

Memory-enhancing effects of 7,3',4'-trihydroxyisoflavone by regulation of cholinergic function and BDNF signaling pathway in mice

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ABSTRACT

7,3',4'-Trihydroxyisoflavone (THIF) is a secondary metabolite derived from daidzein and is abundantly present in soybeans. Daidzein and 7,3',4'-THIF exhibit several pharmacological activities, including antioxidant and anti-atopic properties. However, the effects of 7,3',4'-THIF on cognitive function have not been fully investigated. Here, we evaluated the effects of 7,3',4'-THIF on memory using Y-maze and passive avoidance tests. The positive control groups were given donepezil (5 mg/kg, p.o.) or piracetam (200 mg/kg, i.p.) and the treated groups were given 7,3',4'-THIF (0.25, 0.5 and 1 mg/kg, p.o.). 7,3',4'-THIF at 1 mg/kg and donepezil at 5 mg/kg effectively ameliorated memory impairments induced by scopolamine (0.5 mg/kg, i.p.) in mice. In addition, 7,3',4'-THIF at 1 mg/kg and piracetam at 200 mg/kg significantly enhanced memory in intact mice. To examine the underlying mechanisms of 7,3',4'-THIF on cognition following behavioral experiments, biochemical tests were performed in the whole hippocampus. 7,3',4'-THIF (1 mg/kg, p.o.) significantly recovered scopolamine-induced cholinergic impairments. Moreover, brain-derived neurotrophic factor (BDNF), postsynaptic density protein-95 (PSD-95), and synaptophysin, along with phosphorylation of extracellular signal-regulated kinase 1/2 (ERK1/2) and cAMP response element binding (CREB), were significantly increased by 7,3',4'-THIF (1 mg/kg, p.o.). Our findings indicate that 7,3',4'-THIF improves cognitive function by regulating cholinergic system and BDNF signaling.

1. Introduction

Alzheimer's disease (AD), the most common type of dementia, is a progressive and age-associated neurodegenerative disorder characterized by cognitive impairment and memory decline (Cutler and Sramek, 2001). The key cause of AD is accumulation of amyloid beta protein in the form of senile plaques in the brain (Swomley et al., 2014). Moreover, alteration of cholinergic neurons is associated with learning and memory deficits in AD (Ferreira-Vieira et al., 2016). Based on these findings, acetylcholinesterase inhibitors such as donepezil, tacrine, and rivastigmine are the common form of drug therapy used to ameliorate cognitive impairment in AD (Mufson et al., 2008). However, these agents have side effects, including diarrhea and nausea (Colović et al., 2013). Therefore, further studies are needed to develop alternative therapies for preventing dementia symptoms without negative side effects.

Synaptic loss or dysfunction is another major pathological feature in AD. Brain-derived neurotrophic factor (BDNF), a member of the neurotrophin family, is correlated with neuronal proliferation and synaptic

plasticity (Lu et al., 2013). Furthermore, BDNF plays an important role in cognition through activation of intracellular signaling cascades, including the mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK) pathway (Gutiérrez-Vargas et al., 2015). In addition, cAMP-response element binding protein (CREB), a downstream molecule of the pathway, is phosphorylated by activated ERK and promotes BDNF transcription (Lu et al., 2018). The BDNF/ERK/CREB pathway is strongly associated with neural survival, synaptic function, and memory enhancement (Cunha et al., 2010). Additionally, the BDNF pathway modulates the expression of essential synaptic proteins such as postsynaptic density-95 (PSD-95) and synaptophysin (Tartaglia et al., 2001). Therefore, BDNF signaling offers a potential therapeutic target in treatment of AD due to its involvement in neurogenesis in the hippocampus.

The hippocampus has been recognized as a major brain region related to learning and memory, and there have been many reports on the correlation between neurogenesis and cognition in the hippocampus (Jarrard, 1993; Stuchlik, 2014). It is broadly accepted that neurogenesis in the adult brain mainly occurs in the dentate gyrus (DG) of the

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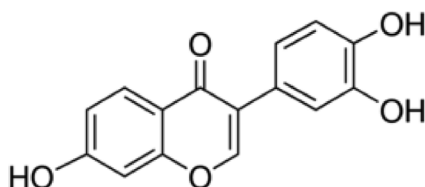


Fig. 1. Chemical structure of 7,3',4'-trihydroxyisoflavone (THIF).

hippocampus (Gonçalves et al., 2016). Moreover, it was reported that activation of the cAMP cascade increases proliferation and survival of new neurons in the mouse hippocampus (Nakagawa et al., 2002). Moreover, at early stages of AD, impaired memory function is accompanied by declined neurogenesis in the hippocampus (Mu and Gage, 2011). Therefore, in our current study, we performed biochemical experiments using the hippocampal tissue that is most closely associated with cognitive function.

Isoflavones are abundantly found in soybean-based foods and are effective in preventing progression of various diseases (Huang et al., 2016). In particular, daidzein, the main active compound of soybeans, exerts many pharmacological properties, including antioxidant, anti-inflammatory, and anticancer effects (Choi and Kim, 2014; Magee et al., 2014). In addition, it shows potential cognitive-enhancing effects on memory impairments in several animal models (Kim et al., 2010; Zeng et al., 2010). Daidzein is further bioconverted to its major metabolites, 7,3',4'-trihydroxyisoflavone (7,3',4'-THIF; chemical structure in Fig. 1), 6,7,4'-THIF, and 7,8,4'-THIF, by enzymatic reaction in the human body (Kulling et al., 2001). In addition to various therapeutic effects on esophageal cancer and atherosclerosis, our previous studies showed that 6,7,4'-THIF and 7,8,4'-THIF have a memory-enhancing activity in memory impaired mouse models (Ko et al., 2018a, 2018b; Lee et al., 2018; Lim et al., 2017). Meanwhile, the effects of 7,3',4'-THIF on cognition and memory have not been evaluated previously.

Thus, the present study sought to explore the effects of 7,3',4'-THIF on cognition and memory function in animal models. Behavioral experiments were conducted to assess the effect of 7,3',4'-THIF on learning and memory using the Y-maze and passive avoidance tests. However, there are currently no appropriate behavioral or clinical studies using 7,3',4'-THIF. Therefore, to select the optimal doses of 7,3',4'-THIF for animal tests, we performed preliminary experiments using 7,3',4'-THIF at most effective doses of other metabolites of daidzein in our previous results. Our preliminary results indicated that the maximum enhancing effect of 7,3',4'-THIF on memory function occurred at 1 mg/kg. Based on these observations, we used less than 1 mg/kg of 7,3',4'-THIF in the present study. In addition, to examine the possible mechanisms of 7,3',4'-THIF, we measured levels of acetylcholinesterase and choline acetyltransferase (ChAT) in the whole hippocampus. Further, in the whole hippocampus, expression levels of BDNF, PSD-95, and synaptophysin, as well as phosphorylation of ERK and CREB, were evaluated using Western blot. Finally, in the DG of the hippocampus, the immunopositive cells of phosphorylation of ERK and CREB were analyzed.

