Therapeutic potential of *Gastrodia elata* Blume for the treatment of Alzheimer’s disease

Guang-Biao Huang¹, Tong Zhao¹, Sushma Shrestha Muna¹, Hong-Mei Jin¹, Jong-Il Park¹, Kyu-Sik Jo², Bo-Hee Lee², Soo-Wan Chae³, Sun-Young Kim³, Soo-Hyun Park³, Eun-Ock Park³, Eun-Kyung Choi³, Young-Chul Chung¹,²

1 Department of Psychiatry, Chonbuk National University Medical School, Jeonju 561-756, Republic of Korea
2 Muju Chunma Native Local Industrial Center, Muju 568-844, Republic of Korea
3 Department of Pharmacology, Chonbuk National University Medical School, Jeonju 561-756, Republic of Korea
4 Department of Pharmacology, Chonbuk National University Medical School, Jeonju 561-756, Republic of Korea
5 Research Institute of Clinical Medicine of Chonbuk National University-Biomedical Research Institute of Chonbuk National University Hospital, Jeonju 561-756, Republic of Korea

Abstract

Several studies have demonstrated that the Chinese herb *Gastrodia elata* Blume can protect against amyloid beta-peptide (Aβ)-induced cell death. To investigate the possible therapeutic effects of *Gastrodia elata* Blume on Alzheimer’s disease, we established a rat model of Alzheimer’s disease by injecting Aβ25-35 into bilateral hippocampi. These rats were intragastrically administered 500 or 1 000 mg/kg *Gastrodia elata* Blume per day for 52 consecutive days. Morris water maze tests showed that *Gastrodia elata* Blume treatment significantly improved the spatial memory of Alzheimer’s disease rats. Congo red staining revealed that *Gastrodia elata* Blume significantly reduced the number of amyloid deposits in the hippocampus of these rats. Western blot analysis showed that choline acetyltransferase expression in the medial septum and hippocampus was significantly increased by the treatment of *Gastrodia elata* Blume, while Ellman method showed significant decrease in the activity of acetylcholinesterase in all three regions (prefrontal cortex, medial septum and hippocampus). These findings suggest that long-term administration of *Gastrodia elata* Blume has therapeutic potential for Alzheimer’s disease.

Key Words

neural regeneration; neuroprotective effect; Traditional Chinese medicine; *Gastrodia elata* Blume; Alzheimer’s disease; Morris water maze test; choline acetyltransferase; acetylcholinesterase; grants-supported paper; neuroregeneration

Research Highlights

2. Aβ25-35 had detrimental effects on choline acetyltransferase expression and acetylcholinesterase activity in the brain.
3. Long-term administration of *Gastrodia elata* Blume partially reversed these effects in Alzheimer’s disease rats.
INTRODUCTION

Gastrodia elata Blume, a saprophytic, perennial herb in the Orchidaceae family, is native to several Asian countries, including China, Korea and Japan. The dry tuber of Gastrodia elata Blume (Gastrodiae rhizome; "Tianma" in Chinese) has been used for centuries in traditional Chinese medicine to treat dizziness, paralysis and epilepsy, and is listed in the Chinese Pharmacopoeia[1]. Recently, Tianma and its main bioactive component, gastrodin, have been shown to have diverse effects, including cognition enhancing[2-4], neuroprotective[5-6], antidepressant[7] and anti-inflammatory[8] effects. Hsieh et al[9] reported that a single administration of gastrodin and p-hydroxybenzyl alcohol, an aglycone of gastrodin, prolonged cycloheximide- and apomorphine-induced step-through latency in a passive avoidance task, but did not prolong scopolamine-induced step-through latency in the same task. Their conclusion was that gastrodin and p-hydroxybenzyl alcohol can facilitate the consolidation and retrieval of memories, but not the acquisition of memories. However, the administration of gastrodin or p-hydroxybenzyl alcohol for 1 week significantly prolonged the shortened step-through latency induced by scopolamine in the passive avoidance task[2-10]. In addition, Gastrodia elata Blume improves d-galactose[11] and aluminum chloride-induced[12] impairment in the passive avoidance test. Interestingly, Gastrodia elata Blume has also been reported to protect against amyloid β-peptide (Aβ)-induced cell death, suggesting that Gastrodia elata Blume has therapeutic potential for Alzheimer’s disease[3, 12]. The strong hydroxyl radical scavenging activity of Gastrodia elata Blume has been suggested to underlie the neuroprotective effect of the herb[3]. However, the mechanisms by which Gastrodia elata Blume improves memory remain poorly understood.

