

# Long-term administration of ginsenoside Rh<sub>1</sub> enhances learning and memory by promoting cell survival in the mouse hippocampus

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**Abstract.** Ginsenosides, the secondary plant metabolites produced by *Panax ginseng* are responsible for the enhancing effects on learning observed following treatment with *Panax ginseng*. A number of studies have provided correlational evidence that cell proliferation and survival are closely associated with hippocampal-dependent learning tasks. In this study, to investigate the beneficial effects of ginsenoside Rh<sub>1</sub> on hippocampal cells and learning, mice (6 months old) were administered ginsenoside Rh<sub>1</sub> at a dose of 5 and 10 mg/kg/day for a period of 3 months. Saline-treated mice were used as controls. The enhancement of memory and learning in the mice was evaluated by hippocampal-dependent tasks (passive avoidance tests and Morris water maze tests) and the immunohistochemical marker of cell proliferation, bromodeoxyuridine (BrdU). In addition, the levels of brain-derived neurotrophic factor (BDNF) were measured following treatment. Based on our data, the Rh<sub>1</sub>-treated group (5 and 10 mg/kg) showed a significantly improved learning and memory ability in the passive avoidance tests compared with the control group; however, only treatment with 10 mg/kg ginsenoside Rh<sub>1</sub> significantly promoted spatial learning ability in the Morris water maze test. Ginsenoside Rh<sub>1</sub> significantly enhanced cell survival in the dentate gyrus of mice, although it did not enhance hippocampal cell proliferation. In addition, ginsenoside Rh<sub>1</sub> upregulated the expression of BDNF. These findings address the potential therapeutic significance of ginsenoside Rh<sub>1</sub> as a nutritional supplement in memory loss and neurodegenerative diseases.

## Introduction

A wide range of food or dietary supplements that are derived from plants have been shown to be able to modify the functions of the central nervous system. Ginsenosides, the secondary plant metabolites produced by *Panax ginseng*, are classified into two major groups in terms of the number and position of sugar moieties: 20 (S)-protopanaxadiol (PPD) and 20 (S)-protopanaxatriol (PPT) saponins. The diversity of individual ginsenosides may be responsible for their specific pharmacological effects (1). Increasing evidence has indicated the beneficial effects of ginsenosides on the central nervous system (2-6). However, the majority of these studies have focused on the beneficial effects of ginsenosides Rg<sub>1</sub> and Rb<sub>1</sub>.

Over the past three decades, an increasing number of studies have focused on the correlation between neurogenesis and memory formation (7-9). The vast majority of these studies describe neurogenesis in the subregions of the hippocampus [the subventricular zone and subgranular zone (SGZ)] (10,11), whereas only a few studies have investigated neuronal survival (12). Several factors that affect hippocampal neurogenesis cause corresponding changes in cognitive performance. For example, voluntary running improves performance in the Morris water maze task by increasing cell proliferation in the SGZ (13). Aged animals and animals under stress display impaired memory and learning in hippocampal-dependent tasks (14-16). Therefore, the putative function of neurogenesis in the SGZ in learning and memory is considered an index for the evaluation of substrates that exert beneficial effects. On the other hand, brain-derived neurotrophic factor (BDNF) has been shown to promote the differentiation and survival of neurons in the adult brain (17).

In this regard, only a few studies have reported the effects of the oral administration of metabolites (18,19) of the two ginsenosides, Rg<sub>1</sub> and Rb<sub>1</sub>, such as ginsenosides Rh<sub>1</sub>, PPT, compound K and PPD. Thus, the question remains of whether ginsenoside Rh<sub>1</sub> can affect learning and memory ability. This issue is not only of academic interest but also has a number of practical implications for future research and product development. Therefore, in the present study, we investigated the effects of the long-term administration of ginsenoside Rh<sub>1</sub> on memory and learning in the adult mouse brain.

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## Materials and methods

**Animals and housing conditions.** Male ICR mice, 6 months of age were housed in a temperature-controlled animal room with a 12-h light-dark cycle and allowed access to food and water *ad libitum*. All experiments were performed in strict accordance with the Guide for the Care and Use of Laboratory Animals issued by the National Institutes of Health (USA), and were approved and monitored by the Ethics Committee of Animal Experiments at Chungnam National University, Daejeon, Korea. Prior to the experiments, the mice were left undisturbed for 7 days and were randomly assigned to 1 of 4 experimental groups: i) the saline (0.9% NaCl)-treated group ( $n=16$ ); ii) the group treated with 5 mg/kg Rh<sub>1</sub> ( $n=16$ ); iii) the group treated with 10 mg/kg Rh<sub>1</sub> ( $n=16$ ); and iv) the behavioral test group ( $n=30$ , passive avoidance test;  $n=30$ , water maze test). The behavioral test group was subdivided into 3 groups (control, 5 mg/kg Rh<sub>1</sub> and 10 mg/kg Rh<sub>1</sub>; 10 mice per group) and the other 3 groups were subdivided into 2 groups ( $n=8$  per group) for the evaluation of neurogenesis and cell survival in the hippocampus.

