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PROTECTIVE EFFECT OF PREGNENOLONE SULFATE AGAINST SCOPOLAMINE-INDUCED MEMORY IMPAIRMENT IN AN EXPERIMENTAL ANIMAL MODEL

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RESEARCH ARTICLE

ABSTRACT. AIM: To investigate the effect of pregnenolone sulfate (PS) on scopolamine-induced memory impairment in mice and also to probe the possible mechanisms of its action. METHODS: In all experiments, the pharmacological model of amnesia in mice was induced by i.p. administration of scopolamine (1 mg/kg). The number of errors and the latency in the step-down task and the latency in the step-through task were measured 24 hours after the training. The activity of acetylcholinesterase (AChE) and choline O-acetyltransferase (ChAT) was also measured in the cortex and hippocampus 24 hours following drug administration. RESULTS: In the step-down task experiments, scopolamine increased the step-down number of errors ($P < 0.05$) and reduced the step-down latency ($P < 0.01$). Pretreatment with PS (1 mg/kg, s.c.) reduced scopalamine-induced step-down errors ($P < 0.05$), and it also reversed a decrease in the step-down latency ($P < 0.05$). In the step-through task experiments, while scopolamine reduced the step-through latency ($P < 0.01$), pretreatment with PS (1 mg/kg, s.c.) reversed the decrease ($P < 0.01$). In addition, scopolamine administration increased the AChE activity and decreased the ChAT activity in the cortex and hippocampus, but pretreatment with PS (1 mg/kg, s.c.) significantly diminished the effects of scopolamine on the activity of AChE and ChAT in the brain. CONCLUSION: Administration of PS partially protected against the memory impairment in mice induced by scopolamine. The mechanisms of this protective effect likely relate to an inhibition of the AChE activity and an increase of the ChAT activity in the cortex and hippocampus.

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1. INTRODUCTION

Alzheimer's disease (AD), the most common form of senile dementia, is a slowly progressive neuropsychiatric disorder, characterized by neurodegeneration and memory loss. A large number of clinical as well as laboratory studies have suggested that a marked decrease in the CNS cholinergic transmission is involved in the development of cognitive deterioration and memory deficits in AD patients [1-3]. Accordingly, one of the widely-used treatment strategies for AD centers on the restoration of the CNS cholinergic neurotransmission. Several specific strategies have already been developed, which include the use of acetylcholine precursors, acetylcholinesterase (AChE) inhibitors, and cholinergic receptor agonists [4].

Notably, several neurosteroids, such as pregnenolone, dehydroepiandrosterone and their sulfate derivatives, which can be biosynthetically formed in the central nervous system (CNS), have also been suggested to play a role in the regulation of various CNS functions [5-7]. Results from a number of preliminary studies have demonstrated that pregnenolone sulfate (PS) acts as a non-competitive antagonist at the $GABA_A$ receptors [8], and it can also function as an allosteric agonist for the NMDA receptor [7]. These observations have led to the earlier suggestion that these neurosteroids might serve as endogenous modulating agents of the learning and memory processes. In line with this suggestion, it has been demonstrated that PS can partially protect against the memory deficits induced by the administration of an NMDA receptor competitive antagonist [9]. However, very little is known at present about the possible effects of PS on cholinergic neurotransmission in the CNS [10-11]. Our present study is thus designed to determine the protective effect of PS on the cholinergic hypofunction-associated memory impairment and also to probe its possible mechanism of action in an experimental animal model.

2. MATERIALS AND METHODS

2.1. DRUGS AND REAGENTS

PS was supplied by Sigma Chemical Co. (batch number 102K1088), scopolamine was supplied by Shanghai Harvest Pharmaceutical Corporation Ltd. (batch number 030701), and the AChE activity determination kit was purchased from Nanjing Jiancheng Bioengineering Institute (batch number 20040706). [^{3}H]Acetyl-CoA (88.8 GBq/mmol, batch number 203) was obtained from Amersham Biosciences UK Ltd. (Buckinghamshire, England). PS was dissolved in dimethylsulfoxide (Me_2SO) and then diluted in distilled water, with 5% Me_2SO in the final solution. Scopolamine was diluted in normal saline. PS (0.5 or 1 mg/kg) was injected s.c. 45 min before the start of the pretraining. Scopolamine (1 mg/kg) was administered i.p. 20 min before pretraining. All the animal experiments were carried out concurrently, and the drugs and vehicle were administered at a volume of 10 mL/kg body weight.