Alzheimer’s disease is a multifaceted neurodegenerative disorder of the central nervous system characterized by progressive cognitive dysfunction. A major pathologic hallmark of Alzheimer’s disease is the presence of numerous senile plaques, neurofibrillary tangles in brain regions, and cholinergic dysfunction. The degree of cognitive dysfunction in Alzheimer’s disease patients significantly correlates with a decline in choline acetyltransferase activity and the loss of cholinergic neurons[13-14]. Interestingly, it has been shown that the G4 globular form of acetylcholinesterase is selectively decreased in the brain of Alzheimer’s disease patients, whereas levels of the monomeric forms of the protein are increased in the vicinity of amyloid plaques[15]. Direct injection of Aβ25-35 into the hippocampus has been used successfully to create animal models of Alzheimer’s disease[16-18], although some studies have yielded conflicting results[19-20]. The potential benefits of Gastrodia elata Blume for the treatment of Alzheimer’s disease have never been investigated in vivo.

The aim of this study was to investigate the effects of Gastrodia elata Blume on spatial memory in a rat Alzheimer’s disease model using the Morris water maze test. To explore the mechanisms behind these putative effects, we examined Alzheimer’s disease model rats treated with cellulose or Gastrodia elata Blume for choline acetyltransferase expression and acetylcholinesterase activity in several brain regions.

RESULTS

Quantitative analysis of experimental animals

A total of 43 rats were initially included in this study, and divided into four groups. Some rats (Alzheimer’s disease model rats) received bilateral injections of Aβ25-35 into the hippocampus (n = 31) and were then treated daily with oral administration of either 0.5% cellulose (Aβ25-35/cellulose group, n = 9) (control), 500 mg/kg Gastrodia elata Blume (Aβ25-35/GEB group, n = 11) or 1 000 mg/kg Gastrodia elata Blume (Aβ25-35/GEB group, n = 11) for 52 days (GEB: Gastrodia elata Blume). The vehicle group (n = 12) consisted of vehicle-injected rats treated daily with oral administration of 0.5% cellulose for 52 days. All 43 rats were included in the final analysis.

Effects of Gastrodia elata Blume on spatial memory in Alzheimer’s disease rats

Typical swim paths are shown in Figure 1, and the water maze data latency and distance data are shown in Figure 2. The acquisition day had a significant effect on latency and on the distance traveled to the hidden platform [latency: F(3, 32) = 85.04, P < 0.000 1; distance: F(3, 33) = 101.43, P < 0.000 1], indicating that all of the rats readily learned the task. There was also a main effect of treatment on latency and on distance traveled to the hidden platform [latency: F(3, 32) = 4.21, P = 0.013; distance: F(3, 33) = 3.27, P = 0.033]. No significant interactions between acquisition day and treatment were found for latency [F(3, 32) = 1.01, P > 0.05] or distance traveled [F(3, 33) = 0.45, P > 0.05]. Post hoc analysis for
the main effect of treatment during acquisition revealed a significant difference in the escape latencies of the control and Aβ25-35/GEB1000 groups ($P = 0.00142$) and revealed a trend towards a difference in escape latencies between the control and Aβ25-35/cellulose and between the control and Aβ25-35/GEB1000 groups. It also revealed a significant difference in the distances traveled by the control and Aβ25-35/cellulose groups as well as in the distances traveled by the control and Aβ25-35/GEB1000 groups ($P < 0.05$).

In the probe trial, both the time spent [$F(3, 37) = 2.79$, $P = 0.54$] and the distance traveled [$F(3, 37) = 3.29$, $P = 0.031$] in the target quadrant varied significantly among the four groups. Fisher’s post hoc comparisons demonstrated significant differences in time spent and distance traveled in the target quadrant between the Aβ25-35/cellulose group and the control ($P = 0.018$ and $P = 0.012$, respectively), Aβ25-35/GEB1000 ($P = 0.033$ and $P = 0.008$, respectively) and Aβ25-35/GEB1000 ($P = 0.017$ and $P = 0.029$, respectively) groups. In the working memory test, all animals exhibited an improvement in performance with repeated trials in terms of latency and distance traveled to the hidden platform [latency: $F(3, 35) = 307.41$, $P < 0.001$; distance: $F(3, 32) = 285.63$, $P < 0.001$]. However, no significant main effects of treatment on latency [$F(3, 35) = 0.33$, $P > 0.05$] or distance traveled [$F(3, 32) = 1.93$, $P > 0.05$] were observed, nor were any significant interactions between trial number and treatment for latency [$F(3, 35) = 1.93$, $P > 0.05$] or distance traveled [$F(3, 32) = 2.94$, $P > 0.05$] observed.

