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# The ameliorating effect of 1-palmitoyl-2-linoleoyl-3-acetylglycerol on scopolamine-induced memory impairment via acetylcholinesterase inhibition and LTP activation

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ABSTRACT

In the present study, we investigated whether 1-palmitoyl-2-linoleoyl-3-acetylglycerol (PLAG), a component of antlers of *Cervus nippon* Temminck, would have memory-ameliorating properties against cholinergic blockade-induced memory impairment in mice. In the passive avoidance task to investigate the effects of PLAG on long-term memory, PLAG (10 mg/kg, p.o.) administration ameliorated scopolamine-induced memory impairment. PLAG also reversed the impairments of working memory in the Y-maze task and spatial memory as shown in the Morris water maze. To identify the mechanism of the memory-ameliorating effect of PLAG, acetylcholinesterase (AChE) inhibition assay and the Western blot analysis were conducted. In the AChE inhibition assay, PLAG inhibited the AChE activity in mice and PLAG increased the expression levels of phosphorylated CaMKII, ERK, and CREB in the hippocampus. Additionally, long-term potentiation (LTP) of synaptic strength occurred by PLAG treatment in the hippocampal cultures. Overall, the present study suggests that PLAG reversed memory deficits in an animal model and that it affects biochemical pathways related to learning and memory.



## 1. Introduction

Alzheimer's disease (AD) is the most common form of dementia. It is estimated that 35.6 million people are living with dementia worldwide, with an anticipated increase to 115.4 million by 2050 [1]. The hallmark of AD is an impaired cholinergic neurotransmission, which contributes to global and progressive cognitive dysfunction, thus current symptomatic therapies for mild to moderate AD aim to increase the activity of cholinergic neurotransmitter system by the inhibition of acetylcholinesterase (AChE) [2,3]. Up to date, approved treatments by US Food and Drug Administration includes five drugs that are used to treat the cognitive function of AD are as below: rivastigmine (Exelon), galantamine (Razadyne, Reminyl), tacrine (Cognex), and donepezil (Aricept) and NMDA receptor antagonist memantine (Namenda). Each drug acts to delay the breakdown of acetylcholine or inhibit neurotoxicity. They have all been shown to modestly slow the progression of cognitive symptoms and reduce problematic behaviors in some people, but at least half of the people who take these drugs do not respond to them or have severe side effects [4,5]. Thus, much effort is being directed towards the discovery of disease cure therapies which can block the progression of the disease and drugs targeting various molecular pathways [6–9]. Therefore, it would be necessary to explore the

*Abbreviations:* PLAG, 1-palmitoyl-2-linoleoyl-3-acetylglycerol; AChE, acethylcholinesteras; CaMKII, $Ca^{2+}$/calmodulin-dependent protein kinase II; ERK, extracellular signal-regulated kinase; CREB, cAMP response element-binding protein; LTP, long-term potentiation.

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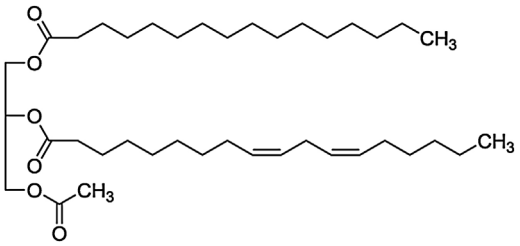

**Fig. 1.** The chemical structure of PLAG (1-palmitoyl-2-linoleoyl-3-acetylglycerol).

new therapeutic agents that increase cholinergic activity, reverse the cognitive dysfunction with fewer side effects.

Antlers from *Cervus nippon* Temminck (Cervidae) have been used to increase the vital function in the traditional oriental medicine. Recent pharmacological approaches also revealed that deer antler is known to have hematopoiesis regulatory effect, immunity, anti-oxidant, anti-inflammatory, or tonic effect [10,11]. Recently, it has been reported that deer antler extracts ameliorate scopolamine-induced cognitive dysfunction in mice [12]. Until yet, it is not known which compound(s) is active one to show such pharmacological activities. Recently, Shin et al. suggested that 1-palmitoyl-2-linoleoyl-3-acetylglycerol (PLAG, called as EC-18) would be an active compound of a deer antler [13]. PLAG has been reported to have several pharmacological efficacies like a potent activity in hematopoiesis, anti-tumor activities in biliary cancer model, or a modulator of immune-related factors from both *in vivo* and *in vitro* studies [14–17,13]. However, the effect of PLAG on learning and memory has not been studied, yet.

Here, we investigated the effects of PLAG on cognitive function using scopolamine-induced memory impairment mice model as an animal model for AD [18–21]. For evaluation, the passive avoidance, the Y-maze, and the Morris water maze test were employed. In addition, to understand the mechanism of action of PLAG, we performed AChE inhibition activity in *ex vivo* as well as analyzed the expression level of memory-related proteins and electrophysiological studies.

## 2. Materials and methods

### 2.1. Animals

Male ICR mice (6 weeks old; 25–30 g body weight) used in the experiments were purchased from the Orient Co., Ltd., which is a branch of the Charles River Laboratories (Gyeonggi-do, Korea). Arrived mice were kept 5 per cage in University facility for additional 1 week before the experiment. Mice were provided food and water *ad libitum* and kept in a 12 h light/dark cycle (the light was on from 07:30–19:30 h) at constant temperature ($23 \pm 1\,^{\circ}C$) and humidity ($60 \pm 10\%$). We used 100 mice for the passive avoidance task, 60 mice for the Y-maze, 40 mice for the Morris water maze, 28 mice for the open-field test, 35 mice for AChE activity assay and 12 mice for the Western blotting. A total number of animals was 275. We used the different animals in each behavioral or biochemical test. All behavioral experiments were in progress to 10:00 to 16:00, animal treatment and care were performed in accordance with the Animal Care and Use Guidelines, and the experimental protocols were approved by the Institutional Animal Care and Use Committee (IACUC) (approved No. KHP-2014-02-01).

