Metabolic alteration of neuroactive steroids and protective effect of progesterone in Alzheimer’s disease-like rats

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Abstract
A correlation between metabolic alterations of neuroactive steroids and Alzheimer’s disease remains unknown. In the present study, amyloid beta (Aβ) 25–35 (Aβ25-35) injected into the bilateral hippocampus CA1 region significantly reduced learning and memory. At the biochemical level, hippocampal levels of pregnenolone were significantly reduced with Aβ25-35 treatment. Furthermore, progesterone was considerably decreased in the prefrontal cortex and hippocampus, and 17β-estradiol was significantly elevated. To our knowledge, this is the first report showing that Aβ25-35, a main etiological factor of Alzheimer's disease, can alter the level and metabolism of neuroactive steroids in the prefrontal cortex and hippocampus, which are brain regions significantly involved in learning and memory. Aβ25-35 exposure also increased the expression of inflammatory mediators, tumor necrosis factor-α and interleukin-1β. However, subcutaneous injection of progesterone reversed the upregulation of tumor necrosis factor-α and interleukin-1β in a dose-dependent manner. Concomitant with improved cognitive abilities, progesterone blocked Aβ-mediated inflammation and increased the survival rate of hippocampal pyramidal cells. We thus hypothesize that Aβ-mediated cognitive deficits may occur via changes in neuroactive steroids. Moreover, our findings provide a possible therapeutic strategy for Alzheimer's disease via neuroactive steroids, particularly progesterone.

Key Words
neural regeneration; neurodegenerative disease; neuroactive steroids; Alzheimer’s disease; progesterone; amyloid beta; cognition; neuroprotection; neuroregeneration
INTRODUCTION

Alzheimer’s disease (AD), characterized clinically by progressively cognitive impairment and the appearance of senile plaques and neurofibrillary tangles, is the most prevalent senile dementia. Amyloid beta (Aβ), a β-sheet peptide fragment produced by the proteolytic cleavage of amyloid precursor protein by γ- and β-secretases, is the major component of senile plaques. Both in vivo and in vitro studies have shown that accumulation of Aβ fibrils triggers neurodegeneration, supporting the view that Aβ aggregation plays an important role in AD. Aβ causes oxidative damage, inflammatory responses, and memory impairment, all of which lead to neuronal dysfunction and degeneration responsible for cognitive deficits observed in AD.

Steroid hormones and their metabolites within the central nervous system are commonly defined as neuroactive steroids or neurosteroids. They can be synthesized de novo from cholesterol by glial cells and neurons, or synthesized in the periphery by the adrenal glands and gonads. The concentration of neuroactive steroids is higher in the central nervous system than in the periphery. Neuroactive steroids mainly include pregnenolone and dehydroepiandrosterone (their sulfate derivatives, pregnenolone sulfate and dehydroepiandrosterone sulfate), and progesterone, 5α-dihydroprogesterone, 3α, 5α-tetrahydroprogesterone (allopregnenolone), deoxycorticosterone, tetrahydrodeoxycorticosterone, and estradiol. Neuroactive steroids play an important role as rapid endogenous modulators of neuronal excitability. However, no specific receptor has been reported for neuroactive steroids thus far. Most of their actions in the nervous tissue were reported as a modulation of membrane neurotransmitter receptors, such as γ-amino butyric acid (GABA), N-methyl-D-aspartate (NMDA), and sigma 1 receptors, thus affecting neuronal plasticity, anxiety, responses to stressful stimuli, and neuropsychiatric symptoms represented during AD. Some neuroactive steroids have been shown to improve learning and memory ability and protect against Aβ peptide-induced neurotoxicity, thus exerting neuroprotection. Few studies have addressed changes of neuroactive steroid levels in the central nervous system of AD patients. Moreover, limitations in the methodology are evident in these studies. For subject limitations: (1) the content of neuroactive steroids in plasma or cerebrospinal fluid of AD patients may not directly reflect their levels in brain tissue, and (2) the origin of the studied corpse is very restricted. For methodology limitations: (1) despite studies of neurosteroid steroidogenic enzymes, the metabolism of neuroactive steroids is complex, and moreover, changes of steroidogenic enzyme levels are inconsistent with those of neuroactive steroids, and (2) the accuracy and sensitivity of radioimmunoassays used in the studies are lower than high performance liquid chromatography-tandem mass spectrometry. These studies focused on the correlation between neuroactive steroid levels and the prevalence of AD, and not the effect of Aβ on neuroactive steroid levels and the role of neuroactive steroids in Aβ-induced learning and memory impairment.