2. Materials and methods

2.1. Chemicals and reagents

7,3',4'-THIF (purity > 98.1%) was purchased from Indofine Chemical Company, Inc. (San Mateo, CA, USA). Donepezil hydrochloride, piracetam, acetylthiocholine iodide, 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB), sodium bicarbonate, scopolamine hydrobromide, anti-β-actin antibody, paraformaldehyde, sucrose, and dimethyl sulfoxide (DMSO) were purchased from Sigma Chemicals (St. Louis, MO, USA). Rabbit anti-BDNF antibody was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Rabbit anti-ChAT

antibody was purchased from Abcam Company (Cambridge, UK). Rabbit anti-phospho CREB, anti-CREB, anti-phospho ERK, anti-ERK, anti-PSD-95, and anti-synaptophysin antibodies were purchased from Cell Signaling Technology Inc. (Danvers, MA, USA). Secondary antibodies were purchased from Jackson ImmunoResearch Laboratories Inc. (West Grove, PA, USA). All other chemicals were of analytical grade.

2.2. Animals

Male CD-1 mice at four weeks of age (weighing 21–23 g) were used in the present study. Animals were purchased from Koatech Co., Ltd. (Pyongtaek, Republic of Korea). Mice were housed 5–6 per cage with free access to water and food and habituated for one week under constant temperature ($23 \pm 1^\circ\text{C}$) and humidity ($55 \pm 5\%$) and a 12-h light/dark cycle (lights on 07:00 to 19:00 h). All mice euthanized using carbon dioxide (CO_2) to perform biochemical assays after the behavioral experiments. All animal experiments were carried out in accordance with the NIH Guide for the Care and Use of Laboratory Animals and with the approval of the Institutional Animal Care and Use Committee of Sungkyunkwan University (approval number of the animal study: SKKUIACUC2018-12-22-3).

2.3. Drug treatment and experimental design

To examine the effects of 7,3',4'-THIF on intact cognitive function, mice were randomly divided into the following five groups ($n = 12$ each group): control group (10% tween 80 with 5% DMSO in distilled water, p.o.), 7,3',4'-THIF 0.25 mg/kg (p.o.) group, 7,3',4'-THIF 0.5 mg/kg (p.o.) group, 7,3',4'-THIF 1 mg/kg (p.o.) group, and piracetam 200 mg/kg (i.p.) group. The Y-maze test and training trial of the passive avoidance test were performed 1 h after a single administration of 7,3',4'-THIF in intact mice, respectively. We used piracetam as a positive control for cognitive enhancement (Mukherjee and Banerjee, 2013). In addition, to examine the memory ameliorating effects of 7,3',4'-THIF on memory impairments, mice were randomly divided into six groups ($n = 10$ each group): control group (0.9% saline, i.p. + 10% tween 80 with 5% DMSO in distilled water, p.o.), scopolamine 0.5 mg/kg (i.p.) group, 7,3',4'-THIF 0.25 mg/kg (p.o.) + scopolamine 0.5 mg/kg (i.p.) group, 7,3',4'-THIF 0.5 mg/kg (p.o.) + scopolamine 0.5 mg/kg (i.p.) group, 7,3',4'-THIF 1 mg/kg (p.o.) + scopolamine 0.5 mg/kg (i.p.) group, and donepezil 5 mg/kg (p.o.) + scopolamine 0.5 mg/kg (i.p.) group. Memory impairments were induced using scopolamine (0.5 mg/kg, i.p.) 30 min after a single administration of donepezil and 7,3',4'-THIF (Kwon et al., 2010). And then, the Y-maze test and training trial of the passive avoidance test were performed 30 min after the scopolamine or saline administration. Since intraperitoneal injection has a faster rate of absorption into the blood stream than oral administration, it is quite common to set a schedule in order from p.o. to i.p. prior to behavioral experiments (Shimizu, 2004). Also, the experimental schedule was based on our previous research (Ko et al., 2018a) and other studies reporting damaged memory function after 30 min of scopolamine injection and memory recovery after 1 h of flavonoid injection (Maher et al., 2006; Riekkinen et al., 1990). Donepezil was used as a positive control for the memory test (Kwon et al., 2010). In all animal studies, the experimenter was blind to the experimental treatment conditions.

2.4. Y-maze test

To evaluate spatial working memory, spontaneous alternation was assessed by conducting the Y-maze test as previously described (Ko et al., 2018a). The Y-maze is a 3-arm maze with each arm placed at 120° angles to the others. The arms were 30 cm long, 5 cm wide, and 12 cm high, and the arms of the maze were designated as A, B, and C. Mice were placed in one arm of the maze, and the behaviors were monitored

for 8 min using a video camera. An arm entry was recorded when the mouse entered an arm of the maze with all four paws. The entry sequences and total numbers of arm entries were measured manually. In experiments with the scopolamine model, 1 h before each behavioral test, the mice were given 7,3',4'-THIF (0.25, 0.5, or 1 mg/kg, p.o.). After 30 min, memory impairments were induced by administering scopolamine (0.5 mg/kg, i.p.). In addition, to assess the effect of 7,3',4'-THIF alone, intact mice were treated with 7,3',4'-THIF (0.25, 0.5, or 1 mg/kg, p.o.) 1 h before each behavioral test. Prior to use and between test sessions, the apparatus was cleaned with 10% ethanol. A spontaneous alternation referred to the mouse entering a different arm of the maze on three consecutive choices (i.e., in order of ABC, CBA, or CAB but not ABA). The spontaneous alternation percentage (%) was determined using the following equation: spontaneous alternation (%) = [(number of alternations)/(total arm entries - 2)] × 100.

2.5. Passive avoidance test

The passive avoidance test is a fear-motivated test to evaluate long-term memory in rodents, according to previously described methods (Ko et al., 2018a). The apparatus consists of two same size compartments (12 × 10 × 12 cm). One is a white, brightened chamber with a 50-W light bulb at a height of 1 m from the chamber, and the other is a black chamber without light; the chambers are divided by a guillotine door. Both floors are made of 2-mm stainless steel tubes spaced 0.5 cm apart, and only the floor of the black compartment is connected to an electric shock (direct current) device (PA-2010A, O' Hara & Co., Tokyo, Japan). In the training session, the mouse was placed in the light compartment and allowed to enter the dark compartment. Immediately after the mouse passed into the dark side, the door closed, and a 0.8 mA or 0.5 mA mild electric foot-shock was applied to the floor for 3 s. In scopolamine-induced memory impaired mice, a more intense shock (0.8 mA) was used to induce fear-motivated memory. Meanwhile, in intact mice, a low-intensity shock (0.5 mA) was used to examine natural memory. The high-intensity could induce a narrow behavioral window to measure any memory-enhancing effects of 7,3',4'-THIF in intact mice. The intensities of each experimental condition of electrical foot shock for animal study were based on our preliminary results. Then, the test session was performed only 24 h after the training session in both the experiments with intact mice and scopolamine model. In the test session, the mouse was placed in the light side again. In experiments with the scopolamine model, mice were given 7,3',4'-THIF (0.25, 0.5, or 1 mg/kg, p.o.) 1 h before the training trial. After 30 min, memory impairments were induced by administering scopolamine (0.5 mg/kg, i.p.). In addition, to assess the effect of 7,3',4'-THIF alone, intact mice were treated with 7,3',4'-THIF (0.25, 0.5, or 1 mg/kg, p.o.) 1 h before the training trial. The session continued until the mouse moved into the dark side or until 300 s had elapsed. Latency to move to the dark side was measured. If the mouse did not cross the arm boundary by the end of the session, the latency was recorded as 300 s.

