) administration.

(A) The place navigation test: Time to find the hidden platform. The escape latency in the cerebral ischemia-reperfusion group was significantly longer than that in the sham group. *P < 0.05, vs. sham group; †P < 0.05, vs. cerebral ischemia-reperfusion group.

(B) Platform-passing times in the probe trial test. *P < 0.05, vs. sham group; †P < 0.05, vs. cerebral ischemia-reperfusion group.

Data are expressed as mean ± SD, n = 10. Statistical analysis was carried out by one-way analysis of variance followed by the least significant difference test or Student Newman-Keuls test. Sham: Sham operation group; ischemia: cerebral ischemia-reperfusion group; ischemia + Aβ: cerebral ischemia-reperfusion + Aβ group.

In the probe trial test, the platform-passing times in the cerebral ischemia-reperfusion group were significantly fewer than those in the sham operation group (P < 0.05; Figure 1B). The platform-passing times in the combination group were significantly fewer than those in the cerebral ischemia-reperfusion group (P < 0.05). However, the platform-passing times in the Aβ administration group...
Effects of cerebral ischemia-reperfusion and Aβ administration on hippocampal neural cells

The effects of cerebral ischemia-reperfusion and Aβ administration on the survival of hippocampal neural cells were examined using cresyl violet staining of rat brain sections. As shown in Figure 2, the number of surviving hippocampal CA1 neurons in the cerebral ischemia-reperfusion group was significantly lower than that in the sham operation group.

The number of surviving hippocampal CA1 neurons in the combination group was also significantly lower than that in the sham operation group (P < 0.05). There was no statistical difference in the number of surviving hippocampal CA1 neurons between the cerebral ischemia-reperfusion and combination groups (P < 0.05). However, in the cerebral ischemia-reperfusion group, the loss of neurons was mainly observed in the hippocampal CA1 region (P < 0.05). Additionally, in the combination group, the loss of neurons appeared in both the CA1 and CA2 regions.

The phosphorylation of tau in rats with cerebral ischemia-reperfusion injury subjected to Aβ administration

Next, the level of phosphorylated tau was examined by western blot. As shown in Figure 3, the level of tau phosphorylated at paired helical filament-1 (Ser[396,404]) was not significantly different between the cerebral ischemia-reperfusion group and sham operation group. The level of phosphorylated tau in the combination group was significantly higher than that in the sham operation group (P < 0.05). Moreover, the level of phosphorylated tau in the Aβ administration group was significantly higher than that in the sham operation group, but lower than the combination group (P < 0.05).

Protein levels of phosphorylated glycogen synthase kinase 3β and protein phosphatase 2A in rats subjected to cerebral ischemia-reperfusion and Aβ administration

Considering that glycogen synthase kinase 3β is the major tau kinase, the activity of glycogen synthase kinase 3β during cerebral ischemia-reperfusion combined with Aβ administration was determined by western blot analysis. Protein levels of glycogen synthase kinase 3β when phosphorylated at Ser[9] showed no significant difference between the cerebral ischemia-reperfusion group and sham operation group (Figure 4).
Aβ group.

(B) Semiquantitative representations of \( p \)-administration groups were determined using western blot.

(A) Effect of amyloid β-peptide (Aβ) administration on phosphorylated tau protein (p-tau) levels in the rat hippocampus following cerebral ischemia-reperfusion.

(Figure) Western blot assay of tau phosphorylation in the sham group, cerebral ischemia-reperfusion group, cerebral ischemia-reperfusion + Aβ group and Aβ administration group.

(B) Semiquantitative representations of protein levels of \( p \)-administration groups were determined using western blot.

Sham: Sham operation group; ischemia: cerebral ischemia-reperfusion group; ischemia + Aβ: cerebral ischemia-reperfusion + Aβ group.

Figure 3 Effect of amyloid β-peptide (Aβ) administration on phosphorylated tau protein (p-tau) levels in the rat hippocampus following cerebral ischemia-reperfusion.

(A) Western blot assay of tau phosphorylation in the sham group, cerebral ischemia-reperfusion group, cerebral ischemia-reperfusion + Aβ group and Aβ administration group.

(B) Semiquantitative representations of p-tau. Bands were scanned and the intensities were determined by absorbance (A). \( ^{a}P < 0.05 \), vs. sham group; \( ^{b}P < 0.05 \), vs. cerebral ischemia-reperfusion group. Data are expressed as mean ± SD, \( n = 8 \). Statistical analysis was carried out by one-way analysis of variance followed by the least significant difference test or Student-Newman-Keuls test.