2.2. ANIMALS

Male Kunming strain mice (clean grade, certification No. SCXK hu 2002-0002), weighing 20-25 g, were obtained from the Department of Laboratory Animal Sciences of Fudan University. In the behavioral experiments, the mice were randomly divided into five groups (N = 10), which included one control group, one model group, one vehicle group, and two PS (0.5 and 1 mg/kg, s.c.) treatment groups. For the measurement of the AChE activity, the mice were randomly divided into four groups (N = 10), namely, the control group, the model group, the vehicle group, and the PS (1 mg/kg, s.c.) treatment group. In the measurement of the ChAT activity, the mice were randomly divided into three groups (N = 7), namely, the control group, the model group, and the PS (1 mg/kg, s.c.) treatment group. All the animals were housed in plastic cages and allowed free access to laboratory food and water under standardized laboratory conditions. In the behavioral experiments, the mice were used following a 4-day period of adaptation to the laboratory conditions. Behavioral experiments were carried out in a sound-proof experimental room between 9:00 h and 15:00 h at the ambient temperatures.

2.3. STEP-DOWN PASSIVE AVOIDANCE TASK EXPERIMENTS

A step-down passive avoidance task was employed using the apparatus consisting of a

Perspex box (25 cm × 15 cm × 20 cm), a floor with bars (0.3-cm diameter) spaced at 1.0 cm intervals, and a rubber platform (4-cm diameter, 4-cm height) set on the bars in the center. Electric shock was given through the bars connected with a scrambled shock generator. During the training, each mouse was placed gently on the platform and allowed to freely adjust to the surroundings for 3 min, and then electric shock (at 30 V, 50 Hz) was delivered through the bars. When the mouse stepped down from the platform onto the bar floor, the electric shock was delivered to the animal. The cutoff time was 5 min. The retention trial was performed 24 h following the training. Electrical shock was also delivered during this trial session. Each mouse was again placed on the platform. The time (step-down latency) that elapsed until the mouse stepped down from the platform was recorded. If the mouse did not step down from the platform within 300 s, the retention trial was terminated and the maximal step-down latency of 300 s was recorded. An error was counted whenever the mouse stepped down from the platform and the number of errors made in 5 min was recorded.

2.4. STEP-THROUGH PASSIVE AVOIDANCE TASK EXPERIMENTS

The apparatus for this experiment consisted of a Perspex box (50 cm × 15 cm × 20 cm) and it was divided into two compartments, including an illuminated box and a dark box. The floor consists of bars (0.3 cm diameter) spaced 1.0 cm apart. The bars of the dark compartment floor could be electrified. During the training, each mouse was first placed in the illuminated compartment. When it stepped into the dark compartment, the mouse received a shock punishment (at 40 V, 50 Hz). The mouse could escape from the shock only by stepping back into the safe illuminated compartment. The response latency before the first footshock within 3 min was measured in the training session. The mice with a latency >180 s were abandoned. Twenty-four hours after the training, each mouse was introduced to the illuminated compartment, and the latency to step-through to the dark compartment was recorded as a passive avoidance behavior indicating the memory levels. Electrical shock was not delivered during this test session. If a mouse did not cross into the dark compartments within 5 min, the retention session was ended and a score of 300 s was recorded.

2.5. MEASUREMENT OF BRAIN AChE ACTIVITY

Forty mice were randomly divided into four groups (N = 10) as aforementioned. Twenty-four hours after drugs given, mice were sacrificed by decapitation and the brain was rapidly removed and kept on ice. Cortex and hippocampus were separated, weighed, and homogenized in ice-cooled normal saline to make a 20% (w/v) and 10% (w/v) homogenates, respectively. The homogenates were centrifuged at 480 × g for 10 min at 4°C and stored at –20°C until use within 3 days. The measurement of AChE activity in the cortical and hippocampal homogenates was carried out according to the procedures of the assay kit provided by the Nanjing Jiancheng Bioengineering Institute (www.njjcbio.com). Protein concentrations were determined using the Coomassie blue protein-binding method using bovine serum albumin (BSA) as standard.