2.6. BDNF enzyme-linked immunosorbent assay (ELISA)

Total BDNF level was detected using a BDNF sandwich ELISA kit according to the manufacturer's instructions (Elabscience Biotechnology, Houston, TX, USA). Mice were subjected to behavioral experiments 60 min after treatment, and the whole hippocampus of each brain was isolated immediately after the behavioral experiments. This timing from drug treatment to behavioral experiments is sufficient to allow drug transport to the mouse brain (Spencer, 2010; Yang et al., 2012). Six brains were used per assay. All subsequent hippocampal assays were performed using the same samples. The whole hippocampus of each group was promptly excised and homogenized in a rotary homogenizer with 200 µl of ice-cold lysis T-per tissue protein extraction buffer (Thermo Scientific, Rockford, IL, USA) containing protease and phosphatase inhibitor cocktails (Roche Diagnostics,

GmbH, Mannheim, Germany). The homogenate was incubated on ice for 30 min. After centrifugation at 10,000 × g for 15 min, the supernatant was obtained and stored at -70 °C. The plate was read at 450 nm with a microplate reader (SpectraMax M2, Molecular Devices, Sunnyvale, CA, USA). The protein concentration was normalized by using a protein assay kit (Thermo Scientific). The BDNF level was calculated as the percentage of the corresponding value of the vehicle-treated group.

2.7. Acetylcholinesterase activity assay

The acetylcholinesterase activity assay was performed as previously described (Ko et al., 2018a). Mice were subjected to behavioral experiments 60 min after treatment, and the whole hippocampus of each brain was isolated immediately after the behavioral experiments. This timing from drug treatment to behavioral experiments is sufficient to allow drug transport to the mouse brain (Spencer, 2010; Yang et al., 2012). Four brains were used per assay. The whole hippocampus was rapidly homogenized in sodium phosphate buffer (0.1 mM, pH 7.4) with a rotary homogenizer (MagNa Lyser, Roche Diagnostics, GmbH, Germany). The homogenates were centrifuged at 14,000 × g for 15 min at 4 °C. Acetylthiocholine iodide solution (25 µl, 75 mM) was added to 100 µl of buffered Ellman's reagent (10 mM DTNB and 15 mM sodium bicarbonate) at room temperature for 10 min. Sodium bicarbonate was used as a stop solution to stop the enzymatic reaction of the assay. The test mixture was then incubated for 5 min to trigger the reaction after addition of the enzyme source (8 µl) obtained from the supernatant. Acetylcholinesterase activity was measured at a wavelength of 412 nm using a UV spectrometer (UV-1700 PharmaSpec, Shimadzu Co. Ltd., Japan).

2.8. Western blot

The Western blot method was carried out as previously described (Ko et al., 2018a). In brief, mice were subjected to behavioral experiments 60 min after treatment, and the whole hippocampus of each brain was isolated immediately after the behavioral experiments. This timing from drug treatment to behavioral experiments is sufficient to allow drug transport to the mouse brain (Spencer, 2010; Yang et al., 2012). Four brains were used per assay. The whole hippocampus of each group was promptly excised and homogenized in a rotary homogenizer with 200 µl of ice-cold lysis T-per tissue protein extraction buffer (Thermo Scientific, Rockford, IL, USA) containing protease and phosphatase inhibitor cocktails (Roche Diagnostics, GmbH, Mannheim, Germany). The homogenate was incubated on ice for 30 min. After centrifugation at 10,000 × g for 15 min, the supernatant was obtained and stored at -70 °C. Protein samples were subjected to 8–10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred onto a polyvinylidene difluoride (PVDF) membrane (Pall Corporation, Pensacola, FL, USA) in transfer buffer [25 mM Tris-HCl buffer (pH 7.4) containing 192 mM glycine and 20% v/v methanol] and blocked with 5% non-fat milk in Tris-buffered saline with 0.1% Tween-20 (TBST; 0.5 mM Tris-HCl [pH 7.5], 150 mM NaCl, and 0.1% Tween-20) for 1 h at room temperature. Each membrane was incubated in primary antibodies overnight at 4 °C: anti-BDNF (1:500), anti-ChAT (1:1000), anti-PSD-95 (1:1000), anti-synaptophysin (1:1000), anti-phospho ERK (1:2000), anti-ERK (1:2000), anti-phospho CREB (1:1000), and anti-CREB (1:1000). After rinsing the membranes in TBST, blots were incubated in horseradish peroxidase-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA) for 1 h at room temperature. The band density was determined via enhanced chemiluminescence (ECL) by immersing the probed membrane in a 1:1 mixture of ECL reagents A and B (Animal Genetics Inc., Suwon, Republic of Korea) for 5 min. Membranes were then exposed to photographic film for a few minutes. Protein bands were analyzed using ImageJ software from NIH (Bethesda, MD, USA).

2.9. Immunohistochemistry

The mice were deeply anesthetized with pentobarbital (50 mg/kg, i.p.) after the behavioral experiments and intracardially perfused with a solution containing 4% paraformaldehyde. Four brains were used per assay. Each brain was post-fixed in 4% paraformaldehyde and then placed in 30% sucrose. The tissue was then frozen and sectioned coronally at 30 μ m using a cryostat (Leica, Nussloch, Germany). The brain sections were incubated with anti-phospho ERK (1:100) and anti-phospho CREB (1:100) primary antibodies. For immunoperoxidase staining, biotinylated secondary antibody (7 mg/ml; Vector Laboratories Inc., Burlingame, CA, USA) was applied, followed by incubation with an avidin-biotin horseradish peroxidase complex (Vector Laboratories). The peroxidase label was detected by incubating the tissue sections in 3,3'-diaminobenzidine (Sigma). Images were produced using a DP digital camera (Olympus Optical, Tokyo, Japan) connected to a microscope (BX 51, Olympus). The analysis for counting immunopositive cells was performed only in the DG of the hippocampus [Stereotaxic coordinates: anteroposterior (AP) -2.0 mm, mediolateral (ML) $+1.5$ mm, dorsoventral (DV) -2.0 mm].