Sham: Sham operation group; ischemia: cerebral ischemia-reperfusion group; ischemia + Aβ: cerebral ischemia-reperfusion + Aβ group.

Figure 4 Effect of amyloid β-peptide (Aβ) administration on phosphorylated glycogen synthase kinase 3 (p-GSK-3β) protein levels in the rat hippocampus following cerebral ischemia-reperfusion.

(A) Protein levels of p-GSK-3β in the sham, cerebral ischemia-reperfusion, cerebral ischemia-reperfusion + Aβ, and Aβ administration groups were determined using western blot.

(B) Semiquantitative representations of protein levels of p-GSK-3β. Bands were scanned and the intensities were determined by absorbance (A). \( ^{a}P < 0.05 \), vs. sham group; \( ^{b}P < 0.05 \), vs. cerebral ischemia-reperfusion group. Data are expressed as mean ± SD, \( n = 8 \). Statistical analysis was carried out by one-way analysis of variance followed by the least significant difference test or Student-Newman-Keuls test.

Sham: Sham operation group; ischemia: cerebral ischemia-reperfusion group; ischemia + Aβ: cerebral ischemia-reperfusion + Aβ group.

Figure 5 Effect of amyloid β-peptide (Aβ) administration on phosphorylated protein phosphatase 2A (p-PP2A) protein levels in the rat hippocampus following cerebral ischemia-reperfusion.

(A) Protein levels of p-PP2A in the sham, cerebral ischemia-reperfusion, cerebral ischemia-reperfusion + Aβ, and Aβ administration groups were determined using western blot.

(B) Semiquantitative representations of p-PP2A protein levels. Bands were scanned and the intensities were determined by absorbance. \( ^{a}P < 0.05 \), vs. sham group; \( ^{b}P < 0.05 \), vs. cerebral ischemia-reperfusion group. Data are expressed as mean ± SD, \( n = 8 \). Statistical analysis of the results was carried out by one-way analysis of variance followed by the least significant difference test or Student-Newman-Keuls test.

Sham: Sham operation group; ischemia: cerebral ischemia-reperfusion group; ischemia + Aβ: cerebral ischemia-reperfusion + Aβ group.
The level of phosphorylated glycogen synthase kinase 3β in the combination group was significantly lower than that in the sham operation group ($P < 0.05$), which suggested the activity of glycogen synthase kinase 3β was increased in the combination group. The level of phosphorylated glycogen synthase kinase 3β in the Aβ administration group was also significantly lower than that in the sham operation group, but higher than that in the combination group ($P < 0.05$).

In addition, the activity of protein phosphatase 2A, the major tau phosphatase, was detected using western blot. The level of protein phosphatase 2A phosphorylated at Tyr$^{307}$ appeared to have no significant difference between the cerebral ischemia-reperfusion group and sham operation group (Figure 5). The level of phosphorylated protein phosphatase 2A in the combination group was significantly higher than that in the sham operation group ($P < 0.05$), which means the activity of protein phosphatase 2A was reduced in the combination group. The level of phosphorylated protein phosphatase 2A in the Aβ administration group was also not significantly different from that of the sham operation group.

**DISCUSSION**

Aβ is produced by the proteolytic processing of the amyloid precursor protein. Under normal physiological conditions, Aβ is enzymatically degraded and metabolized. The two specific forms of Aβ, Aβ1–40 and Aβ1–42, are prone to adopt a β-sheet conformation and aggregate as amyloid fibrils in the brain. Increasing evidence suggests that the β-sheet conformation of Aβ has neurotoxic effects.

Yankner et al.[32] reported that Aβ could induce neuronal cell death in cultured neurons, and May et al.[29] suggested that the toxicity of Aβ is associated with β-sheet conformation. In addition, Pike et al.[33] found that Aβ solution incubated for 24 hours at 37°C has much stronger neurotoxicity than newly dissolved Aβ. Owing to the hydrophobic nature of Aβ, Aβ monomers aggregate into polymers in aqueous solution to form insoluble aggregates, which is probably responsible for the β-sheet structure.

Many studies have found that Aβ fibrils can induce neuronal death in vitro. Here, we conducted an in vivo study and found that fibrillar Aβ alone failed to impair spatial learning ability and induce neuronal loss. However, combination of brain ischemia and Aβ administration could further aggravate neuronal injury. Using western blot analysis, we found that the activity of glycogen synthase kinase 3β was remarkably increased in the combination group compared with the cerebral ischemia-reperfusion group or Aβ treatment group.