Although neuroactive steroids are known to be neuroprotective, changes in their level during AD and their role in Aβ-mediated cognitive impairment remain elusive given the limitation in sample sizes and analysis methods. We have reported previously that the concentration of progesterone decreases in the media of Aβ25-35-induced primary rat cortical neurons, and progesterone treatment inhibits Aβ25-35-induced cell toxicity as well as apoptosis. To gain a better understanding on the role of neuroactive steroids in the pathology of AD, the present study investigated the effect of progesterone administration against Aβ25-35-induced impairment in vivo.

RESULTS

Quantitative analysis of experimental animals
In experiment 1, 30 rats were randomly divided into three groups (sham, vehicle, or Aβ25–35; 10 rats per group). Aβ25–35 (2 g/L) or vehicle (sterile distilled water) was slowly injected into the bilateral CA1 region of the rat hippocampus (except the sham group). In experiment 2, 60 rats were randomly divided into three groups (sham, vehicle, or Aβ25–35; 20 rats per group), and treated the same as in experiment 1. In experiment 3, 50 rats were randomly divided into five groups: (1) sham, (2) Aβ25–35, or Aβ25–35 + (3) progesterone (P) low dose (P1) (4 mg/kg), (4) P medium dose (P2) (8 mg/kg) or (5) P high dose (P3) (16 mg/kg) (10 rats per group). Sham and Aβ25–35 groups received sesame oil injection daily between 6 and 12 days after Aβ25–35 injection instead of progesterone.

### Injection of Aβ25–35 into hippocampal CA1 impaired spatial learning and memory

In experiment 1, the Morris water maze (MWM) behavioral task was used to examine hippocampus-dependent spatial learning and memory. The mean escape latency in the MWM trials was significantly (*P* < 0.01) prolonged in the Aβ25–35 group compared with the vehicle group (Figure 1A). Moreover, the number of platform crossings and the retention time ratio in the platform quadrant of Aβ25–35 rats in the probe test were considerably (*P* < 0.01) lower than those of vehicle animals (Figure 1B, C), thus confirming spatial learning and memory impairment in Aβ25–35 rats. Overall, no difference of mean escape latency, the number of platform crossings, and the retention time ratio in the platform quadrant (quadrant IV) was observed between sham and vehicle groups.

### Aβ25–35 affected hippocampal morphology and increased cytokine production

In experiment 1, histological examination showed that Aβ25–35 significantly (*P* < 0.01) decreased the number of intact pyramidal cells in the hippocampal CA1 compared with the vehicle group (Figure 2A, B). At the cellular level, Aβ25–35 significantly (*P* < 0.05) increased the expression of pro-inflammatory cytokines, tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β), compared with the vehicle group (Figure 3A). Similarly, Aβ25–35 significantly (*P* < 0.05) up-regulated gene expression of TNF-α and IL-1β expression (Figure 3B).

### Aβ25–35 altered the level of neuroactive steroids in the prefrontal cortex and hippocampus

In experiment 2, the level of allopregnanolone in prefrontal cortex and hippocampus of the Aβ group remained unchanged compared with the sham group, and no difference of neuroactive steroids was found for both regions between sham and vehicle groups (Figure 4). The level of pregnenolone in the hippocampus of the Aβ25–35 group at 7 and 12 days after Aβ25–35 injection was significantly (*P* < 0.05) decreased compared with the sham group. The level of progesterone in the prefrontal cortex and hippocampus of the Aβ25–35 group at 7 and 12 days after Aβ25–35 injection was significantly (*P* < 0.01) decreased compared with sham animals (Figure 4). The level of 17β-estradiol in the prefrontal cortex of the Aβ25–35 group at 7 days after Aβ25–35 injection and in hippocampus of Aβ group at 7 and 12 days after Aβ25–35 injection was significantly (*P* < 0.05) increased compared with the sham group (Figure 4).