### 2.2. Materials

PLAG (Fig. 1) was donated from ENZYCHEM Lifesciences Co. (Seoul, Korea). Donepezil hydrochloride monohydrate, (−)-scopolamine hydrobromide, acetylthiocholine iodide and DTNB (5,5′-dithiobis [2-nitrobenzoic acid]) were purchased from the Sigma Chemical Co. (St. Louis, MO). Scopolamine and donepezil were dissolved in 0.9% saline and PLAG was suspended in 10% Tween 80 solution. Rabbit polyclonal antibodies of $Ca^{2+}$/calmodulin-dependent protein kinase II (CaMKII), extracellular signal-regulated kinase (ERK), anti-cAMP response element-binding protein (CREB) and HRP-conjugated secondary antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Rabbit polyclonal antibodies of phospho-CaMKII (pCaMKII) and phospho-ERK1/2 (pERK) were purchased from Cell Signaling Technology (Cell Signaling, MA). The rabbit polyclonal anti-phosphorylated CREB (pCREB) antibody was purchased from Millipore (Temecula, CA). All other materials were obtained from normal commercial sources and were of the highest grade available.

### 2.3. Passive avoidance task

Passive avoidance task was executed over 2 days that divided into acquisition and retention trials. The passive avoidance box consisted of two identical, light and dark, boxes (20 cm × 20 cm × 20 cm), divided by a guillotine door (5 cm × 5 cm). The bottom of both boxes consisted of 2 mm steel rods spaced 1 cm apart and the light box was attached a 50 W bulb as described elsewhere [22]. In an acquisition trial, each mouse was placed in the light box and the door between the two boxes was opened 10 s later. When the mouse entered the dark box, the door automatically closed, and an electrical shock (0.5 mA) was delivered through the steel rods for 3 s. If the mouse did not enter the dark box within 60 s after the door opened, then the mouse was smoothly forced to put into the dark box, and considered the latency as 60 s. Mice were administered PLAG (1, 3 or 10 mg/kg, p.o.), donepezil (5 mg/kg, p.o.) or the same volume of vehicle (10% Tween 80 solution, p.o) 1 h before the acquisition trial, and administration of scopolamine (1 mg/kg, i.p.) or 0.9% saline 30 min before the acquisition trial. Retention trial was conducted by introducing mice again into the light box 24 h after the acquisition trial. The door opens and then record the time when the mice entered the dark box as the step-through latencies in the acquisition and retention trials. Step-through latencies were recorded for up to 300 s.

In a memory enhancement study, a single administration of PLAG (1, 3 or 10 mg/kg, p.o.) was conducted 1 h before the acquisition trial without scopolamine. When the mice entered the dark box, the guillotine door automatically closed and electrical shock was delivered through the steel rods for 3 s at 0.25 mA instead of 0.5 mA to avoid a ceiling effect. Latency time was recorded up to 600 s. Other methods were the same as those described above.

### 2.4. Y-maze

The Y-maze was a three-arm maze with angles of 120° among the arms from dark opaque polyvinyl plastic (40 cm long, 3 cm wide and with walls 12 cm high) as described elsewhere [23]. PLAG (1, 3 or 10 mg/kg, p.o.), donepezil (5 mg/kg, p.o.) or the same volume of vehicle (10% Tween 80 solution, p.o) was administered 1 h before the test. Scopolamine (1 mg/kg, i.p.) was administered 30 min after the PLAG treatment in order to induce memory impairment. The mice were placed on one arm at the first time and then started recording the sequence (i.e., ABCAB, etc) and a number of arm entries manually for each mouse over an 8 min. At the end of each test, the maze was cleaned with 70% ethanol spray to remove odors or residues. A real alternation was defined as entries into the arm on consecutive triads (i.e., ACB, CBA, or BAC but not CBC). The number of total arm entries indicates a locomotor activity. The percentage of spontaneous alternation was set in the following way: % alternation = [(number of alternations)/(total arm entries − 2)] × 100.

### 2.5. Morris water maze

The Morris water maze is a circular pool (90 cm in diameter and 45 cm in height) with four visual cues. The pool was filled with water (24 ± 1 °C) 30 cm depth containing black food colors. The water tank was put in a dimly light, soundproof test room. A black platform (6 cm in diameter and 29 cm high) was placed in one of the pool quadrants divided into cues. The first experimental day was conducted to swim training for 60 s in the absence of the platform. During the next 4 consecutive days, the mice were received two trials per session per day in the state with the platform [22]. When a mouse located the platform, it was allowed to remain there for 10 s. If the mouse did not locate the platform within 60 s, it was introduced into the platform and placed on it for 10 s. The time interval between the two trials in a session was 30 min [23]. During each trial session, the time to find the hidden platform, latency, was recorded by a video camera-based Ethovision System (Noldus, Wageningen, The Netherlands). One day after the last training trial session, mice were received to probe trial session in which the platform was removed from the pool. In the probe trial, the mice were allowed to swim for 60 s to explore for it. A record was kept of the swimming time in the pool quadrant where the platform had previously been placed. PLAG (10 mg/kg, p.o.), donepezil (5 mg/kg, p.o.) or the same volume of vehicle (10% Tween 80 solution, p.o.) was administered 1 h before the first trial in each session during the training sessions. Memory impairment of the mice was induced by scopolamine (1 mg/kg, i,p.) at 30 min before the first trial in each session.