2.6. MEASUREMENT OF BRAIN ChAT ACTIVITY

ChAT activity was determined in homogenates using the radioenzymatic method. Cortex and hippocampus were homogenized in 2 mL of 0.32 mmol/L sucrose solution and 900 μL aliquots of homogenate were added to 225 μL of 2% Triton X-100 containing 50 mmol/L disodium EDTA. The samples were centrifuged at 10000 × g for 5 min at 4°C and stored at –20°C until use within 3 days. A 10-microliter aliquot of the sample was incubated for 15 min at 37°C in 10 μl medium containing [^{3}H]acetyl-CoA (1667 Bq/tube, with a final concentration at 0.2 mmol/L acetyl CoA), choline chloride (12.0 mmol/L), eserine hemisulfate (15.0 μmol/L), sodium chloride (0.4 mol/L), sodium phosphate buffer (pH 7.4, 0.1 mol/L), disodium EDTA (10.0 mmol/L) and 1.0 g/L BSA. The reaction was terminated by placing tubes in an ice-water bath and the addition of 25-μL ice-cold double-distilled water to each tube. The extraction solution (250 μL, containing 85% toluene, 15% acetonitrile, 5 g/L sodium tetraphenylboron) was added. The amount of [^{3}H]acetylcholine produced was determined by addition of 1 mL toluene containing 0.05% 2,5-diphenyloxazole and 0.02% 1,4-bis-(5-phenyl-2-oxazoyl)-benzene, and the total radioactivity (d.p.m.) in the organic phase was

TABLE 1. EFFECTS OF PS ON STEP-DOWN LATENCY IN THE STEP-DOWN PASSIVE AVOIDANCE TASK (N = 10).

TREATMENT GROUPS	NUMBER OF ERRORS
Control	1.2 ± 0.6
Scopolamine 1 mg/kg	3.7 ± 2.5[b]
5% Me_2SO 10 mL/kg + Scopolamine 1 mg/kg	2.4 ± 1.5[b]
PS 0.5 mg/kg + Scopolamine 1 mg/kg	1.9 ± 1.0
PS 1 mg/kg + Scopolamine 1 mg/kg	1.3 ± 0.7[e]

[b] $P < 0.05$ vs control group. [e] $P < 0.05$ vs scopolamine-treated group.

determined using LS 6500 multi-purpose scintillation counter (Beckman COULTERTM, USA). All samples were run in triplicate. Background levels were determined in parallel tubes in the absence of the sample protein. The protein content of each sample was determined using the Coomassie blue protein-binding method. The ChAT activity was calculated according the amount of [^{3}H]acetylcholine formed and was expressed as nanomoles of ACh per milligram of protein per hour.

2.7. STATISTICAL ANALYSIS

The data were expressed as mean ± SD. All the data, except the step-down numbers of errors, were analyzed using the *ANOVA* and the Dunnett's multiple comparison test. The Mann-Whitney test was used for comparing the numbers of errors in the step-down task experiments. In all of the statistical analyses, the level for statistical significance was set at $P < 0.05$.

3. RESULTS

3.1. EFFECT OF PS ON THE STEP-DOWN PASSIVE AVOIDANCE TASK

Administration of scopolamine (1 mg/kg) caused a marked increase in the step-down numbers