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*Figure 1* Effect of bilateral hippocampal injection of amyloid β 25–35 (Aβ25–35) on spatial learning and memory. (A) Effect of Aβ25–35 on mean escape latency of rats in navigation trials of the Morris water maze (MWM) behavioral task. A shorter escape latency indicates a stronger learning ability. D1–D5: The time in MWM task. (B) Effect of Aβ25–35 on the number of platform crossings of the probe trial (90 seconds) in the MWM behavioral task. A higher number of platform crossings indicates a stronger memory ability. (C) Effect of Aβ25–35 on IV time ratio in the probe trial (90 seconds) of the MWM behavioral task. A longer retention time in the quadrant indicates a stronger memory ability. Data are presented as mean ± SEM. The mean escape latency of the MWM behavioral task was analyzed by repeated-measures analysis of variance (ANOVA) follow by least significant difference post hoc test. Other data were statistically analyzed by one-way ANOVA, follow by least significant difference post hoc test. *n* = 10 rats per group. *P* < 0.01, vs. vehicle group.
Progesterone reduced Aβ25-35-induced behavioral impairment, cell loss, and cytokine production

In experiment 3, administration of progesterone enhanced the cognitive performance of Aβ25-35-treated rats in a dose-dependent manner. The mean escape latencies of the P_L, P_M, and P_H groups were significantly shortened compared with the Aβ25-35 group; P < 0.05 or P < 0.01; Figure 5A). The number of platform crossings of the P_M and P_H groups was significantly increased compared with the Aβ25-35 group (P < 0.05 or P < 0.01; Figure 5B). The IV time ratio was significantly increased in the P_L, P_M, and P_H groups compared with the Aβ group (P < 0.01; Figure 5C).

Progesterone reversed Aβ25-35-induced cell loss dose-dependently. Compared with the Aβ25-35 group, the number of intact pyramidal cells in the P_L, P_M, and P_H groups was significantly increased (P < 0.01; Figure 6). Progesterone reversed the Aβ25-35-mediated up-regulation of TNF-α and IL-1β. Western blot analysis showed that expression of hippocampal TNF-α was significantly lower in the progesterone-treated than in Aβ25-35-treated rats (P < 0.01; Figure 7A). Similar significant results were found with IL-1β (P < 0.05 or P < 0.01; Figure 7B). Gene expression was significantly down-regulated for TNF-α (P < 0.01; Figure 7C). Similar significant results were found with IL-1β for P_L, P_M, and P_H groups (P < 0.05 or P < 0.01; Figure 7D).

DISCUSSION

The present study shows for the first time that injection of Aβ25-35 into the hippocampal CA1 sub-region of rats reduces levels of pregnenolone and progesterone, and increases levels of 17β-estradiol, in the prefrontal cortex and hippocampus. Furthermore, Aβ25-35 up-regulates the expression of pro-inflammatory cytokines, TNF-α and IL-1β. Progesterone reverses Aβ25-35-mediated impairment and thus, may be a potential therapeutic strategy for AD.

Aβ25-35 has been widely used to investigate Aβ-mediated impairments, under in vivo and in vitro conditions. Aβ25-35-mediated memory impairment in rodents has been confirmed using different behavioral tests, including...
spontaneous alternation, passive avoidance, and MWM[29-33].

Figure 4  Effect of amyloid β 25-35 (Aβ25-35) on the levels of neuroactive steroids in the prefrontal cortex (PFC) and hippocampus (Hipp).

Pregnenolone (A), progesterone (B), allopregnanolone (C) and 17β-estradiol (D) were simultaneously quantified by high performance liquid chromatography-tandem mass spectrometry. Data are presented as mean ± SEM, and analyzed by one-way analysis of variance followed by least significant difference post hoc test. n = 10 rats per group. *P < 0.05, **P < 0.01, vs. vehicle group.

In line with these reports, this study showed that intracerebral injection of aggregated Aβ25-35 into the bilateral hippocampal CA1 region induced cognitive deficits. Moreover, these impairments may have been attributed to cell loss, which has also been observed in other studies[31-38]. However, some studies have failed to find histological impairment, which may be partly due to the low concentration of Aβ25-35[33-34]. Inflammatory mediators secreted by microglial and astrocytic cells exposed to Aβ may also contribute to cognitive deficits[35]. TNF-α and IL-1β are involved in the inflammatory response, and they may also be toxic to neurons and glial cells[36]. The increased level of pro-inflammatory cytokines in the central nervous system was shown to impair cognitive function[37] and play a fundamental role in the pathogenesis of AD[38-39]. The present study showed that Aβ25-35 up-regulated the expression of TNF-α and IL-1β, indicating a possible involvement of these pro-inflammatory cytokines in this AD mode.