### 2.6. Open field test

The open field test was performed in a clear black Plexiglas box (41.5 cm × 41.5 cm × 41.5 cm), recorded with the video-based Ethovision system (Noldus, Wageningen, The Netherlands), as described elsewhere [24]. First, the administration of PLAG (1, 3 or 10 mg/kg, p.o.) or the same volume of vehicle (10% Tween 80 solution, p.o.) in mice was completed 1 h before the test. When the experiment started, mice were placed in the center of the box and their locomotor action was recorded for 25 min using the video-tracking system. A horizontal locomotor activity was shown as the total ambulatory distance. At the end of each test, each box was cleaned with into 70% ethanol spray and paper towel.

### 2.7. Acetylcholinesterase inhibition assay

An analysis of AChE activity was conducted using acetylthiocholine iodide substrate in a colorimetric method [25]. In our *ex-vivo* study, mice were administered PLAG (10 mg/kg, p.o.) or the same volume of vehicle (10% Tween 80 solution, p.o.) and sacrificed 1 h after each administration [26]. Donepezil (5 mg/kg) was used as a positive control. Whole mouse brain except cerebellum was homogenized in a glass Teflon homogenizer (Eyela, Japan) including 3.5 ml (10 times of each tissue weight) volumes of homogenizer buffer (0.1 M phosphate buffer, pH 8.0), and then centrifuged at 3000 g for 10 min at 4 °C. The supernatant was used as an enzyme source for the assay. The supernatant solution was mixed with 144 μl of Buffer A (0.1 M phosphate buffer, pH 8.0), 22 μl of buffered Ellman's reagent (10 mM 5, 5′-dithiobis [2-nitrobenzoic acid] and 15 mM sodium bicarbonate) and 1.1 μl of acetylthiocholine iodide solution, and then mixed with 4.4 μl of neostigmine solution in 96 well after then incubated at room temperature for 10 min. Absorbance was measured at 412 nm using a UV spectrophotometer (OPTIZEN 2120UV, Mecasys Co., Ltd., Korea).

### 2.8. Western blot analysis

For the Western blot analysis, PLAG (10 mg/kg, p.o.) or the same volume of vehicle (10% Tween 80 solution) was administered 1 h before, and scopolamine (1 mg/kg, i.p.) or the same volume of vehicle was also administered 30 min before sacrifice, respectively. Thereafter, the mice were sacrificed, and the brain tissues were removed. Isolated hippocampal tissues were homogenized in an ice-cold Tris-HCl buffer (20 mM, pH 7.4) including 320 mM sucrose, 1 mM EDTA, 1 mM EGTA, 1 mM PMSF, 1 mM sodium orthovanadate and one tablet of protease inhibitor (Roche, Seoul, Korea) per 50 ml of buffer. Tissue samples were centrifuged at 12,000 rpm for 20 min at 4 °C, quantified by the Bradford method using Pierce bicinchoninic acid (BCA) protein assay kit (Thermo scientific, PA). Quantitative homogenized samples (15 μg of protein) were subjected to SDS-PAGE (10% gels) under reducing conditions. The proteins were transferred to a polyvinylidene difluoride (PVDF) membranes in transfer buffer [25 mM Tris-HCl buffer (pH 7.4) including 192 mM glycine and 20% v/v methanol] at 400 mA for 2 h at 4 °C. The membrane was washed five times with a solution of Tris-buffered saline/Tween 20 (TBS-T) and maintained for 2 h in a blocking solution (5% skim milk) at room temperature. Then it was maintained overnight in a 1:3000 dilution of the anti-pCaMKII, anti-CamKII, anti-pERK, anti-ERK, anti-pCREB or anti-CREB antibody at 4 °C. Next day, the membranes were washed again five times with TBS-T and maintained in a 1:5000 dilution of the appropriate horseradish peroxidase-conjugated secondary antibodies for 2 h at room temperature. The membranes were washed five times with TBS-T and developed by enhanced chemiluminescence (Amersham Life Science, Arlington Heights, IL). The immunoblots were visualized using a bio-imaging program on an ImageQuant ™ LAS 4000 mini biomolecular imager (Fujifilm Lifescience U.S.A., Stamford, CT) and analyzed using Multi Gauge version 3.2 software (Fujifilm Holdings Corporation, Tokyo, Japan). The levels of pCaMKII, pERK, or pCREB were normalized to total CaMKII, ERK, or CREB by calculating the ratio from the control group in the same membranes, respectively.

### 2.9. Acute hippocampal slice preparation and electrophysiology

Mouse hippocampal slices were prepared using microvibratome (Lafayette-Campden neuroscience™). The brain was rapidly removed and placed in ice-cold artificial cerebrospinal fluid (aCSF; bubbled with 95% $O_2$/5% $CO_2$) which was comprised of following ingredients: (mM) NaCl, 124; KCl, 3; $NaHCO_3$, 26; $NaH_2PO_4$, 1.25; $CaCl_2$, 2; $MgSO_4$, 1; D-glucose, 10. Transverse hippocampal slices (400 μm thick) were prepared and submerged in aCSF (20–25 °C) for 1 h before transfer to the recording chamber (28–30 °C, flow rate: 3 ml/min) as required. Field recordings were made from stratum pyramidal in the area hippocampal CA1. Stimulating electrodes were placed in the Schaffer collateral-commissural pathway. Stimuli (constant voltage) were delivered at 30 s intervals. To induce LTP, one train of high frequency stimulation (100 pulses at 100 Hz) was delivered. The slope of the evoked field potential responses was averaged from four consecutive recordings (EPSPs) evoked at 30 s intervals. PLAG (~100 μM) was perfused to recording chamber from 30 min before to immediately after HFS treatment.