**Effects of Gastrodia elata Blume on amyloid deposits in Alzheimer’s disease rats**

Congo red staining showed that the CA1 neurons in control rats were arranged in an orderly fashion, with distinct edges and clear nuclei, without Congo red stained deposits. In comparison, stained amyloid deposits in the hippocampus were scarce in the Aβ25-35/GEB1000 and Aβ25-35/GEB1000 groups (Figure 3). Few or no stained amyloid deposits were seen in the prefrontal cortex or medial septum (data not shown).
**Effects of Gastrodia elata Blume on choline acetyltransferase expression**

Significant differences in choline acetyltransferase expression in the medial septum \( F(3, 33) = 4.71, P = 0.008 \) and hippocampus \( F(3, 34) = 3.53, P = 0.025 \) were found among the four groups. Fisher’s post hoc analysis revealed significant differences in medial septum expression between the \( \beta_25-35/ \)cellulose and \( \beta_25-35/ \)Gastrodia elata Blume (GB) groups (\( P < 0.001 \) for both comparisons), as well as significant differences in hippocampal expression between the \( \beta_25-35/ \)cellulose and control, \( \beta_25-35/ \)Gastrodia elata Blume (GB) groups (\( P < 0.05 \) for all comparisons). However, no significant difference in choline acetyltransferase expression in the prefrontal cortex was found among the four groups \( F(3, 34) = 0.32, P > 0.05 \) (Figure 4).

**Effects of Gastrodia elata Blume on acetylcholinesterase activity**

Acetylcholinesterase activity in the prefrontal cortex, medial septum and hippocampus differed significantly among the four groups \( F(3, 29) = 8.37, F(3, 30) = 7.9 \) and \( F(3, 32) = 23.85 \), respectively; \( P < 0.001 \) for all. Fisher’s post hoc analysis showed significantly higher acetylcholinesterase activity in the prefrontal cortex and hippocampus in the \( \beta_25-35/ \)cellulose group compared with the control, \( \beta_25-35/ \)Gastrodia elata Blume (GB)000 and \( \beta_25-35/ \)Gastrodia elata Blume (GB)1000 groups (\( P < 0.001 \) for all). In addition, acetylcholinesterase activity in the medial septum was significantly higher in the \( \beta_25-35/ \)cellulose group than in the control or \( \beta_25-35/ \)Gastrodia elata Blume (GB)000 groups (\( P < 0.05 \) for both) (Figure 5).

![Figure 4 Effects of Gastrodia elata Blume (GB) on choline acetyltransferase (ChAT) levels in the prefrontal cortex (PFC), medial septum (MS) and hippocampus (HIP) of \( \beta_25-35- \)induced Alzheimer’s disease rats. Western blot analysis was repeated twice (each group \( n = 4–5 \) rats), and the absorbance ratio of choline acetyltransferase to \( \beta \)-actin (% vehicle) was used to represent the level of protein expression. Data are expressed as mean \pm SEM and were analyzed by one-way analysis of variance, followed by Fisher’s post hoc test. \( \cdot P < 0.05, \) vs. \( \beta_25-35/ \)cellulose group.

![Figure 5 Effects of Gastrodia elata Blume (GB) on acetylcholinesterase (AChE) activity in prefrontal cortex (PFC), medial septum (MS) and hippocampus (HIP) of \( \beta_25-35- \)induced Alzheimer’s disease rats. The acetylcholinesterase activity assay was repeated three times (each group \( n = 4–5 \) rats), and the ratio of acetylcholinesterase to vehicle was used to represent the activity of acetylcholinesterase expression. Data are expressed as mean \pm SEM and were analyzed by one-way analysis of variance, followed by Fisher’s post hoc test. \( \cdot P < 0.05, \) vs. \( \beta_25-35/ \)cellulose group.