Moreover, the activity of protein phosphatase 2A was significantly reduced in the combination group compared with the cerebral ischemia-reperfusion group or Aβ treatment group. The level of phosphorylated tau protein was ranked as follows: the combination group > Aβ administration group > sham operation group. As glycogen synthase kinase 3β is the major tau kinase and protein phosphatase 2A is the major tau phosphatase, the increased activity of glycogen synthase kinase 3β and reduced activity of protein phosphatase 2A jointly contributed to an increase in tau phosphorylation in the combination group. These findings suggest that Aβ administration synergistically improves transient ischemic brain injury.

According to the “Aβ theory” of Alzheimer’s disease pathogenesis, Aβ lies at the top of the signal pathway. Aβ can activate glycogen synthase kinase 3β and induce increased phosphorylation of tau, which may result in the formation of neurofibrillary tangles. However, the underlying mechanism is not clear. Alzheimer’s disease is characterized by two hallmark brain lesions, extracellular Aβ deposits and intracellular neurofibrillary tangles, but Aβ deposits also exist in the brain of some healthy elderly people, suggesting Aβ is not necessary for dementia. This is consistent with the results of our present study. The ability of learning and memory and the neuronal survival of rats in the Aβ administration group showed no significant difference when compared with the sham operation group. Previous studies have found that neurofibrillary tangles are more relevant to Alzheimer’s disease than Aβ deposits.[36] Neurofibrillary tangles are composed of abnormally hyperphosphorylated tau proteins. Hyperphosphorylation of tau depresses its microtubule assembly activity and its binding to microtubules[34,35], thus causing neurodegeneration in the brain of Alzheimer’s disease patients. In a word, tau may be the main executor for neuronal cell death. The synergistic actions of brain ischemia and Aβ may induce tau hyperphosphorylation, but the underlying mechanism needs further study.

In recent years, many researchers have found that there is a close association between brain ischemia and Alzheimer’s disease. Brain ischemia can result in Alzheimer’s disease-like brain injuries, such as increased production of Aβ and hyperphosphorylation of tau protein.
Some studies propose an “ischemia-reperfusion theory” of Alzheimer’s disease pathogenesis. The results from this study also support this new theory. Recent progress in Alzheimer’s disease research has begun to unravel the pathogenesis of the disease, but few in vivo animal models have been shown to be suitable for therapeutic drug studies.

In this study, we created a rat model of dementia by combining intracerebroventricular administration of Aβ and brain ischemia. This model could aggravate spatial memory impairment and neuronal cell death. Therefore, this model may be useful for studying the pathogenesis of Alzheimer’s disease and ischemic stroke, and be useful for the development of new therapeutic drugs.

In conclusion, the combination of brain ischemia and Aβ administration aggravates spatial memory impairment and neuronal cell death. These findings suggest that brain ischemia is an important factor that facilitates the development of Alzheimer’s disease. This in vivo model may be useful for therapeutic drug development in the treatment of Alzheimer’s disease and other neurodegenerative diseases.

MATERIALS AND METHODS

Design
A parallel controlled, comparative, in vivo experiment.

Time and setting
Experiments were performed from May 2008 to July 2010 at the School of Life Sciences, Tsinghua University, China.

Materials
A total of 80 healthy, clean male Sprague-Dawley rats, 7 weeks of age, were purchased from the Vital River Corporation, Beijing, China. Experimental procedures for animals were in strict accordance with the Guidance Suggestions for the Care and Use of Laboratory Animals, issued by the Ministry of Science and Technology of China[34].

Methods
Establishment of a rat model of cerebral ischemia-reperfusion
Transient brain ischemia followed by reperfusion was induced by the four-vessel occlusion method, as described by Pulsinelli et al[35]. Briefly, rats were anesthetized intraperitoneally (i.p.) with 300 mg/kg chloral hydrate (Sigma-Aldrich, St. Louis, MO, USA) and immobilized in a stereotaxic apparatus (RWD Life Science Co., Ltd., Shenzhen, Guangdong Province, China). The bilateral vertebral arteries were electrocauterized with a bipolar coagulator. On the next day, the bilateral common carotid arteries were occluded with aneurysm clips to induce brain ischemia. After 15 minutes of occlusion, the aneurysm clips were removed followed by 7 days of reperfusion. Rats in the sham operation group received the same surgical procedures except the carotid arteries were occluded. The body temperature of rats was maintained at 37°C using a heating pad. Rats that did not exhibit loss of their righting reflex during brain ischemia were excluded from the subsequent experiment.