### 2.10. Statistics

All of the data represented as the means ± S.E.M. The latency time in the passive avoidance task, the spontaneous alternations, the number of total arm entry in the Y-maze task, swimming time in target quadrant, the number of hidden platform crossing, speed of mice in the probe trial of the Morris water maze task, the total

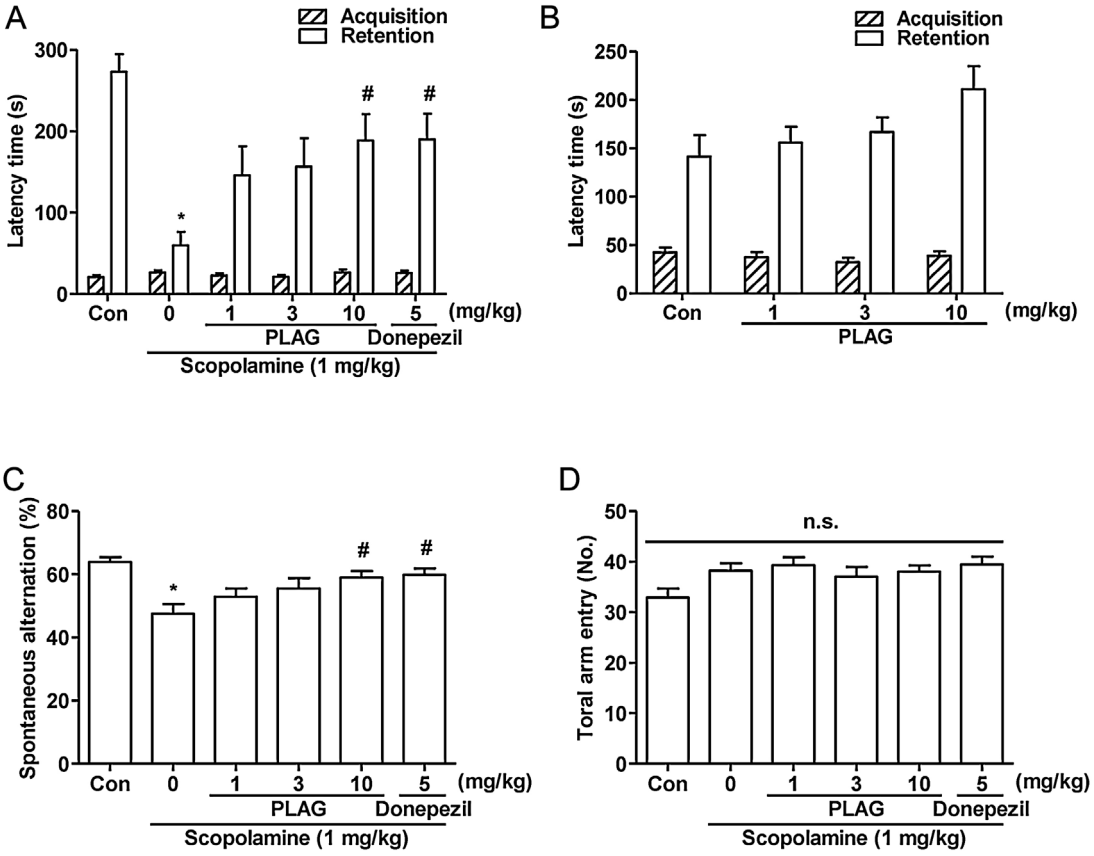


**Fig. 2.** (A) The effects of PLAG on scopolamine-induced memory impairment in the passive avoidance task. PLAG (1, 3 or 10 mg/kg, p.o.), donepezil (DNZ, 5 mg/kg, p.o.) or the same volume of vehicle (10% Tween 80 solution, p.o.) was administered to mice 1 h before the acquisition trial. Memory impairment was induced by scopolamine (1 mg/kg, i.p.) 30 min before the acquisition trial. The retention trial was conducted up to 300 s 24 h after the acquisition trial. (B) The effect of PLAG on memory enhancement of normal naïve mice in the passive avoidance task. PLAG (1, 3 or 10 mg/kg, p.o) or the same volume of vehicle (10% Tween 80 solution, p.o.) was administered to mice 1 h before the acquisition trial. In the acquisition trial, mice were received a mild electrical shock (0.25 mA). The retention trial was conducted 24 h after the acquisition trial and observed for 10 min. (C–D) The effects of PLAG on scopolamine-induced memory impairment in the Y-maze task. PLAG (1, 3 or 10 mg/kg, p.o.), donepezil (DNZ, 5 mg/kg), or the same volume of vehicle (10% Tween 80 solution, p.o.) was administered to mice 1 h before the Y-maze task. Memory impairment was induced by scopolamine (1 mg/kg, i.p.) 30 min before the task. Spontaneous alternation behavior (C) and the number of total arm entry (D) were recorded during a period of session time, 8 min. Data represent means ± S.E.M. (n = 9-10/group) ($^{*}P < 0.05$, versus the vehicle-administered control group; $^{\#}P < 0.05$, versus the scopolamine-administered group) Con, control. n.s., no significance.

distance moved in the open field test, and Western blots were analyzed by one-way analysis of variance (ANOVA) followed by Newman-Keuls test for multiple comparisons. Escape latency time of training day in the Morris water maze task and the distance moved in period of 5 min in the open field test were analyzed by a two-way ANOVA, followed by Bonferroni's post hoc analysis using the day (Morris water maze task) or time (open field task) as one variable and the treatment as a second factor. For the electrophysiological studies, the data were analyzed using one-way analysis of variance (ANOVA) followed by Student-Newman-Keuls test for multiple comparisons during the last five minutes of the one-hour recording. The statistical significance was set at $P < 0.05$. All statistical analyses were performed using Prism 5.0 software (GraphPad, La Jolla, CA).