2.10. Statistical analysis

All results are expressed as mean $\pm$ S.E.M. and were analyzed using Prism software, version 6.0 (GraphPad Software, San Diego, CA, USA). Behavioral experiments were subjected to one-way analysis of variance (ANOVA), following by the Fisher's LSD test. Biochemical experiments and Western blot and immunohistochemistry assays were analyzed by ANOVA, followed by the Newman-Keuls *post hoc* test for multiple comparisons. Statistical significance was defined as $p < 0.05$.

3. Results

3.1. Effects of 7,3',4'-THIF on cognitive function of intact mice in the Y-maze and passive avoidance tests

The effects of 7,3',4'-THIF on spatial and fear-motivated memory in intact mice were examined using the Y-maze and passive avoidance tests, respectively. As shown in Fig. 2A, 7,3',4'-THIF (1 mg/kg, p.o.) and piracetam (200 mg/kg, i.p.) increased spontaneous alternative behavior in intact mice [Fig. 2A, $F(4, 55) = 2.16$, $p < 0.05$ and $p < 0.05$, respectively]. However, 7,3',4'-THIF did not affect the number of arm

entries (locomotor activity) of mice [Fig. 2B, $F(4, 55) = 1.28$]. Moreover, in the test trial of the passive avoidance test, 7,3',4'-THIF (1 mg/kg, p.o.) and piracetam (200 mg/kg, i.p.) significantly increased step-through latency time compared to the vehicle-treated mice [Fig. 2C, $F(4, 45) = 3.20$, $p < 0.05$ and $p < 0.05$, respectively]. In comparison between 7,3',4'-THIF groups, the step-through latency of mice treated with 7,3',4'-THIF 1 mg/kg and piracetam 200 mg/kg was significantly higher than the 7,3',4'-THIF 0.25 mg/kg group (Fig. 2C, $p < 0.05$ and $p < 0.05$, respectively). Also, we confirmed that there were no significant differences between the latency of the training trial in each group [Fig. 2C, $F(4, 45) = 1.41$]. Thus, these results indicated that 7,3',4'-THIF has a memory-enhancing effects in mice. However, this effect may be not due to the decreased locomotor activity or impaired motor function in mice.

3.2. Effects of 7,3',4'-THIF on BDNF level and expression of PSD-95 and synaptophysin in the hippocampus of intact mice

To investigate the underlying mechanisms of the effects of 7,3',4'-THIF on intact memory function in mice, BDNF level and PSD-95 and synaptophysin expression were assessed using ELISA and Western blot analysis in the hippocampus. As shown in Fig. 3A, ELISA results indicated that 7,3',4'-THIF (1 mg/kg, p.o.) and piracetam (200 mg/kg, i.p.) significantly increased BDNF level to 111.6% and 110.9% of the control groups, respectively, in the hippocampus of intact mice [Fig. 3A, $F(4, 25) = 3.36$, $p < 0.05$ and $p < 0.05$, respectively]. In comparison between 7,3',4'-THIF groups, BDNF level of the hippocampus of mice treated with 7,3',4'-THIF 1 mg/kg and piracetam 200 mg/kg was significantly higher than the 7,3',4'-THIF 0.25 mg/kg group ($p < 0.05$ and $p < 0.05$, respectively). In addition, our Western blot analysis showed that 7,3',4'-THIF (1 mg/kg, p.o.) and piracetam (200 mg/kg, i.p.) markedly increased the expression of PSD-95 to 131.9% and 130.6% of the control groups, respectively [Fig. 3B, $F(2, 9) = 8.79$, $p < 0.01$ and $p < 0.01$, respectively]. Also, 7,3',4'-THIF (1 mg/kg, p.o.) and piracetam (200 mg/kg, i.p.) dramatically increased the expression of synaptophysin to 121.9% and 128.9% of the control groups, respectively, in the hippocampus [Fig. 3C, $F(2, 9) = 6.52$, $p < 0.05$ and $p < 0.01$, respectively].

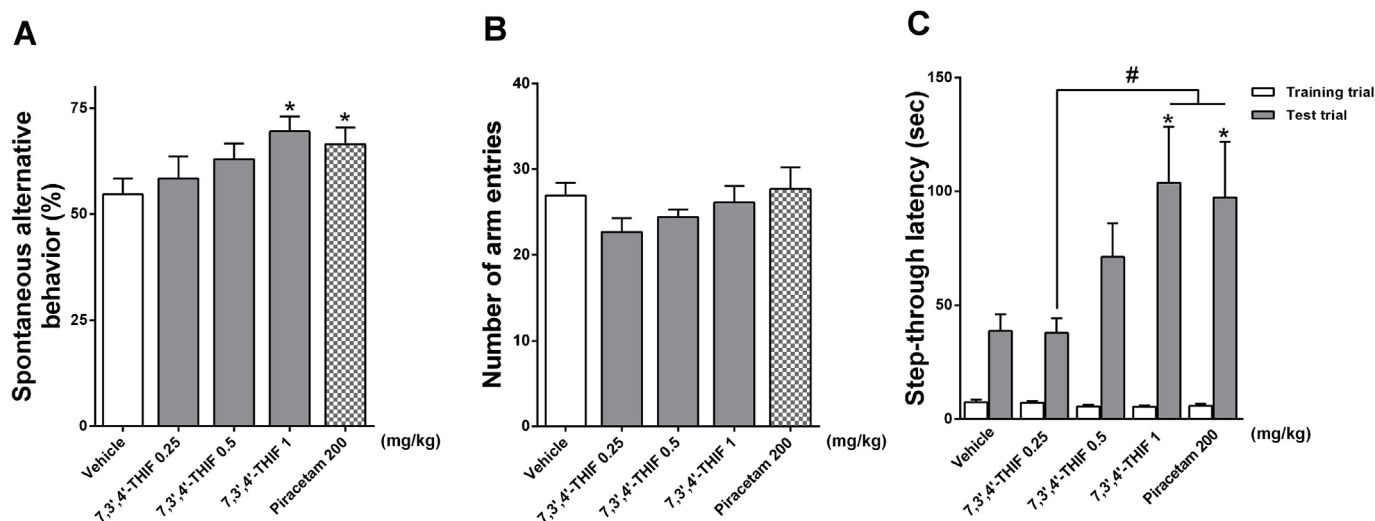


Fig. 2. Effects of 7,3',4'-THIF on cognitive function of intact mice in the Y-maze and passive avoidance tests. Spontaneous alternative behavior (A), number of arm entries (B), and step-through latency (C) were assessed 1 h after administration of 7,3',4'-THIF (0.25, 0.5, and 1 mg/kg, p.o.) or piracetam (200 mg/kg, i.p.) in mice. Data are expressed as the mean $\pm$ S.E.M. ($n = 12$). * $p < 0.05$ compared with the vehicle group. # $p < 0.05$ compared with the 7,3',4'-THIF 0.25 mg/kg group.