Figure 5  Effect of progesterone on amyloid β 25-35 (Aβ)-induced deficits of spatial learning and memory.

(A) Mean escape latency in navigation trials of the Morris water maze behavioral task. A shorter escape latency indicates a stronger learning ability.

(B) Number of platform crossings in the probe trial. A higher number of platform crossings indicates a stronger memory ability.

(C) Effect of Aβ25-35 on IV time ratio in the probe trial. A longer retention time in the quadrant indicates a stronger memory ability.

Data are presented as mean ± SEM, and analyzed by repeated-measures analysis of variance followed by least significant difference post hoc test. n = 10 rats per group. *P < 0.05, **P < 0.01, vs. Aβ group. P1: Aβ25-35 + 4 mg/kg progesterone (P) (low dose); P2: Aβ25-35 + 8 mg/kg P (medium dose); P3: Aβ25-35 + 16 mg/kg P (high dose). D1–5: The time in Morris water maze task.
Previous findings have indicated that neuroactive steroids are associated with the pathological processes of several disorders, including diabetes mellitus, social isolation, and traumatic brain injury. Recently, several studies have reported the alterations of levels of neuroactive steroids in AD patients. However, the relationship between Aβ and neuroactive steroids remains elusive. A general trend of lower levels of neuroactive steroids was observed in different brain regions of AD patients. The current study confirmed these findings by using high-speed liquid chromatography (HPLC)/tandem mass spectrometry (MS), which enabled the quantification of neuroactive steroids. Findings from this analysis revealed that pregnenolone and progesterone were decreased in Aβ25–35-treated rats but 17β-estradiol was increased. Caruso et al. also found increased levels of 17β-estradiol in both young adult and aged 3× transgenic model of AD mice compared with wild type aged-matched controls. Reduced levels of 17β-estradiol have been reported to be low in brains of postmenopausal women with AD. However, other reports have found no changes between control and AD cases. This discrepancy may be a result of the subjects chosen or different analytical methods. Previous studies on postmortem brain tissue of AD patients have suggested that reduced levels of neuroactive steroids may increase the risk of AD. The present study demonstrated that using Aβ25–35 as an etiological factor altered the level of neuroactive steroids. This alteration may be a result of Aβ25–35-induced impairment of cell viability, thus contributing to a decrease in the synthesis of neuroactive steroids. The change in the level of neuroactive steroids may, in turn, render the cell more vulnerable to Aβ25–35 injury. Increased levels of progesterone in the prefrontal cortex and hippocampus were found with a concomitant reversal of both cognitive impairment and cell loss in progesterone treatment, thus further strengthening our hypothesis of a relationship between impaired cognition and altered levels of neuroactive steroids. Apart from its reproductive function, progesterone may play a possible protective role of progesterone in the central nervous system as been given more attention over the past decade. Previous studies have investigated the effect of progesterone on its ability to reverse cognitive deficits. However, some studies have failed to find this protective effect.

Figure 6 Effect of progesterone on hippocampal CA1 pyramidal cells in rats exposed to amyloid β 25–35 (Aβ25–35).

(A) Histological examination of the hippocampal CA1 region stained with thionine (Nissl staining, × 400). (B) The number of intact pyramidal cells (per 1 mm linear length of the same section of hippocampal CA1 region). Data are presented as mean ± SEM, and analyzed by one-way analysis of variance followed by least significant difference post hoc test. n = five rats per group. *P < 0.01, vs. Aβ25–35 group.

Figure 7 Effect of progesterone on hippocampal expression of tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) in rats exposed to amyloid β 25–35 (Aβ).

(A–D) Western immunoblot analysis and reverse transcription-PCR analysis of TNF-α and IL-1β expression. Data are presented as mean ± SEM, and analyzed by one-way analysis of variance followed by least significant difference post hoc test. *P < 0.05, **P < 0.01, vs. Aβ group. n = five rats per group.
This discrepancy may have arisen from the use of different animal species, varying administration dosages, and behavioral tests used. The protective mechanisms of progesterone include reduced neuronal vulnerability to excitotoxic or ischemic damage\(^{50}\), decreased peroxidation of lipid\(^{35}\), and a suppressed inflammatory response\(^{50}\). In line with these reports, this study showed that progesterone decreased the expression of TNF-α and IL-1β in the hippocampus of Aβ\(_{25-35}\)-treated rats. Because progesterone is converted into its metabolites, allopregnanolone and estradiol, future studies could explore whether progesterone itself or its metabolites are responsible for the protective effect. Both metabolites have been studied in rodent paradigms and clinical experiments\(^{53-56}\).