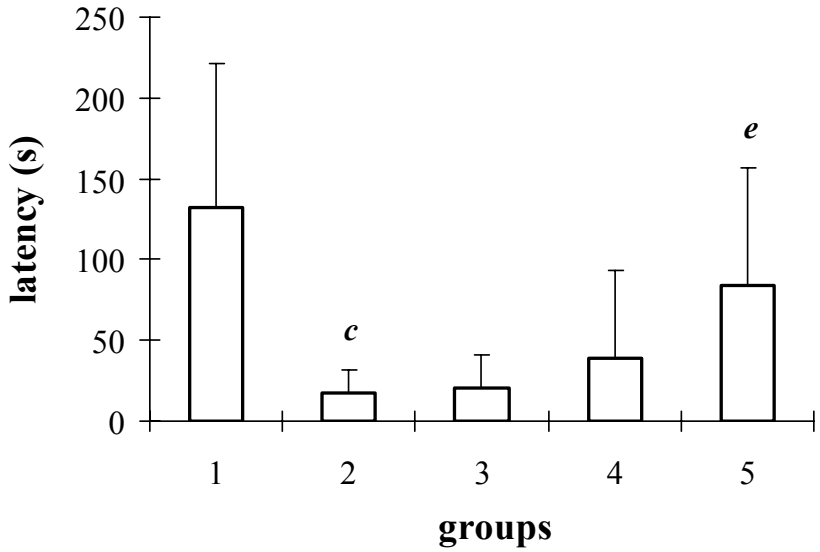

FIGURE 1. EFFECT OF PS ON STEP-DOWN LATENCY OF MICE IN THE PASSIVE AVOIDANCE-TASK (N = 10).
[c] $P < 0.01$ vs control group. [e] $P < 0.05$ vs scopolamine-treated group.
Different groups: 1: Control. 2: Scopolamine 1 mg/kg. 3: Scopolamine 1 mg/kg + 5% Me_2SO 10 mL/kg.
4: Scopolamine 1 mg/kg + PS 0.5 mg/kg. 5: Scopolamine 1 mg/kg + PS 1 mg/kg.

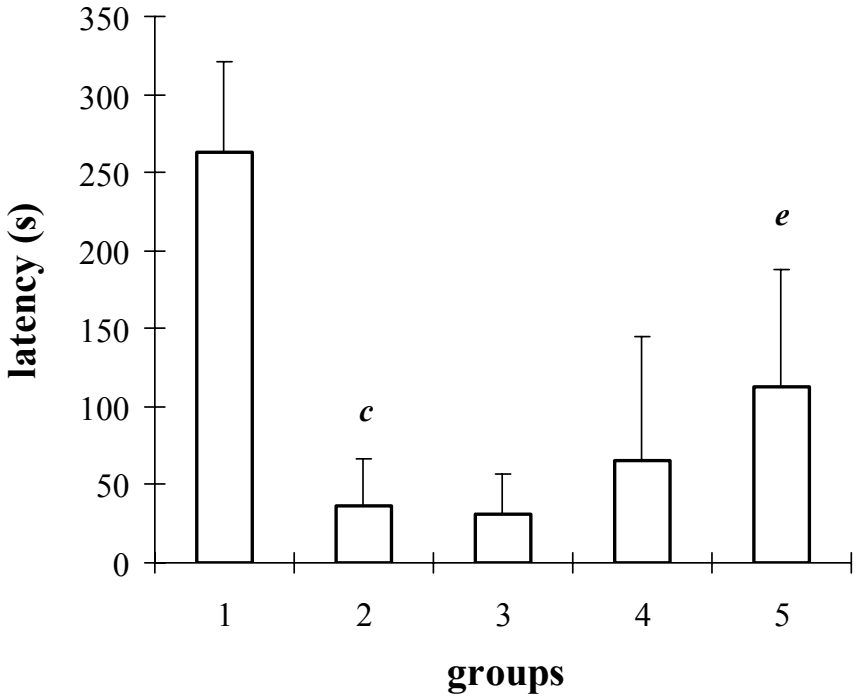

FIGURE 2. EFFECT OF PS ON STEP-THROUGH LATENCY OF MICE IN THE PASSIVE AVOIDANCE-TASK (N = 10).
[c] $P < 0.01$ vs control group. [e] $P < 0.05$ vs scopolamine-treated group.
Different groups: 1: Control. 2: Scopolamine 1 mg/kg. 3: Scopolamine 1 mg/kg + 5% Me_2SO 10 mL/kg.
4: Scopolamine 1 mg/kg + PS 1 mg/kg. 5: Scopolamine 1 mg/kg + PS 1 mg/kg.

of errors ($P < 0.05$, TABLE 1), and it also significantly reduced the step-down latency ($P < 0.01$, FIG. 1) 24 h after training. Pretreatment with PS at 0.5 mg/kg (s.c.) decreased the step-down numbers of errors, and it also increased step-down latency. However, the difference was not statistically significant compared to the scopolamine-treated group. Pretreatment with PS (at 1 mg/kg, s.c.) decreased the step-down errors significantly ($P < 0.05$, TABLE 1) and also reversed scopolamine-induced reduction in the step-down latency ($P < 0.05$, FIG. 1).

3.2. EFFECT OF PS ON THE STEP-THROUGH PASSIVE AVOIDANCE TASK

Administration of scopolamine (1 mg/kg, i.p.) caused a significant reduction in the step-through latency ($P < 0.01$). Pretreatment with PS (0.5 mg/kg, s.c.) increased the step-through latency, but the effect was not statistically significant compared with the scopolamine-treated animals. Pretreatment with PS (1 mg/kg, s.c.) reversed the reduction in the step-through latency significantly ($P < 0.05$, FIG. 2).