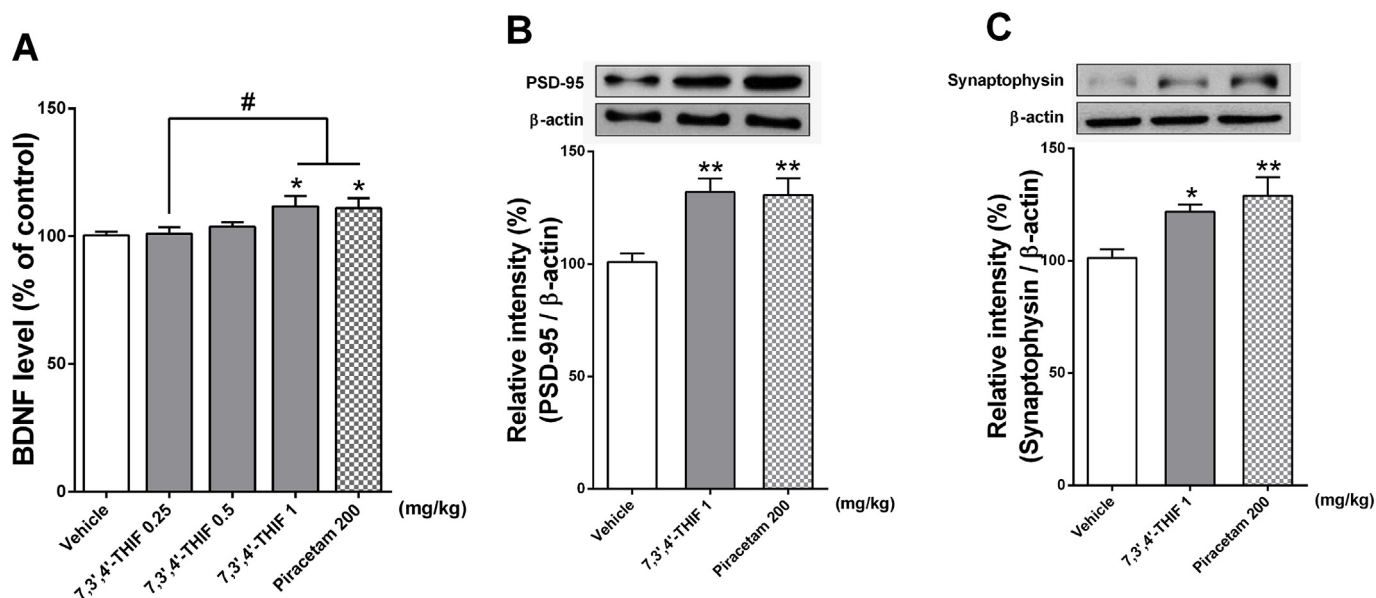


Fig. 3. Effects of 7,3',4'-THIF on BDNF level (A) and expression of PSD-95 (B) and synaptophysin (C) using ELISA and Western blot analysis in the hippocampus of intact mice. After the behavioral experiments, each brain was removed to obtain the hippocampus. Data are expressed as the mean $\pm$ S.E.M. (n = 4–6). * p < 0.05 and ** p < 0.01 compared with the vehicle group. # p < 0.05 compared with the 7,3',4'-THIF 0.25 mg/kg group.

3.3. Effects of 7,3',4'-THIF on phosphorylation of ERK and CREB in the hippocampus of intact mice

Next, in the hippocampus of intact mice, the effects of 7,3',4'-THIF on phosphorylation of ERK and CREB were assessed using Western blot and immunohistochemistry analysis. As shown in Fig. 4A and D, the expression of phospho ERK and CREB in the hippocampus of mice receiving 7,3',4'-THIF (1 mg/kg, p.o.) and piracetam (200 mg/kg, i.p.) was significantly increased compared to the control groups (Fig. 4A and D). As shown in Fig. 4B and C, there were significant increases in number of immuno-positive cells for phospho ERK [Fig. 4B, F(2, 9) = 5.96, p < 0.05 and p < 0.05, respectively] and CREB [Fig. 4C, F(2, 9) = 7.03, p < 0.01 and p < 0.05, respectively] in the DG of the hippocampus of mice exposed to 7,3',4'-THIF and piracetam. In addition, our Western blot data indicated that 7,3',4'-THIF (1 mg/kg, p.o.) and piracetam (200 mg/kg, i.p.) significantly increased the expression levels of phospho ERK to 122.6% and 120.3% of the control values, respectively [Fig. 4E and F(2, 9) = 10.41, p < 0.01 and p < 0.01, respectively]. Moreover, 7,3',4'-THIF (1 mg/kg, p.o.) and piracetam (200 mg/kg, i.p.) significantly increased the levels of phospho CREB to 129.3% and 127.4% of the control values, respectively, in the whole hippocampus [Fig. 4F, F(2, 9) = 3.84, p < 0.05 and p < 0.05, respectively].

3.4. Effects of 7,3',4'-THIF on scopolamine-induced memory impairment in mice in the Y-maze and passive avoidance tests

The effects of 7,3',4'-THIF against scopolamine-induced memory impairment were tested next. In this experiment, scopolamine (0.5 mg/kg, i.p.) significantly decreased spontaneous alternative behavior compared to the control groups [Fig. 5A, F(5, 66) = 4.09, p < 0.001]. However, 7,3',4'-THIF (1 mg/kg, p.o.) and donepezil (5 mg/kg, p.o.) significantly reversed cognitive impairment compared to the scopolamine-treated groups (Fig. 5A, p < 0.01 and p < 0.05, respectively). The number of arm entries was significantly higher in all scopolamine-treated groups compared to the control groups [Fig. 5B, F(5, 66) = 7.05, p < 0.001, respectively]. In addition, in the passive avoidance test, we evaluated the effects of 7,3',4'-THIF on long-term and fear-motivated memory function of mice. As shown in Fig. 5C, scopolamine resulted in significant memory decline compared to the

vehicle-treated groups [Fig. 5C, F(5, 66) = 3.74, p < 0.001]. However, compared with scopolamine-treated groups, a significant improvement of memory function was observed in the groups receiving 7,3',4'-THIF (1 mg/kg, p.o.) or donepezil (5 mg/kg, p.o.) (Fig. 5C, p < 0.05 and p < 0.05, respectively). There were no differences in latency time across treatment groups during the training trial of the passive avoidance tests [Fig. 5C, F(5, 66) = 0.39]. Thus, this observation indicated the memory ameliorating effects may be not due to the decreased locomotor activity or impaired motor function in mice.