In summary, the present study shows that Aβ\(_{25-35}\) impairs learning and memory abilities of rats, accompanied by reduced levels of progesterone. Treatment of these AD rats with progesterone reverses cognitive impairment. Therefore, this study provides a possible therapeutic strategy for AD.

### MATERIALS AND METHODS

#### Design
A randomized, controlled animal study and comparative observation pertaining to histocytology.

#### Time and setting
Experiments were performed at Bethune International Peace Hospital of Chinese PLA, China from December 2011 to January 2013.

#### Materials

**Animals**
A total of 140 male healthy Sprague-Dawley rats, of clear grade, weighing 210–230 g, aged 3 months, were purchased from the Animal Corporation of Hebei Province, China (license No. SCXK (Ji) 2008-1003). The animals were housed per cage under a 12-hour light/dark cycle, at 25°C, with food and water *ad libitum*. The animals were acclimatized 7 days before experiments. All experiments complied with the *Guidance Suggestion for the Care and Use of Laboratory Animals*, formulated by the Ministry of Science and Technology of China\(^{57}\).

**Drugs**
Pregnenolone, progesterone, allopregnanolone, 17β-estradiol, methyltestosterone and Aβ\(_{25-35}\) were all purchased from Sigma-Aldrich (St. Louis, MO, USA). The stock solution of Aβ (2 mmol/L) was prepared in sterile distilled water and incubated for 7 days at 37°C for ageing and aggregation before use.

#### Methods

**Experiment 1: Effect of Aβ\(_{25-35}\) on spatial learning and memory in rats**
After rats were anesthetized with 2% pentobarbital sodium (40 mg/kg, intraperitoneal injection), they were placed in a stereotaxic apparatus (Jiangwai Type 1, Suixi, Anhui Province, China). A total of 5 μmol/L aggregated Aβ\(_{25-35}\) or vehicle (sterile distilled water) was slowly (over 10 minutes) injected (using a 10 μL microsyringe) into the bilateral CA1 region of the hippocampus\(^{59}\) (anterior/posterior –3.5 mm, media/lateral ± 2.0 mm, and dorsal/ventral –2.8 mm ventral to the skull surface), followed by an additional 5 minutes to allow diffusion of the injected content. The rectal temperature was maintained at 36–37°C for all animals throughout the surgery. Animals in the control group underwent no surgery. Seven days after treatment surgery, rats were assessed for learning and memory for the following 6 days using the MWM behavioral task. Immediately following the final behavioral test, five rats in each group were sacrificed (via decapitation), and the hippocampi were removed (on ice) and stored at –80°C. The remaining animals were anesthetized with pentobarbital sodium and perfused through the ascending aorta with normal saline followed by 4% paraformaldehyde. The brain tissues were dissected and fixed for histological examination.

**Experiment 2: Effect of Aβ\(_{25-35}\) on the levels of neuroactive steroids in the brain**
The treatment regime was the same as in Experiment 1. Between 16:00 and 18:00 on days 7 and 12 of Aβ\(_{25-35}\) exposure, 10 rats from each group were sacrificed (via decapitation), and the prefrontal cortex and hippocampus were removed (on ice) and stored at 80°C until analysis. The remaining animals were anesthetized with pentobarbital sodium and perfused through the ascending aorta with normal saline followed by 4% paraformaldehyde. Brain tissue was dissected and fixed for histological examination.

**Experiment 3: Effect of progesterone on Aβ\(_{25-35}\)-induced cognition impairment**
This experiment mainly explored the effect of progester-
terone on cognitive impairment induced by Aβ25-35 microinjection. Between 6 and 12 days after surgery, Aβ25-35 rats were injected subcutaneously with 4 (P↓1), 8 (P↓2), or 16 (P↓3) mg/kg progesterone (Sigma-Aldrich) (dissolved in sesame oil) daily at 13:30. Behavioral tests were conducted (at 14:30) between 7 and 12 days after progesterone treatment. Immediately following behavioral tests, five rats from each group were decapitated, and the hippocampi were removed (on ice) and stored at −80°C. The remaining animals were anesthetized with pentobarbital sodium and perfused through the ascending aorta with normal saline followed by 4% paraformaldehyde. Brain tissue was dissected and fixed for histological examination.