3.3. EFFECT OF PS ON THE BRAIN ACHE ACTIVITY

Scopolamine administration (1 mg/kg, i.p.) increased the AChE activity in the cortex and hippocampus of the animals significantly ($P < 0.01$). Pretreatment with PS (1 mg/kg, s.c.) inhibited the AChE activity significantly compared with the scopolamine-treated group ($P < 0.01$, FIG. 3).

3.4. EFFECT OF PS ON THE BRAIN CHAT ACTIVITY

Scopolamine administration reduced the ChAT activity in the cortex and hippocampus of the animals ($P < 0.01$). Pretreatment with PS (1 mg/kg, s.c.) increased the ChAT activity in cortex ($P < 0.05$) and hippocampus ($P < 0.01$, FIG. 4).

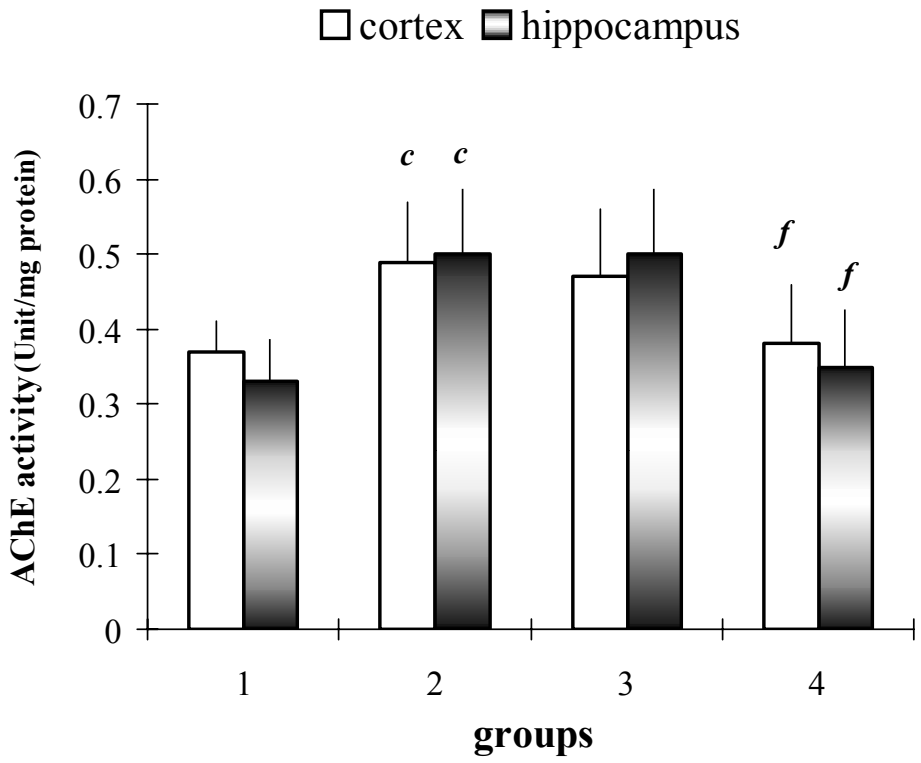

FIGURE 3. EFFECT OF PS ON AChE ACTIVITY IN MICE (N = 10).
[c] P < 0.01 vs control group. [f] P < 0.01 vs scopolamine-treated group.
Different groups: 1: Control. 2: Scopolamine 1 mg/kg. 3: Scopolamine 1 mg/kg + 5% Me_2SO 10 mL/kg.
4: Scopolamine 1 mg/kg + PS 1 mg/kg.

4. DISCUSSION

A commonly-encountered problem in the development of therapeutic drugs for AD is the lack of suitable animal models. Scopolamine is a muscarinic cholinergic receptor antagonist that can cause cognitive deficits similar to those seen in AD patients. On the basis of the widely-accepted premise that cholinergic deficiency is an important etiological factor in the development of amnesic and cognitive deficits in AD, we thus chose to use the scopolamine-induced cholinergic hypofunction and memory impairment as an animal model for evaluating the protective effects of PS.