**DISCUSSION**

To produce the rat model of Alzheimer’s disease, we used bilateral hippocampal injections of \( \beta_25-35- \). Significant differences between the control (vehicle-injected, cellulose-treated non-Alzheimer’s disease rats) and the \( \beta_25-35/ \)cellulose groups were observed in the probe trial and in the distance traveled in the acquisition trial. In a preliminary experiment, significant differences in both escape latency and distance traveled were demonstrated between the control and \( \beta_25-35/ \)cellulose groups in the probe and acquisition trials (data not shown). These findings suggest that bilateral hippocampal injection of \( \beta_25-35- \) produces a valid rat model of Alzheimer’s disease. The validity of this model is supported by our findings of a significant decrease in choline acetyltransferase expression and a significant increase in acetylcholinesterase activity in the \( \beta_25-35/ \)cellulose group relative to the control group, and by our observation of Congo red staining, revealing amyloid deposition in the hippocampus of the...
Acetylcholinesterase levels reported to be neurotoxic, resulting in increased amyloid deposits. The deposition of Aβ increase in acetylcholinesterase expression induced by control rats in all three areas. The deposition of Aβ might be more sensitive than acetylcholinesterase to levels comparable to those in Gastrodia elata. Injection of Aβ peptide on choline acetyltransferase expression induced by amyloid deposition. This report is the first to demonstrate that hippocampal injection of Aβ peptide induces impairment of spatial memory and has detrimental effects on choline acetyltransferase expression and acetylcholinesterase activity, and that Gastrodia elata Blume treatment partially restores spatial memory function through its ability to restore these critical cholinergic enzymes to normal levels. Similar evidence supporting the detrimental effects of Aβ peptide on the cholinergic system comes from reports that the injection of Aβ peptide into rat brain impairs learning as a result of a hypofunction of the septohippocampal cholinergic system caused by a reduction in acetylcholine release mechanisms and as a consequence of cholinergic neuronal degeneration. The detrimental effect of Aβ peptide on cholinergic markers may be related to its ability to self-aggregate and form Ca2+-permeable channels in membranes, resulting in excessive Ca2+ influx and the induction of neurotoxic cascades.

Thus, the protective effect of Gastrodia elata Blume on spatial memory function may be related to its ability to counteract the harmful changes in levels of critical cholinergic enzymes induced by Aβ peptide. The mechanisms underlying the neuroprotective effects of the herb have been investigated in several studies, and include the following: suppressing tumor necrosis factor-α-induced vascular inflammation, increasing Bcl-2 expression and inhibiting caspase-3 activation, antioxidative effect and GABAergic neuromodulation, suppressing nitric oxide synthase and microglial activation, and increasing blood supply to the vertebral-basilar artery. Taken together, these findings suggest that Gastrodia elata Blume preserves choline acetyltransferase and acetylcholinesterase activities from Aβ toxicity through various neuroprotective mechanisms, resulting in partial recovery from spatial memory impairment.
Several methodological considerations should be mentioned. First, in the present study, Gastrodia elata Blume was administered through gastric gavage, not through spontaneous oral intake. This method was selected because the Gastrodia elata Blume solution had a peculiar odor that might have caused inter-individual variation in intake and because forced feeding might have affected study parameters. In addition, contrary to the manufacturer’s instructions, ethopropasine-HCI was not added to the incubation mixture in the QuantiChrom acetylcholinesterase assay. Since ethopropasine-HCI inhibits nonspecific cholinesterase activity, our acetylcholinesterase activity results represent total cholinesterase activity. Even though the physiological role of the nonspecific cholinesterase has not been established, it comprises approximately 10% of the total cholinesterase activity\(^4\). Therefore, this limitation should be considered in interpreting our acetylcholinesterase activity results. Furthermore, we did not investigate how preservation of choline acetyltransferase expression by Gastrodia elata Blume impacts acetylcholine levels. In future studies, other cholinergic markers such as acetylcholine and vesicular acetylcholine transporter, as well as central nervous system inflammatory markers, should be examined to clarify the mechanisms by which Gastrodia elata Blume enhances cognitive performance.

In conclusion, the present study demonstrated that (i) intrahippocampal injection of Aβ peptide impairs spatial reference memory and has detrimental effects on choline acetyltransferase expression and acetylcholinesterase activity in the rat brain; and (ii) long-term administration of Gastrodia elata Blume partially reverses these effects. These findings suggest that Gastrodia elata Blume has potential therapeutic value for Alzheimer’s disease.