## 3. Results

### 3.1. Effect of PLAG on scopolamine-induced memory impairment in the passive avoidance task

To investigate the memory-ameliorating effect of PLAG on cholinergic blockade-induced memory impairment, the passive avoidance task was conducted using scopolamine-treated model. The administration of PLAG exerted a significant group effect on step-through latency in the retention trial [$F$ (5, 52) = 5.019, $P < 0.05$, Fig. 2A]. The reduction of the step-through latency induced by scopolamine (1 mg/kg) was significantly ameliorated in the PLAG-administered group (10 mg/kg) as observed in the donepezil-administered group (5 mg/kg, $P < 0.05$). According to the above result, we applied the selected dose of PLAG (10 mg/kg) to further studies. In case of memory enhancement experiment using normal naïve mice, PLAG seemed to be effective in the memory improvement in a dose-dependent manner, but failed to show the significant result [$F$ (3, 33) = 2.345, $P > 0.05$, Fig. 2B]. These results suggest that PLAG would be effective in the memory amelioration not in the improvement under normal condition.

### 3.2. Effect of PLAG on scopolamine-induced memory impairment in the Y-maze task

The Y-maze task was conducted to investigate the effect of PLAG on spontaneous alternation behavior. The administration of PLAG exerted a significant group effect on spontaneous alternation behavior [$F$ (5, 49) = 5.517, $P < 0.05$, Fig. 2C]. The lowered spontaneous alternation in the scopolamine-treated group was significantly ameliorated by PLAG (10 mg/kg) or donepezil (5 mg/kg) administration. The average numbers of total arm entry were likeness among all experimental groups ($P > 0.05$, Fig. 2D), suggesting

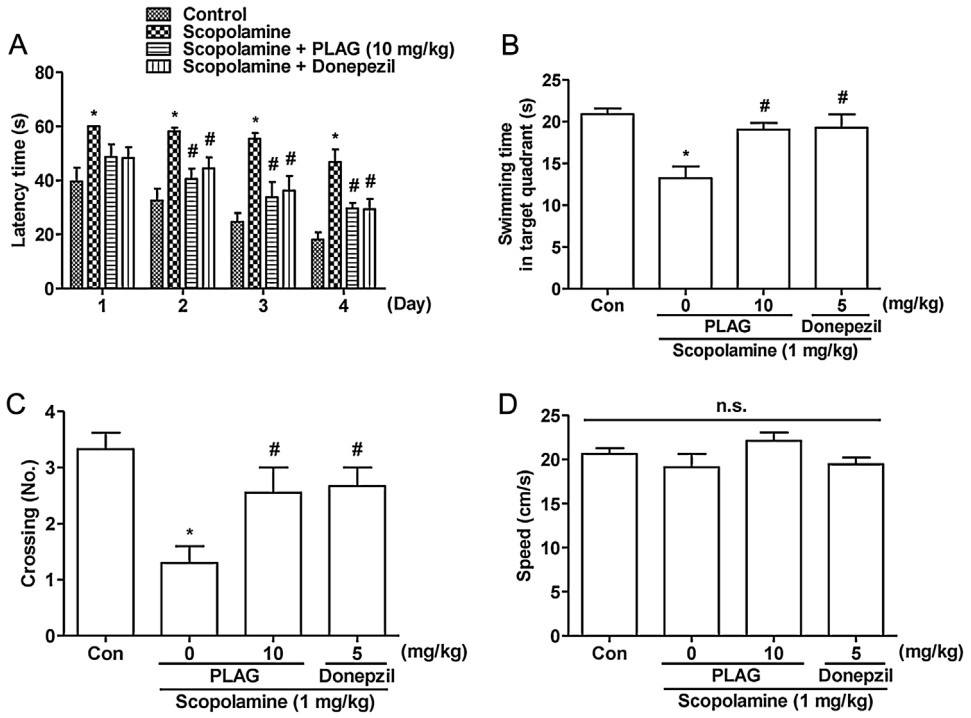


**Fig. 3.** The effects of PLAG on scopolamine-induced memory impairment in the Morris water maze task. The escape latency time throughout training trial sessions for 4 days (A), the swimming time in the target quadrant (B), the number of crossing in area where hidden platform was located (C), and the swimming speed (D) during the probe trial section on day 5 in the Morris water maze task were measured. PLAG (10 mg/kg, p.o.), donepezil (5 mg/kg, p.o.) or the same volume of vehicle (10% Tween 80 solution, p.o.) was administered to the mice 1 h before the first training trial of each session. Memory impairment was induced by scopolamine administration (1 mg/kg, i.p.) 30 min before the first training trial of each session. The training trial and probe trial sessions were conducted over 60 s, described in Materials and Methods. Data represent means ± S.E.M. (n = 9-10/group) ($^{*}P < 0.05$, versus the vehicle-administered control group; $^{\#}P < 0.05$, versus the scopolamine-administered group) Con, control. n.s., no significance.