Intracerebroventricular injection of Aβ
Aβ1-42 was purchased from ANASPEC, San Jose, CA, USA. Aβ1-42 (100 μg) was dissolved in 100 μL saline and incubated for 72 hours at 37°C. Twenty-four hours after cerebral ischemia-reperfusion, the rats were anesthetized with chloral hydrate (300 mg/kg, i.p.; Sigma) and placed in a stereotaxic apparatus (RWD Life Science Co., Ltd.). Aβ1-42 (10 μL; 1 μg/μL) was administrated unilaterally to rats every 24 hours for 6 days through the left ventricle of the brain (anteroposterior, –0.8 mm; lateral, 1.5 mm; depth, 3.5 mm from the bregma). Rats in the Aβ administration group received the same surgical procedures except for cerebral ischemia-reperfusion.

Western blot analysis of protein samples
Rats were decapitated immediately after 15 minutes brain ischemia followed by 7 days reperfusion. The hippocampi were quickly separated and dipped into liquid nitrogen, and then stored at –80°C. Hippocampi were homogenized in ice-cold homogenization buffer. The homogenates were centrifuged at 800 × g for 15 minutes at 4°C to collect the supernatant, and the protein concentrations were determined by the method of Lowry. For western blot analysis, proteins were separated by 10% (w/v) sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and then electrotransferred onto nitrocellulose membrane (pore size, 0.45 μm). The membrane was probed with a rabbit anti-phospho-GSK3β (Ser9) monoclonal antibody (1:1 000, Cell Signaling Technology, Inc., MA, USA), rabbit anti-GSK3β monoclonal antibody (1:1 000, Cell Signaling), mouse anti-tau monoclonal antibody (1:1 000, Cell Signaling), mouse anti-PHF-1 monoclonal antibody (1:1 000, provided by Dr. Peter Davies, Albert Einstein College of Medicine, Bronx, NY, USA), rabbit anti-phospho-protein phosphatase 2A (Tyr205) monoclonal antibody (1:1 000, Epitomics, Inc., Hangzhou, Zhejiang Province, China and

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rabbit anti-protein phosphatase 2A monoclonal antibody (1:1 000, Epitomics) overnight at 4°C. Detection was performed using alkaline phosphatase conjugated goat-anti-rabbit IgG (1:10 000) or goat anti-mouse IgG (1:10 000).

The bands on the membrane were scanned and analyzed by an image analyzer (Labworks Software, UVP Inc., Upland, CA, USA). The intensities of the bands were determined by the absorbance values. The results were expressed as a fold vs. sham control.

**Morris water maze test**

The Morris water maze was used to test spatial learning and memory in rats. The water maze test was performed in a black circular pool (diameter 1.5 m, height 60 cm, filled with water of 22–23°C to a height of 30 cm). After 15 minutes brain ischemia followed by 3 days reperfusion in rats, the test was conducted. In the hidden-platform trials, a 10-cm-diameter platform was placed 2.0 cm below the water line in the southeastern quadrant of the pool. The rat was placed in the water facing the wall at one random start location of four quadrants. Each rat was allowed to find the submerged platform within 120 seconds, and rest on it for 20 seconds. If rats failed to find the hidden platform within 120 seconds, the rat was placed on the platform for 20 seconds. Two sessions of four trails were conducted on the first testing day, and the interval was 4 hours. The first session was considered as the training procedure. Then, one session of four trails was conducted daily within the next 3 days. Four hours after the last trial, a probe trial was given within 120 seconds in which the platform was removed from the pool.

The escape latency (time to find the platform) and platform-passing times were monitored by a camera (Beijing New World Technology Co., Ltd., Beijing, China) directly above the pool.

**Histological staining of rat brain sections**

Rats were deeply anesthetized with chloral hydrate and perfused transcardially with chilled normal saline for 3 minutes followed by treatment with 4% (w/v) paraformaldehyde. Brains were removed and stored in the same paraformaldehyde solution overnight. Frozen sections (10 µm) were cut coronally and stained with cresyl violet (Sigma).

The sections were examined under a light microscopy (Olympus, Tokyo, Japan) and the surviving pyramidal cells in the hippocampal CA1 per 1 mm length were counted as the density of neurons.

**Statistical analysis**

Values were expressed as mean ± SD from at least eight independent animals. Statistical analysis of the results was conducted using Origin 7.0 software (OriginLab Corporation, USA), and one-way analysis of variance followed by the least significant difference test or Student-Newman-Keuls test. \( P < 0.05 \) was considered significant.

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