## MATERIALS AND METHODS

### Design
A randomized, controlled animal experiment.

### Time and setting
Experiments were performed from February 2011 to December 2011 at the Research Institute of Clinical Medicine, Chonbuk National University-Biomedical Research Institute, Republic of Korea.

### Materials
Male Wistar rats (8 weeks old, 220–250 g) were purchased from Orient Bio Inc. (Seoul, Republic of Korea) and were used throughout the study. The rats were housed in a temperature-controlled room under a 12-hour light-dark cycle (lights on from 7:00 a.m. to 7:00 p.m.) with food and water ad libitum. Experiments were carried out between 9:00 a.m. and 4:00 p.m. All possible efforts were made to minimize animal suffering and the number of animals used, in accordance with the Guidelines for Animal Experiments of Chonbuk National University Medical School, Republic of Korea.

### Methods
**Intrahippocampal injection of Aβ\(_{25-35}\) and administration of Gastrodia elata Blume and cellulose**

Aβ\(_{25-35}\) (Sigma-Aldrich, St. Louis, MO, USA) was dissolved in sterile distilled water at a concentration of 5 µg/µL. To obtain the aggregated form of Aβ\(_{25-35}\), the peptide solution was placed in an incubator at 37°C for 96 hours. Light microscopic observation demonstrated the precipitation of insoluble globular aggregates (Figure 6).

The rats were anesthetized with intraperitoneal injection of a 4:1 mixture of ketamine (Yuhan, Seoul, Korea) and xylazine (Bayer, Seoul, Korea) and placed in a David Kopf stereotaxic instrument (IWOO Scientific Corporation, Seoul, Korea) with the incisor bar set 3.3 mm below the interaural line. Holes were drilled in the skull, and 2 µL of Aβ\(_{25-35}\) (10 µg) or vehicle was bilaterally infused into the hippocampus (CA1 region) through a stainless-steel 25-gauge cannula (Small Parts Inc., Seattle, WA, USA) connected via polyethylene tubing to a 10 µL Hamilton microsyringe. The coordinates used were –3.6 mm anteroposterior from the bregma, +2.0 mm bilateral from the midline, and –2.7 mm ventral from the skull according to the stereotaxic atlas\(^3\). The correct placement of the cannula was verified in preliminary experiments by the injection of India ink. The skull holes were filled with bone wax (Lukens Bone Wax; Angiotech Inc, Vancouver, BC, Republic of Canada).
Canada) to prevent outflow, and the skin was sutured. One day after the injection of Aβ_{25-35} or vehicle, rats received their first dose of powdered Gastrodia elata Blume (Muju Tianma Native Local Industrial Center, Korea) dissolved in water (500 or 1 000 mg/2 mL per kg body weight) or 0.5% cellulose (2 mL/kg) via gastric gavage. Dosing was continued daily throughout the experimental period of 52 days (6 weeks plus 10 days for the Morris water maze test).

**Gastrodia elata Blume preparation**
The rhizome of Gastrodia elata Blume (Tianma) was collected from Muju, Korea and was provided by Muju Tianma Native Local Industrial Center. Whole dried tubers of Gastrodia elata Blume were hammered into smaller pieces and ground to a fine powder. Gastrodia elata Blume was dissolved in deionized water (1.25 or 2.5 g powder in 5 mL deionized water) to yield stock solutions of 250 and 500 mg/mL for use in experiments.

**Morris water maze test**
Morris water maze testing was initiated after 6 weeks of daily Gastrodia elata Blume or cellulose dosing, which continued throughout the 10-day water maze experiment. The water maze consisted of a circular pool (diameter: 180 cm; height: 60 cm) with a black (painted) interior. A small (diameter: 10 cm), black (painted), non-slippery, Plexiglas platform was immersed 1 cm below the water surface so that it was invisible in the black tank. The water temperature was maintained at 25 ± 2°C with a Thermo Heater (Warmtone, Shenzhen, Guangdong, China). Three extra-maze cues (a black, white, or black/white-striped circle, square or triangle, each 60 cm × 80 cm) were hung on the surrounding black curtain in a dimly lit (40 lx at water level) and soundproof test room. A digital camera mounted above the water maze captured five images per second and transmitted them to a personal computer running Smart software (Panlab, Barcelona, Spain) for calculation of the escape latency, distance traveled and average swim speed for each trial. Four points, equally spaced along the circumference of the water maze, were arbitrarily designated north (N), east (E), south (S) and west (W) to create NE, SE, NW and SW quadrants of equal size.