3.5. Effects of 7,3',4'-THIF on acetylcholinesterase activity and expression of ChAT, BDNF, and phospho ERK in the hippocampus of scopolamine-induced mice

As shown in Fig. 6A, scopolamine significantly increased acetylcholinesterase activity in the hippocampus compared to the control group [Fig. 6A, F(3, 20) = 7.14, p < 0.01]. However, treatment with 7,3',4'-THIF (1 mg/kg, p.o.) or donepezil (5 mg/kg, p.o.) significantly decreased the level of acetylcholinesterase compared with that in the scopolamine group (Fig. 6A, p < 0.01 and p < 0.05, respectively). Next, to further examine the molecular mechanisms of 7,3',4'-THIF, Western blot analysis was carried out in the hippocampus of scopolamine-induced mice (Fig. 6B). The results indicated that expression of ChAT [Fig. 6C, F(3, 12) = 4.30, p < 0.05], BDNF [Fig. 6D, F(3, 12) = 4.31, p < 0.05], and phospho ERK [Fig. 6E, F(3, 12) = 5.87, p < 0.05] in the hippocampus of scopolamine-treated mice was significantly decreased to 50.1%, 66.6%, and 49.7% of the control values, respectively. Meanwhile, treatment with 7,3',4'-THIF (1 mg/kg, p.o.) or donepezil (5 mg/kg, p.o.) significantly restored levels of ChAT to 87.1% and 92.1% of the control values, respectively (Fig. 6C, p < 0.05 and p < 0.05, respectively). Also, treatment with 7,3',4'-THIF (1 mg/kg, p.o.) or donepezil (5 mg/kg, p.o.) significantly reversed BDNF to 91.1% and 109.0% of the control values, respectively (Fig. 6D, p < 0.05 and p < 0.01, respectively). Finally, in the hippocampus of scopolamine-treated mice, 7,3',4'-THIF (1 mg/kg, p.o.) or donepezil (5 mg/kg, p.o.) significantly increased phospho ERK to 137.9% and 107.1% of the control values, respectively (Fig. 6E, p < 0.01 and p < 0.05, respectively).

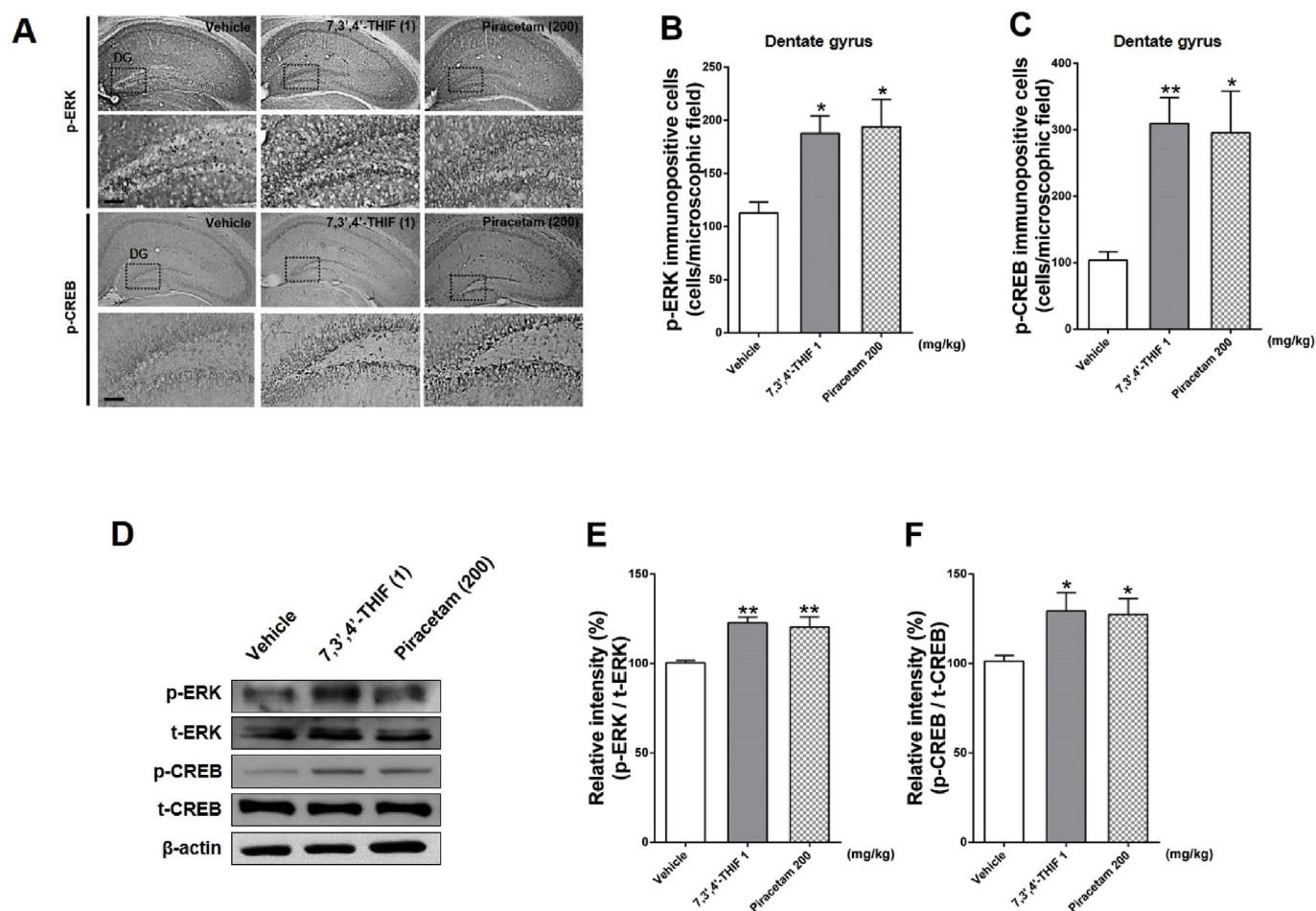


Fig. 4. Effects of 7,3',4'-THIF on phosphorylation of ERK and CREB using immunohistochemistry (A, B, and C) and Western blot analysis (D, E, and F) in the hippocampus of intact mice. After the behavioral experiments, each brain was removed to obtain the hippocampus. The scale bar of immunohistochemistry data represents 50 μ m (magnifications: $\times 100$ and $\times 400$, respectively). Data are expressed as the mean $\pm$ S.E.M. ($n = 4$). * $p < 0.05$ and ** $p < 0.01$ compared with the vehicle group.