**MWM for the assessment of spatial learning and memory**

The MWM task was performed in a pool (180 cm diameter, 60 cm high) consisting of a circular black platform submerged 1 cm below the water (24 ± 1°C) surface (40 cm level). Rats were trained over four trials each day for 5 consecutive days (navigation trials) to detect the hidden platform. If the platform was found within 60 seconds, the animals were allowed to stay for an additional 10 seconds. If rats failed to find the platform within 60 seconds, they were guided to the platform and allowed to stay for 10 seconds. The time needed to reach the platform (escape latency) was analyzed as an index of spatial learning. The probe trial was conducted on day 6, when each animal was allowed 90 seconds to search for the platform, which had been removed. The number of times that the animal crossed this area (number of platform crossings) and time ratio in the platform quadrant (quadrant IV were used as an index of spatial memory retention). A camera was used to track the time to find the platform as well as to measure the swim speed and distance travelled.

**HPLC-MS analysis for neuroactive steroids**

Samples of prefrontal cortex or hippocampus (100 mg) were added with 10 μL of 30 ng/mL methyltestosterone (Sigma-Aldrich) as internal standards, homogenized in 1 mL PBS (pH 7.4) using an ultrasonic homogenizer (Heidolph, Germany), mixed with ethyl acetate/n-hexane (HPLC grade; Concord, Tianjin, China; 9:1, v/v) and centrifuged (13 800 × g for 10 minutes) and then transferred to autosampler vials for HPLC-MS (Thermo Fisher Scientific, Waltham, MA, USA).

The HPLC-MS system consisted of the Surveyor MS Pump Plus, Surveyor AS Plus, TSQ Quantum Access triple quadruple mass spectrometer and Xcalibur Data Systems. Separation was achieved on a XDB C18 analytical column (4.6 x 50 mm (Agilent, Palo Alto, CA, USA) fitted with a XDB C18 guard column (4.6 x 12 mm (Agilent). The HPLC mobile phases were: (A) H2O/0.1% formic acid (HPLC grade) (Concord) and (B) MeOH/0.1% formic acid H2O. The gradient (flow rate 0.5 mL/minute) was: T0 36% A, T6.5 36% A, T6.6 30% A, T16 10% A, T17 10% A, and T18 36% A. The column temperature was 40°C and the injection volume was 30 μL. A MSD quadrapole mass spectrometer equipped with an atmospheric pressure chemical ionization source (Thermo Fisher Scientific, Waltham, MA, USA) was used for the detection of analytes in the positive ion mode. The optimized conditions were as follows: spray voltage (4 500 V), vaporizer temperature (400°C), sheath gas (30 L/min), aux gas (5 L/min), and capillary temperature (270°C). Quantification was performed using the multiple-reaction monitoring method with the transitions (m/z) of 254.97 → 132.9 and 158.86 for 17β-estradiol, 299.03 → 158.86 and 280.9 for pregnenolone, 301 → 188.9 and 282.85 for allopregnanolone (Sigma-Aldrich), 315.03 → 97 and 109.01 for progesterone, and 303.1 → 97.04 and 109.06 for methyltestosterone.

**Histological changes of hippocampal CA1 by Nissl staining**

Brain tissue was fixed in 4% paraformaldehyde in PBS, dehydrated in a series of differing ethanol concentrations, embedded in wax and sectioned (5 μm thickness). Tissue sections were then deparaffinized, dehydrated, and stained with thionine, followed by differentiation, dehydration and clearance before they were mounted. The number of intact pyramidal cells (per 1 mm linear length of the same section of hippocampal CA1) was then counted under an Olympus (Tokyo, Japan) microscope (by three experimenters).