**Visible platform training**
An initial visual-training day was used to familiarize each animal with the water maze and allow between-treatment comparison of possible differences in visual discrimination and motor control. This training was composed of three trials using a 10-cm-diameter visible cued platform positioned in the center of the water maze. For each trial, the animal was positioned against the maze wall at a randomly chosen start position (N, E, S or W) and released into the water maze. The rat was allowed a maximum of 90 seconds to locate the escape platform. Animals failing to locate the platform were guided to the platform by the experimenter. The animals were then allowed to spend 15 seconds on the platform and 45 seconds in the home cage before commencement of the next trial. The inter-trial interval was therefore approximately 1 minute. All animals acquired the swimming response and were able to climb onto the escape platform by the end of the third trial.

**Reference memory acquisition**
After the initial training day, each animal was tested for reference memory using the submerged invisible platform with a visual cue placed in a fixed position (SW). Each animal participated in five trials per day for 5 consecutive days using a procedure similar to that used for the training task. When an animal failed to locate the platform within the 90-second time limit, an escape latency of 90 seconds was assigned. Between trials, the water was strained of feces and gently swirled to diffuse odor cues. A different starting position was used for each trial performed on the same day, and the sequence of starting positions was varied from day to day. Acquisition of reference memory was indicated by a gradual reduction in escape latency and the distance traveled as the training progressed.

The day after the acquisition training period ended (the 6th day), a probe test was performed, in which the platform was removed and the animals were allowed to search for it for 90 seconds. The time spent and distance traveled in the target quadrant (i.e., where the platform was located during the acquisition training sessions) were computed (SMART software, Panlab, Barcelona, Spain).

**Working memory performance**
Working memory was tested after the reference memory experiment using new extra-maze cues placed in a novel spatial arrangement. The animals were tested over 4 consecutive days, with four trials per day, using a procedure similar to that used for the visual task. The submerged platform was placed at a new location on each test day, but remained unchanged between the four trials on a given day. The start positions were counterbalanced across days and trials in a given day. The escape latency and distance traveled values for each trial number (i.e., trials 1–4) over the 4 days were averaged, and these average values were used as a measure of the working
memory performance for that trial number.

**Preparation of brain tissue**

After behavioral tests were completed, rats were killed by decapitation under ether anesthesia. The brains were quickly removed and frozen in liquid nitrogen. From the appropriate coronal sections in a pre-cooled cryostat (Richard-Allan Scientific, Kalamazoo, MI, USA), the prefrontal cortex, medial septum, and CA1 and dentate gyrus regions of the hippocampus were excised bilaterally using a 2.0-mm Harris Uni-Core micropunch (Electron Microscopy Sciences, Hatfield, PA, USA). The approximate bregma levels from which the punches were acquired were 3.70–2.20 mm and 0.70–0.20 mm for the prefrontal cortex and medial septum, respectively, and –3.60 to –4.30 mm for the hippocampus. Tissues were stored at –80°C before use.

Two rats selected randomly from each group for Congo red staining were transcardially perfused under deep anesthesia with ketamine/xylazine (4:1), then with 200 mL of calcium-free Tyrode’s solution, and thereafter with 200 mL of ice-cold 4% paraformaldehyde in 0.1 M PBS. Each brain was removed immediately after perfusion, placed in fresh fixative for at least 4 h, and then transferred sequentially to 20% sucrose in PBS for 12 and 24 h, respectively, and –3.60 to –4.30 mm for the hippocampus. Tissues were stored at –80°C before use.

Congo red staining

Congo red staining[35] was used to identify amyloid deposits in the prefrontal cortex, medial septum and CA1 region of the hippocampus. Briefly, brain sections mounted on slides were hydrated in water (5 minutes), stained in 0.5% Congo red reagent (Sigma-Aldrich Inc.) for 15–20 minutes, rinsed in distilled water, quickly differentiated (5–10 dips) in alkaline alcohol solution, rinsed in tap water (1 minute), counterstained with Gill’s hematoxylin (30 seconds), rinsed in tap water (2 minutes), dehydrated with 95% and 100% ethanol, cleared in xylene, covered with balsam, and cover-slipped. Under a light microscope, the amyloid deposits were red to brown, whereas the nuclei were blue.