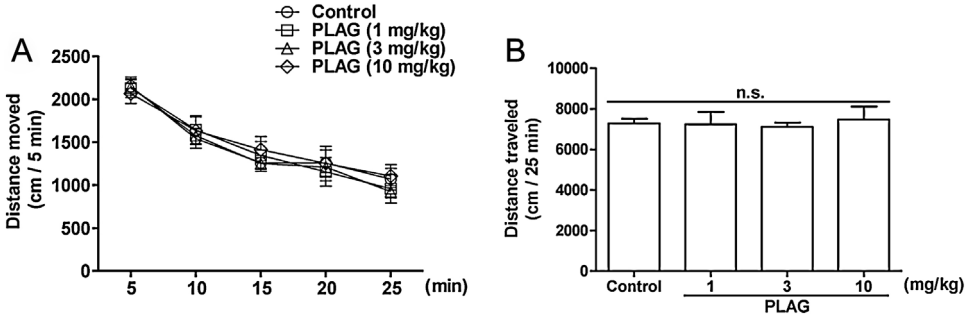


**Fig. 4.** The effects of PLAG on locomotor activity in the open field test. The spontaneous locomotor behavior in the open field test was recorded for 25 min. PLAG (1, 3 or 10 mg/kg, p.o.) or the same volume of vehicle (10% Tween 80 solution, p.o.) was administered 1 h before the test in mice. These data showed the distance moved at an interval of 5 min (A) and the total distance moved for 25 min (B). Data represent means ± S.E.M. (n = 7/group). Con, control. n.s., no significance.

that PLAG did not bring alteration in the general locomotor activity. These results implied that PLAG exerts ameliorating effect against cholinergic blockade-induced short-term or working memory deficit in mice.

### 3.3. Effect of PLAG on scopolamine-induced memory impairment in the morris water maze task

To determine the effect of PLAG on long-term spatial learning and memory, the Morris water maze task was conducted in the scopolamine-induced memory impairment model. As a result of the training of 4 days, both the escape latency of PLAG and donepezil-administered group were significantly reduced compared to that of scopolamine-administered group [2 day, $F(3, 35) = 7.990$, $P < 0.05$; 3 day, $F(3, 34) = 7.813$, $P < 0.05$; 4 day, $F(3, 36) = 12.18$, $P < 0.05$, Fig. 3A]. In the probe trial on day 5, the swimming time in the target quadrant (Fig. 3B) and the number of crossing in area where the hidden platform was located (Fig. 3C) were significantly ameliorated by PLAG (10 mg/kg) [target quadrant, $F(3, 34) = 7.630$, $P < 0.05$, Fig. 3B; number of crossing, $F(3, 33) = 6.287$, $P < 0.05$, Fig. 3C]. However, the average swimming speed showed no significant differences among all experimental groups ($P > 0.05$, Fig. 3D). The results of the water maze task suggested the ameliorating effect of PLAG on the scopolamine-induced cognitive dysfunction.

### 3.4. Effect of PLAG on locomotor activity in the open field test

To investigate whether PLAG exerts stimulatory effects on behavior, the spontaneous locomotor activity of mice were observed in the open field test. Administration of PLAG (1, 3 or 10 mg/kg) did not induce any significant change in the distance moved at each 5 different periods of times ($P > 0.05$, Fig. 4A) and

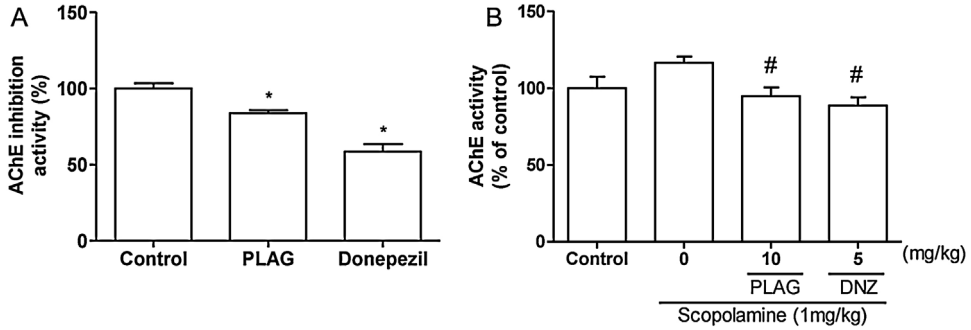


**Fig. 5.** Inhibitory effects of PLAG on acetylcholinesterase (AChE) activity assay in ex vivo with or without scopolamine. In mice, PLAG (10 mg/kg, p.o.), donepezil (5 mg/kg, p.o.) or the same volume of vehicle (10% Tween 80 solution, p.o.) was administered 1 h before and then, the mice were sacrificed (A). In the case of scopolamine-treated group, scopolamine was administered 30 min after each drug treatment (B). All the procedures were the same as described below. Whole brains were removed and homogenized using Buffer A. AChE activities were determined as described in Materials and Methods. Data represent means ± S.E.M. (Control group; n = 5, other groups; n = 10/group) ($^{\#}P < 0.05$, versus the scopolamine-administered group).