**Western blot analysis for hippocampal TNF-α and IL-1β**

Lysates from brain tissue were prepared with lysis buffer (1% Triton X-100, 150 mmol/L NaCl, 10 mmol/L Tris-HCl, pH 7.4, 1 mmol/L EDTA, 1 mmol/L EGTA, 0.2 mmol/L Na2VO3, 0.2 mmol/L phenylmethylsulfonyl fluoride, and 0.5% NP-40 [pH 8.0]). Equal amounts of protein (30 μg) were separated on 10% sodiumdodecyl sulfate poly-acrylamide gels via electrophoresis (90 V, 1.5 hours),
and transferred (20 V, 0.5–1 hour depending on the molecular weight of protein tested) to polyvinylidene difluoride membranes (Millipore, Billerica, MA, USA). Membranes were blocked with 5% bovine serum albumin for 2 hours at room temperature, and incubated with the primary antibodies: rabbit anti-TNF-α polyclonal antibody (1:1 000; Abcam, San Francisco, CA, USA), rabbit anti-IL-1β polyclonal antibody (1:500; Abcam), and mouse anti-β-actin polyclonal antibody (1:5 000; Santa Cruz Biotechnology, Dallas, TX, USA) at 4°C, overnight. Membranes were then incubated with the secondary antibodies: goat anti-mouse IgG-horseradish peroxidase (HRP) (Santa Cruz Biotechnology) for β-actin and goat anti-rabbit IgG-HRP (Abcam) for the other two antibodies, for 2 hours at room temperature. Proteins were detected using the chemiluminescence plus western blot analysis kit (Santa Cruz Biotechnology). The integrated absorbance of each band was measured using a gel image analyzing system (Alpha Image 2200, Alpha, San Leandro, CA, USA). Changes in the relative amounts of protein expression were represented by the ratios of integrated absorbance normalized to β-actin. The experiments were replicated three times.

**Reverse transcription polymerase chain reaction (PCR) for TNF-α and IL-1β**

Total RNA was isolated from hippocampal cells using TRIZol Reagent (Invitrogen, Carlsbad, CA, USA) and equal amounts were reverse transcribed into cDNA using the oligo dT primer. The cDNAs were then used as DNA templates for PCR. GAPDH was used to ensure equal loading. The PCR products were separated on a 1.5% agarose gel and visualized by ethidium bromide. The integrated absorbance of each band was measured using a gel image analyzing system (Alpha Image 2200). Changes in the relative amounts of gene expression were represented by the ratios of integrated absorbance normalized to GAPDH.

**Primers (Sangon Biotech, Shanghai, China):**

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<th>Primer</th>
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<th>Product size (bp)</th>
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<td>IL-1β Sense</td>
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<tr>
<td>Anti-sense</td>
<td>5’-TCC AT CTT TTT GGG TAT TGT T-3’</td>
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**Statistical analysis**

Data are expressed as mean ± SEM. The mean escape latency of the MWM behavioral task were analyzed by repeated-measures analysis of variance followed by least significant difference post hoc test. Other data were statistically analyzed by one-way analysis of variance, followed by least significant difference post hoc test. Significance was reached at values of $P < 0.05$ or $P < 0.01$. Data analyses were performed using SPSS 13.0 (SPSS, Chicago, IL, USA).

**Research background:** Neuroactive steroids participate in the pathogenesis of a variety of neurodegenerative diseases, such as Alzheimer’s disease. However, little is known on the change of neuroactive steroids in the central nervous system of Alzheimer’s disease patients.

**Research frontiers:** The majority of existing studies addressing neuroactive steroids and Alzheimer’s disease focus on the correlation analysis between neuroactive steroid levels and prevalence of Alzheimer’s disease. The impact of amyloid beta (Aβ) (an important etiological factor in Alzheimer’s disease) on the levels of neuroactive steroids, as well as the role of neuroactive steroids on Aβ-mediated learning and memory impairment still remain elusive.

**Clinical significance:** The present study provided a potential therapeutic strategy for the use of neuroactive steroids, particularly progesterone, in learning and memory impairment occurring in Alzheimer’s disease.

**Academic terminology:** Neuroactive steroids: Steroid hormones and their metabolites within the central nervous system. Neuroactive steroids exert sedative, hypnotic, anti-convulsive, anxiolytic, and anti-schizophrenic effects, and improve learning and memory.

**Peer review:** In this study, Alzheimer’s disease-like animal models were established by bilateral hippocampal injection of Aβ25-35. The learning and memory abilities of animals were then detected using the Morris water maze behavioral task. Findings showed that progesterone was effective against Aβ25-35-mediated injury, accompanied by cytoprotective effects.

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(Review ed by Mark F, Liu SX, Zhang B)
(Edited by Yu J, Yang Y, Li CH, Song LP, Liu WJ, Zhao M)