**Western blot analysis**

Tissue samples (prefrontal cortex, medial septum, CA1 and dentate gyrus regions of the hippocampus) were homogenized in 20 mM ice-cold Tris-HCl (pH 7.4) containing 1% protease and phosphatase inhibitor cocktails (Sigma) using a hand homogenizer with a Teflon pestle (Vintage Thomas, Philadelphia, PA, USA). The homogenates were centrifuged for 15 minutes at 14 000 r/min at 4°C, and the resulting supernatant fractions were used for western blot analyses. Protein concentrations were determined using the Bradford assay[27] with commercial reagents (Bio-Rad Laboratories, Foster City, CA, USA) and spectrophotometric measurement at 595 nm. The protein samples (20 µg/lane) were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred electrophoretically to a hydrophobic polyvinylidene difluoride membrane. The membrane was blocked in 5% (w/v) dried non-fat skim milk in PBS containing 0.1% Tween 20 for 120 minutes at room temperature. The membranes were then incubated overnight at 4°C with a polyclonal rabbit anti-choline acetyltransferase antibody (AB143; diluted 1:10 000; Chemicon International Inc., Temecula, CA, USA) or β-actin antibody (4967; diluted 1:1 000; Cell Signaling Technology, Inc., Danvers, MA, USA) in 5% non-fat milk. The next day, the membranes were washed with PBS containing 0.1% Tween 20, and the primary antibody was detected using a horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (PI-1000; Vector; Burlingame, CA, USA) diluted 1:20 000 in PBS (60 minutes, 25°C).

The protein band corresponding to choline acetyltransferase (68 kDa) was detected using an enhanced chemiluminescence immunoblotting detection system (RPN2232; GE Healthcare, UK). The band intensities were evaluated with an LAS-3000plus luminescent image analyzer (Fuji Photo Film Company, Kanagawa, Japan). Band densities were quantified using Multi Gauge version 3.0 (Fujifilm, Tokyo, Japan).

**Acetylcholinesterase assay**

Tissue samples (prefrontal cortex, medial septum, CA1 and dentate gyrus regions of the hippocampus) were homogenized in 20 volumes of cold 0.1 M phosphate buffer (pH 7.5). The homogenates were centrifuged at 14 000 r/min for 15 minutes at 4°C, and 10 µL aliquots of the supernatant fractions were used in a commercial acetylcholinesterase assay (Quantichrom acetylcholinesterase Assay Kit; BioAssay Systems, Hayward, CA, USA). This assay was performed based on an improved Ellman method[38] in which the thiocholine produced by the action of
acetylcholinesterase formed a yellow color with 5,5'-dithiobis(2-nitrobenzoic acid). The intensity of the product color, measured at 412 nm, was proportionate to the enzyme activity in the sample.

Statistical analysis
Results are presented as mean ± SEM. The data were analyzed with SPSS 12.0 (IBM, Armonk, NY, USA) using two-way repeated measures analysis of variance or one-way analysis of variance, followed by Fisher’s post hoc test. In all the cases, differences were considered statistically significant at $P < 0.05$.

Funding: This study was funded by Muju Tianma Native Local Industrial Center, Korea.

Author contributions: Young-Chul Chung participated in the experimental procedures, data analysis and statistical processing, and manuscript writing. Kyu-Sik Jo, Bo-Hee Lee and Soo-Wan Chae provided the funding for the experiments. Sun-Young Kim, Soo-Hyun Park, Eun-Ock Park and Eun-Kyung Choi gave experimental guidance. Guang-Biao Huang, Tong Zhao, Sushma Shrestha Muna, Hong-Mei Jin and Jong-II Park participated in the animal experiment.

Conflicts of interest: None declared.

Ethical approval: The project protocol was approved (#2011-2-0029) by the Animal Ethics Committee of Chonbuk National University, Korea.

Author statements: The manuscript is original, has not been submitted to or is not under consideration by another publication, has not been previously published in any language or any form, including electronic, and contains no disclosure of confidential information or authorship/patent application/funding source disputations.

REFERENCES


(Reviewed by Lakshmana MK, Patel B, Jin XM, Li YW) (Edited by Li CH, Song LP)