The results of our present study showed that i.p. administration of scopolamine at 1 mg/kg effectively induced memory impairment, which was clearly evidenced by our observations of an increased number of errors in the step-down task experiments along with a decreased latency in the step-through experiments. Pretreatment with PS (at 1 mg/kg, s.c.) partially reversed the effects induced by scopolamine treatment. In the step-through task experiments, there was a significant reduction of latency in mice treated with scopolamine compared to the control group. The step-through latency was increased in mice treated with scopolamine in combination with PS (1 mg/kg, s.c.). These data suggest that administration of PS (at 1 mg/kg, s.c.) improved the memory impairment induced by scopolamine both in the step-through and step-down task measurements.

It is known that the cholinergic systems in the cerebral cortex and hippocampus play an important role in various aspects of the cognitive functions [12]. While ChAT is an enzyme for the biosynthesis of acetylcholine, AChE is an enzyme that metabolically degrades acetylcholine. A shift in the

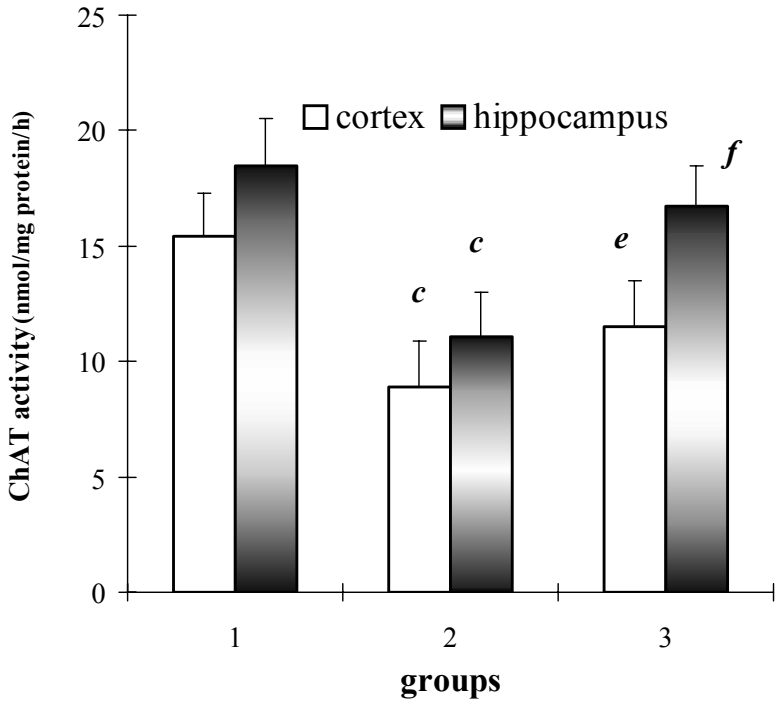

FIGURE 4. EFFECT OF PS ON ChAT ACTIVITY IN MICE (N = 7).
[c] $P < 0.01$ vs control group. [e] $P < 0.05$. [f] $P < 0.01$ vs scopolamine-treated group.
Different groups: 1: Control. 2: Scopolamine 1 mg/kg. 3: Scopolamine 1 mg/kg + PS 1 mg/kg.

balance between these two enzymatic processes favoring an increased accumulation of the acetylcholine neurotransmitter in relevant CNS regions is believed to be highly beneficial for improving the cognitive functions, particularly in an AD patient. In this study, we found that administration of scopolamine (1 mg/kg, i.p.) significantly increased the AChE activity and also decreased the ChAT activity in cortex and hippocampus of mice, both of which would contribute to a significantly reduced level of acetylcholine in these brain regions and thereby may contribute to the memory impairment seen in these animals. However, pretreatment with PS (at 1 mg/kg, s.c.) reversed the scopolamine-induced changes of the AChE and ChAT activities, and it is expected that the combination of these effects of PS would contribute to increased levels of ACh in the cortex and hippocampus. These biochemical observations in the intact animals were consistent with behavioral improvements as observed in this study. We believe that this is an important mechanism that may be partially responsible for the protective effects of PS against the scopolamine-induced memory impairment in the animals.

In summary, administration of PS partially protects against the memory impairment induced by scopolamine. The mechanism relates to its inhibition of the AChE activity and increase of the ChAT activity in the cortex and hippocampus of the animals. These findings suggest that PS may be a potentially useful agent for protection against the cholinergic hypofunction-associated memory impairment in AD patients.

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