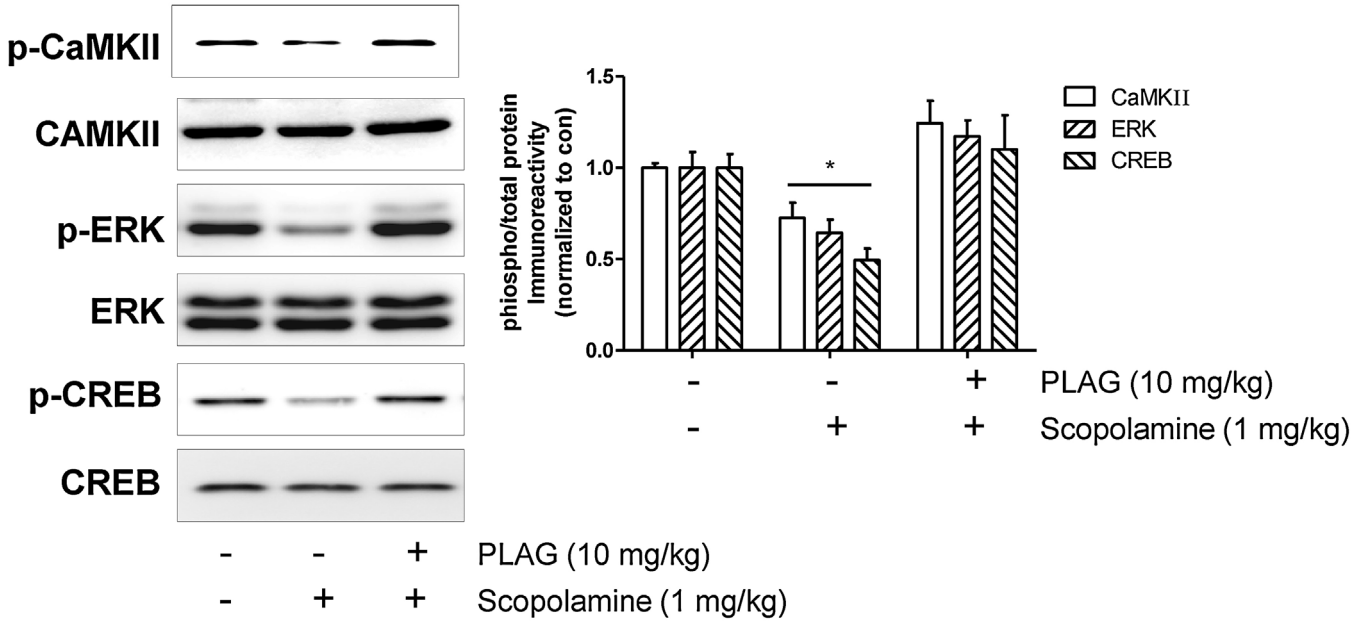


**Fig. 6.** The effects of PLAG on the phosphorylation level of CaMKII, ERK and CREB in the hippocampus. Mice were administered PLAG (10 mg/kg, p.o.) or the same volume of vehicle (10% Tween 80 solution, p.o) 30 min before scopolamine administration (1 mg/kg, i.p.). Sacrificing was conducted 30 min after the final administration. The immunereactivity and quantitative analysis of pCaMKII-CaMKII, pERK-ERK, and pCREB-CREB were examined in the hippocampal tissue. The densitometric analyses of the ratios of pCaMKII/CaMKII, pERK/ERK, and pCREB/CREB were normalized to the control group. Data represent the means ± S.E.M. (n = 4/group) ($^{*}P < 0.05$, $^{\#}P < 0.05$, versus the indicated group). Con, control; Sco, scopolamine.

total distance moved during the test ($P > 0.05$, Fig. 4B). Therefore, we assumed that the ameliorating effects of PLAG against cholinergic blockade-induced cognitive dysfunction were not derived from its increased locomotor behaviors.

### 3.5. *Effect of PLAG on AChE activity in* ex vivo *assay*

Since PLAG reversed the cholinergic activity blockade-mediated cognitive dysfunctions in the several learning and memory tasks, we performed the AChE inhibitory activity assay under the *ex vivo* condition. As a result, PLAG (10 mg/kg) treatment showed significant AChE inhibitory activity against control groups as observed in the donepezil-treated group (5 mg/kg), as a positive control [$F(2, 9) = 33.12$, $P < 0.05$, Fig. 5A]. In addition, PLAG and donepezil inhibited AChE activity in scopolamine-treated mice [$F(3, 29) = 5.652$, $P < 0.05$, Fig. 5B]. Because scopolamine is a nonspecific muscarinic receptor antagonist, it may not affect AChE activity in itself. Our results imply that the positive effect of PLAG on the memory function is resulted from its AChE inhibitory activity, in part.

### 3.6. *Effect of PLAG on the activation of memory-related proteins in the hippocampus*

We performed the Western blot analysis of hippocampal CaMKII and its downstream molecules, ERK and CREB, 1 h after the administration of PLAG in mice. The administration of scopolamine (1 mg/kg) significantly reduced the expression levels of pCaMKII, pERK, or pCREB and those phosphorylation levels were significantly reversed by PLAG administration (10 mg/kg) ($P < 0.05$, Fig. 6). As shown in Fig. 6, PLAG would phosphorylate CaMKII and its downstream ERK and CREB, which are important players in formation and maintenance of learning and memory processing. These results support the possibility that PLAG would ameliorate, in part, the memory dysfunction by the activation of CaMKII-ERK-CREB pathway.

### 3.7. *Effect of PLAG on the hippocampal LTP*

Since our results showed the PLAG activated CaMKII and ERK-CREB pathway, we confirmed the change of hippocampal LTP

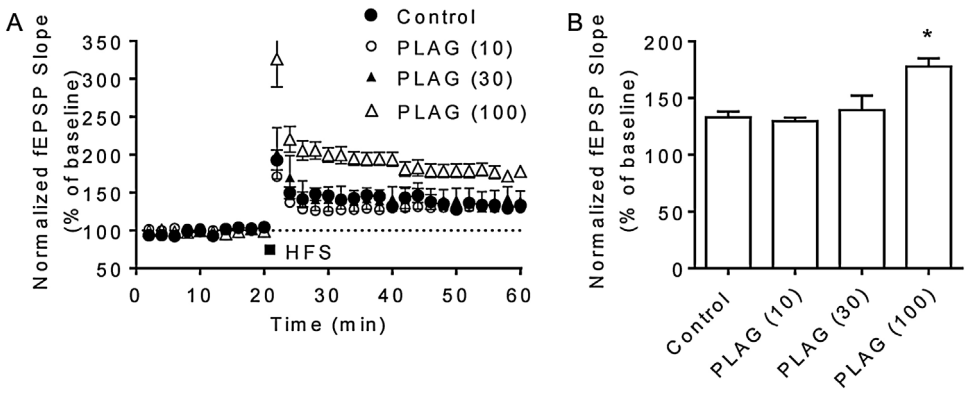


**Fig. 7.** The effect of PLAG on synaptic transmission in the hippocampus. (A) PLAG (10, 30 and 100 μM) was perfused to recording chamber from 30 min before to immediately after HFS. Extracellular field EPSPs (fEPSP) were evoked by stimulating Schaffer collateral CA1 synapse. LTP was evoked by one train of HFS (each 100 Hz, 1 s). (B) Residual potentiation of fEPSPs during the last five minutes of the one-hour recording. Data were represented as mean ± SEM. n = 7/group. $^{*}P < 0.05$. PLAG denotes PLAG treated groups.

in acute hippocampal slices. We measured LTP in the Shaffer-collateral pathway of the hippocampus. In this experiment, PLAG (100 μM) facilitated hippocampal LTP [$F$ (3, 18) = 9.301, $P < 0.05$, $n$ = 5–6/group, Fig. 7C and D], suggesting that this may participate in the effect of PLAG on learning and memory.