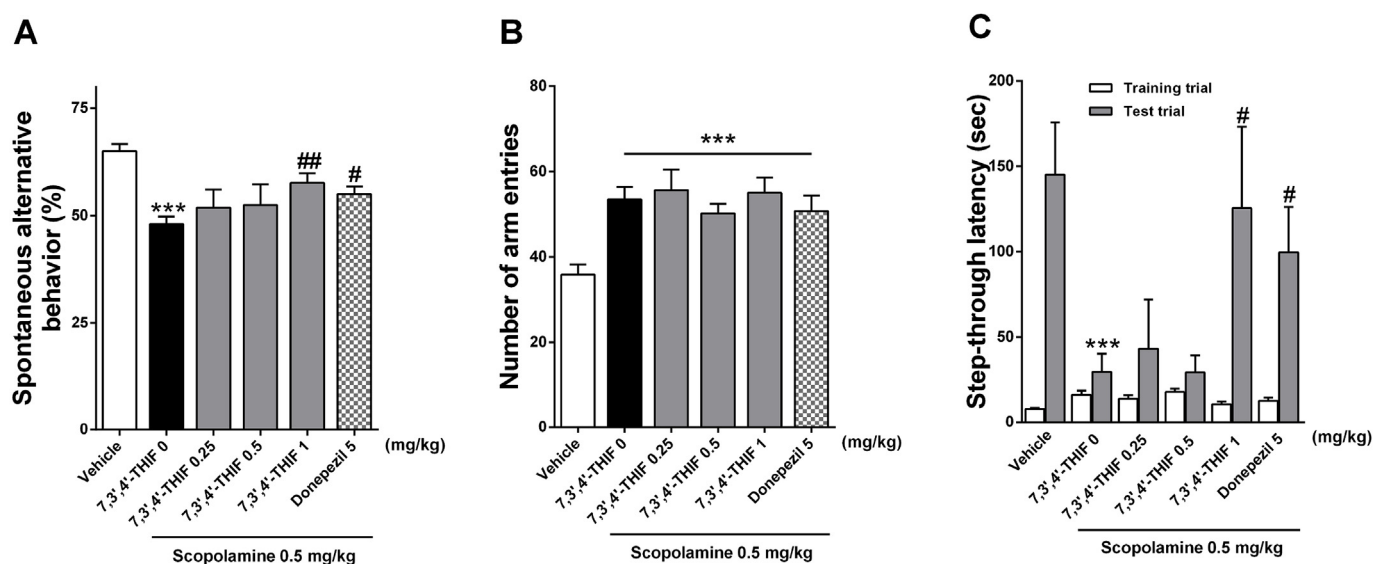


Fig. 5. Effects of 7,3',4'-THIF on scopolamine-induced memory impairments of mice in the Y-maze and passive avoidance tests. Mice were given 7,3',4'-THIF (0.25, 0.5, and 1 mg/kg, p.o.) and donepezil (5 mg/kg, p.o.) 1 h before the behavioral experiments. After 30 min, memory impairment was induced by scopolamine (0.5 mg/kg, i.p.) treatment. Spontaneous alternative behavior (A), number of arm entries (B), and step-through latency (C) were assessed. Data are expressed as the mean $\pm$ S.E.M. ($n = 10$). *** $p < 0.001$ compared with the vehicle group. # $p < 0.05$ and ## $p < 0.01$ compared with the scopolamine-treated group.

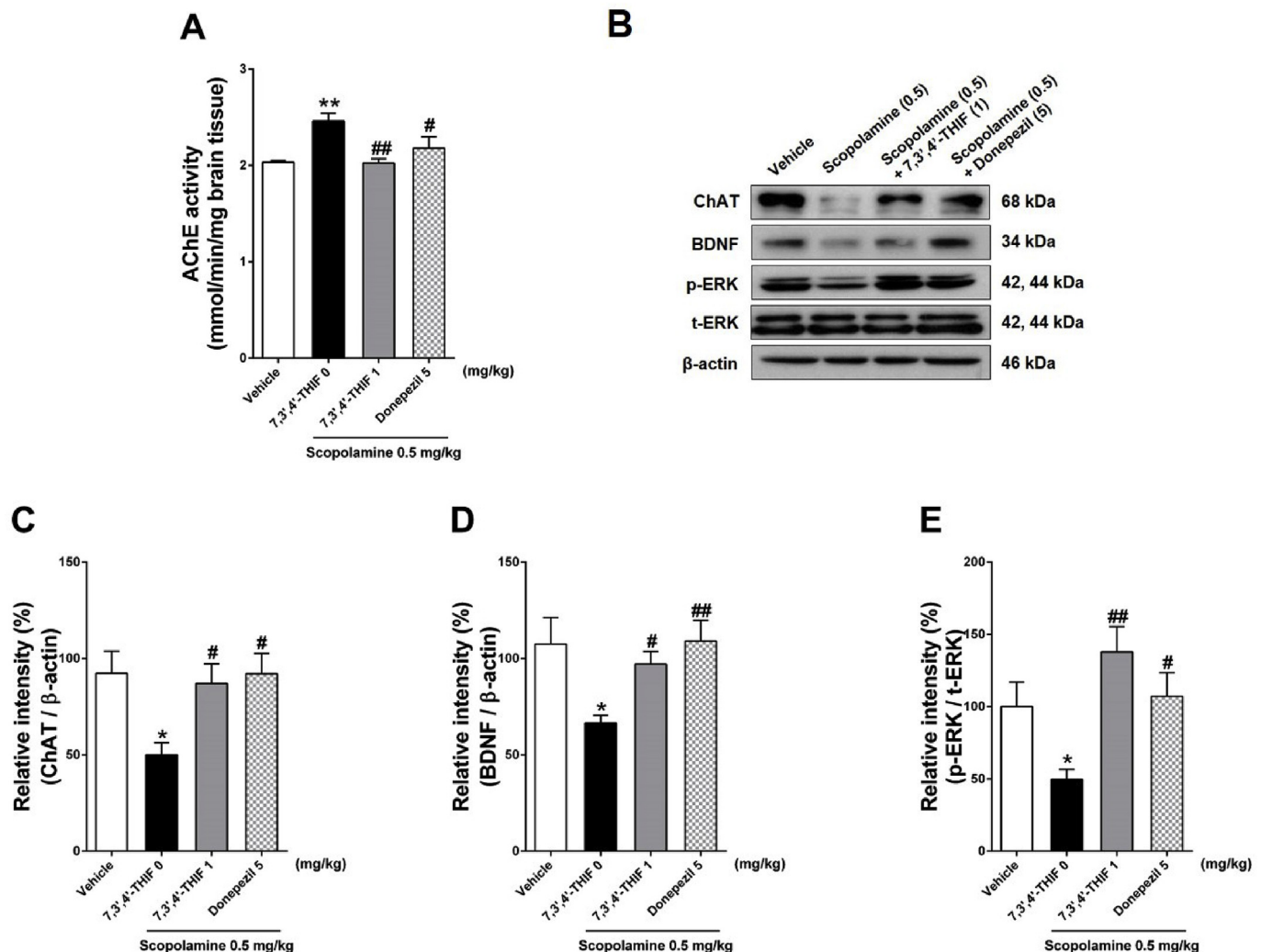


Fig. 6. Effects of 7,3',4'-THIF on acetylcholinesterase activity (A) and expression of ChAT (B and C), BDNF (B and D), and phospho ERK (B and E) in the hippocampus of scopolamine-induced mice. After the behavioral experiments, each brain was removed to obtain the hippocampus. Data are expressed as the mean $\pm$ S.E.M. (n = 4–6). * p < 0.05 and ** p < 0.01 compared with the vehicle group. # p < 0.05 and ## p < 0.01 compared with the scopolamine-treated group.

4. Discussion

In the present study, we hypothesized that 7,3',4'-THIF, a metabolized form of daidzein, would effectively recover memory impairment induced by cholinergic dysfunction. In addition, it could significantly enhance intact cognitive function in mice. These cognitive-enhancing effects of 7,3',4'-THIF may be associated with its role in the cholinergic system, synaptic function, and BDNF signaling in the hippocampus.