## 4. Discussion

In the present study, we found that PLAG reversed cognitive dysfunction induced by cholinergic blockade in several behavioral tasks including the passive avoidance, Y-maze, and the Morris water maze tasks. In addition, CaMKII, ERK or CREB which are relevant to cognitive function and hippocampal LTP were activated and AChE activity was inhibited by PLAG administration.

Since PLAG is a monoacetyldiaglyceride, purified from deer antler, its pharmacological activities are focused on hematopoietic and immunological functions [15]. For example, PLAG is known to be a potent T-helper type 2 modulating factor, suggesting that it could be useful for modulation of immune balance [31]. Deer antler containing PLAG has been used for elder people who suffered from decreased body metabolism and overall strength. Therefore, PLAG, suggested as one of the active compounds of deer antler, may modulate the cognitive dysfunction, especially in elder people. In the present study, PLAG significantly prolonged a step-through latency compared to that of the scopolamine-treated group in the passive avoidance task. PLAG showed the ameliorating effect on decreased spontaneous alternation induced by scopolamine in the Y-maze task. In addition, the escape latency of PLAG-treated group was significantly reduced compared to that of the scopolamine-treated group during training trials in the Morris maze task. On the probe trial, the swimming time in the target quadrant and the number of crossing the zone located hidden platform, were significantly recovered by PLAG administration. Recently, it has been suggested that the deer antler extract ameliorates scopolamine-induced memory impairment, a well-established animal model of dementia Lee et al., 2009. Until yet, there has been no report which compound(s) is active against AChE inhibition in the extract of deer antler. A variety of compounds have been identified in deer antler extract, and among them, PLAG, as monoacetyldiglyceride, is considered to be an active constituent [14]. Here, we observed that PLAG significantly inhibited the AChE activity although the inhibitory activity is not much potent compared to the donepezil-treated group. These results support that PLAG is an active compound of the deer antler extract and imply that PLAG would be a potential candidate for the cholinergic neurotransmitter system-dysregulated disorders like AD. To clarify how PLAG ameliorates the memory impairment by scopolamine, we investigated the molecular mechanisms and the structural specificity of PLAG.

Since many signaling cascades involved in the induction of LTP, including CaMKII or PKC, we assumed that PLAG would play an essential role in learning and memory by enhancing the synaptic plasticity, especially in the hippocampus [27]. In addition, CaMKII plays the main role in the activation of ERK-CREB signaling [28–30]. This evidence suggests that CaMKII, ERK, and CREB activation play an important role in learning and memory. In the present study, we observed that the administration of PLAG increased the phosphorylation level of CaMKII as well as that of ERK or CREB in the hippocampus. Moreover, we observed that PLAG facilitated LTP formation in acute hippocampal slices. However, we did not fully examine the exact mechanism how PLAG activates the CaMKII signaling or LTP formation. Since the AChE inhibitory activity of PLAG is weaker compared to that of donepezil, ameliorating effects of PLAG on cognitive dysfunction may be derived from its synergistic activities of AChE inhibitory activity as well as enhanced synaptic plasticity or the activated CaMKII-ERK-CREB signaling.

Recently, PLAG has been reported to downregulate inducible nitric oxide synthase (iNOS) expression under the immunostimulation status in mice [13]. iNOS plays a role in inflammatory processes including several neurodegenerative diseases. iNOS catalyzes the oxidative deamination of L-arginine to produce nitric oxide at high concentrations, which turns to be neurotoxic to brain cells. Interestingly, the number of iNOS-positive neurons is significantly enhanced in the brain of AD patients, and coincide with the neuronal dysfunction Marail et al., 2009. Thus, iNOS may be associated with the pathogenesis of AD. As PLAG had an ameliorating effect on iNOS expression in the cholinergic blockade (data not shown), we can assume that PLAG may be effective in the learning and memory in the present study, in part. Further study is needed to find out the mechanism of action of PLAG on the cognitive function and is being performed.

In the present study, PLAG showed an inhibitory AChE activity, enhanced synaptic plasticity and anti-inflammatory effects. Thus, PLAG may act to the multiple targets as the therapeutics for AD. In addition, there has been conducted one clinical study for Phase I study (Clinical research information service registration number, KCT0001470, May 7, 2015) by another research group (Yonsei University, Seoul, Korea). Before the above clinical trial, preclinical studies revealed that the NOAEL (no observed adverse effect level) was established >2000 mg/kg for 26 weeks repeated treatment (unpublished data). Together, these results imply that PLAG may be a candidate agent for AD therapy with the strong points such as safety and efficacy.

In conclusion, the present study showed the ameliorating effect of PLAG on scopolamine-induced memory impairment in mice using several behavioral tasks. These effects would be partly due to the AChE inhibitory action, the activation of memory-related proteins, such as CaMKII, ERK, and CREB signaling pathway, and LTP induction.

## Conflict of interest

The authors declared that there is no conflict of interest.

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