In the current study, the effects of 7,3',4'-THIF on cognitive and memory abilities of mice were determined using the Y-maze and passive avoidance tests. Our present results demonstrated that scopolamine dramatically decreased learning and memory function in mice. However, we observed that 7,3',4'-THIF at a dose of 1 mg/kg effectively recovered scopolamine-induced memory impairment. Interestingly, 1 mg/kg 7,3',4'-THIF alone remarkably improved the intact memory function of mice in these behavioral experiments. Moreover, 7,3',4'-THIF did not affect the number of arm entries in the Y-maze test or the latency time of the training trial sessions in the passive avoidance test. The results from the behavioral experiments suggested that 7,3',4'-THIF enhanced intact memory function and ameliorated cognitive deficits in mouse models without other behavioral alterations. In particular, these effects of 7,3',4'-THIF are believed to be involved in the cholinergic system when it restores memory loss from scopolamine. However, in

the Y-maze test, our results showed that there was a slight difference in the spontaneous alternative behavior or the number of arm entries for the two vehicle controls in intact mice (56% and 27 arm entries) and scopolamine model (65% and 36 arm entries), respectively. This slight difference (less than 10%) may exist because of different experimental conditions from the two tests, such as experimenter, experimental environment, time, and animal.

According to the literature, the central cholinergic system plays an essential role in learning and memory function (Brandon et al., 2004). Acetylcholine, an important neurotransmitter for cognitive function, is produced by ChAT in cholinergic neurons, whereas it is degraded by acetylcholinesterase after release (Hut and Van der Zee, 2011). Patients with memory disorders exhibit impairments of cholinergic function such as abnormally elevated acetylcholinesterase activity or reduced ChAT level in the brain (Ferreira-Vieira et al., 2016). Therefore, much research has focused on regulation of these cholinergic systems to enhance memory ability. Hence, in this study, to elucidate the action of 7,3',4'-THIF on cholinergic function, acetylcholinesterase activity and ChAT level were examined in the hippocampus of scopolamine-induced mice. Consistent with previous findings, scopolamine significantly induced cholinergic dysfunction in the mouse hippocampus, as shown by increased acetylcholinesterase activity and decreased ChAT level (Ray et al., 2014). However, 7,3',4'-THIF significantly recovered these

biochemical changes. Thus, based on these findings, 7,3',4'-THIF may recover scopolamine-induced memory impairment by regulating acetylcholinesterase activity and ChAT level in the hippocampus of mice.

As reported previously, BDNF is thought to play a key role in synaptogenesis and synaptic plasticity (Gómez-Pinilla et al., 2007). BDNF signaling influences memory function by promoting neuron survival and differentiation (Babu et al., 2009). In addition, the functions of BDNF in synapse formation are associated with several signaling pathways, including an increase of synaptic vesicle protein synaptophysin (Tartaglia et al., 2001). PSD-95, the important scaffolding protein in excitatory synapses, is also upregulated by BDNF and is required for synapse maturation (Yoshii and Constantine-Paton, 2014). Furthermore, decreased levels of synaptophysin and PSD-95 have been reported to reflect neuronal loss and synaptic disruption in AD (Yuki et al., 2014). In other words, BDNF signaling, which modulates PSD-95 and synaptophysin levels, appears to be a critical factor in cognitive function and memory. For this reason, we investigated the expression of BDNF and two related proteins, PSD-95 and synaptophysin, in the hippocampus of intact mice. In the current study, 7,3',4'-THIF remarkably augmented the levels of BDNF, PSD-95, and synaptophysin in intact mice. These findings provide evidence that 7,3',4'-THIF potentiated cognitive ability by activating BDNF signaling and its downstream targets, PSD-95 and synaptophysin. In addition, in intact mice, 7,3',4'-THIF significantly increased the phosphorylation of ERK and CREB in the hippocampus. ERK1/2 plays an important role in synaptic function and learning and memory performance (Sweatt, 2001). In addition, the transcription factor CREB is involved in hippocampus-dependent learning and memory formation (Yanai et al., 2014). Activation of ERK1/2 could modulate CREB phosphorylation and is necessary for long-term potentiation (LTP) in the hippocampus (Lu, 2003). Moreover, the ERK/CREB pathways are regulated by BDNF expression (Jiang et al., 2017).

Many studies showed that BDNF promotes activation of ERK and CREB and that this pathway could be a therapeutic target for enhancing cognitive function and memory in the hippocampus (Cunha et al., 2010). Moreover, in a mouse model of cholinergic dysfunction, we observed that expression levels of BDNF and phospho ERK were significantly decreased in the hippocampus. However, 7,3',4'-THIF effectively recovered these changes in protein level. Therefore, 7,3',4'-THIF may enhance cognitive function and memory partly by regulating BDNF and its downstream signaling processes in the hippocampus. However, further studies are needed to clarify whether the increased expression of ERK/CREB was directly regulated by BDNF. This can be determined by application of 7,3',4'-THIF into the mice treated with BDNF signaling inhibitors, such as a tyrosine kinase inhibitor, and then confirming alteration of ERK/CREB expression (Rattiner et al., 2004). Nevertheless, the outcomes of this study are in agreement with those of previous studies on other daidzein metabolites, such as 6,7,4'-THIF and 7,8,4'-THIF, which suggests that these metabolites improved cognitive function by regulating BDNF/ERK/CREB signaling. Therefore, the results support our hypothesis that 7,3',4'-THIF activates ERK/CREB partly through BDNF and leads to cognition enhancement. Therefore, there is need for further studies on development of therapeutic agents or functional foods using metabolites of daidzein.

In conclusion, 7,3',4'-THIF improved scopolamine-induced amnesia as well as intact memory function in mice through activation of the cholinergic system and BDNF signaling cascades, and these effects are similar to those of other previously studies metabolites of daidzein, such as 6,7,4'-THIF and 7,8,4'-THIF. However, despite using a lower dose (1 mg/kg) than for other metabolites of daidzein, 7,3',4'-THIF showed a maximum effect on cognitive function. Therefore, 7,3',4'-THIF may be more effective in enhancing memory function than other metabolites of daidzein. In addition, we showed some novel information, specifically the effects on the protein levels of PSD-95 and synaptophysin involved in synaptic function. Our results showed that 7,3',4'-THIF significantly increased the expression of PSD-95 and

synaptophysin in the hippocampus of intact mice. On the other hand, the current study may be considered to have the originality in comparison with the previous published papers (Ko et al., 2018a, 2018b). Thus, we could confirm the potential candidates for development of therapeutic agents of brain-related disorders by measuring the effects of all the major metabolites of daidzein on cognitive function. Therefore, results from the current and previous studies have demonstrated that the major metabolites of daidzein may offer potential therapeutic agents or functional foods for treatment of several neurodegenerative diseases such as AD.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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