**Bromodeoxyuridine (BrdU) and ginsenosides protocol.** Mice in the saline-treated group (0.9% NaCl) and the ginsenoside (FuSong County Natural Biotechnology, Co., Ltd., Fusong, China)-treated groups (Rh<sub>1</sub>, 5 and 10 mg/kg body weight) were orally administrated saline and ginsenoside, respectively for a period of 3 months. Rh<sub>1</sub> doses were converted between adult human (60 kg) and mouse (20 g) body weights, using the body surface area normalization method, as previously described (20). The selected doses corresponded to 2-3 and 5-6 g of ginseng per day in an adult human (60 kg body weight). Considering that the long-term intravenous administration of ginsenosides would cause inflammation or anxiety, we selected oral administration, even though this would be associated with less bioactivity.

The thymidine analogue, BrdU, is incorporated into the DNA of dividing cells and can be detected immunohistochemically in their progeny. The behavioral tests were performed at the end of the drug administration. To determine the effects of ginsenoside Rh<sub>1</sub> on neurogenesis, BrdU (100 mg/kg body weight) was administered to the mice twice per day for 3 consecutive days prior to sacrifice; to determine the effects of ginsenoside Rh<sub>1</sub> on cell survival, BrdU was administered to the mice twice per day for 3 consecutive days (20 days prior to sacrifice) during the 3rd month of the treatment period. Following the evaluation of neurogenesis and cell survival, the animals (8 per group) were sacrificed, the brains were excised and the brain tissue was then subjected to immunohistochemical and protein expression analysis. The overall experimental protocol is presented in Fig. 1.

**Morris water maze test.** Mice from the different groups were subjected to a Morris water maze test for 5 consecutive days in the terminal phase of the administration process. The escape platform (diameter, 10 cm; height, 24 cm) was hidden 1 cm below the surface of the water, which had been made opaque by the addition of non-toxic black paint. Each animal was subjected to 4 experimental trials per day, each lasting 60 sec and each time commencing from 4 different starting points that randomly varied each day. If an animal was not able to

find the platform it was manually place on it at the end of the trail. The animals were allowed to rest on the platform for 15 sec. A probe test was performed on day 6.

**Passive avoidance test.** The passive avoidance test was performed in identical compartments. The illuminated compartment (20x20x20 cm) contained a 100 W bulb, and the floor of the non-illuminated compartment (20x20x20 cm) was composed of 2 mm stainless steel rods at 1 cm intervals. These 2 compartments were separated by a guillotine door (5x5 cm). For the acquisition trials, the mice were initially placed in the illuminated compartment and the door was opened 15 sec later. When the mouse entered the non-illuminated compartment, the door was closed and an electrical foot shock (0.5 mA) of 3 sec in duration was delivered through the stainless steel rods. Twenty-four hours after the acquisition trial, the mice were again placed in the illuminated compartment for the retention trials. The time taken for a mouse to enter the non-illuminated compartment after the opening of the door was termed as the step-through latency time in the retention trials. If a mouse did not enter the non-illuminated compartment within 180 sec, it was assumed that the mouse had remembered the single training trial.

**Immunohistochemistry.** The mice were sacrificed and the brains were removed after the final BrdU injection. The brains were fixed in 4% phosphate-buffered paraformaldehyde for 12 h. The brain tissues were then embedded in paraffin and cut into sections. The sections were mounted on glass slides and stored overnight at 42°C. Following deparaffinization with xylene and rehydration in a graded series of ethanol, the sections were rinsed in 0.01 M phosphate-buffered saline (PBS). BrdU is a widely used S-phase marker of neurogenesis.

For BrdU-immunostaining, the sections were hydrolyzed with 2 N hydrochloride (HCl) in PBS (pH 7.4) at 37°C for 15 min, and then stained using the Invitrogen BrdU staining kit (Invitrogen, Carlsbad, CA, USA). The sections were incubated in serum blocking solution, in a 1:50 dilution of a mouse monoclonal antibody against BrdU (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) and incubated overnight at 4°C in biotinylated secondary antibody at room temperature for 30 min, and finally in streptavidin-peroxidase conjugate at room temperature for 20 min. After each step, the sections were rinsed with PBS. The sections were then incubated in 3,3'-diaminobenzidine (DAB) solution. Subsequently, the sections were incubated in 1% ferric chloride solution at room temperature for 5 min. BrdU-positive nuclei exhibited deposits of dark brown or black-colored precipitates. The sections were counterstained with hematoxylin and cover-slipped with histomount.

**Microscopy and cell counting.** Every 10th section throughout the hippocampus was processed for BrdU immunohistochemistry. Using this spacing ensures that the same neuron will not be counted in two sections. All BrdU-labeled cells in the dentate gyrus (granule cell layer) and hilus were counted in each section. To distinguish single cells within clusters, all counts were performed at  $\times 400$  magnification under a light microscope (Olympus BX-41; Olympus, Tokyo, Japan), omitting cells in the outermost focal plane. A cell was counted as being in the SGZ of the dentate gyrus if it was touching or in

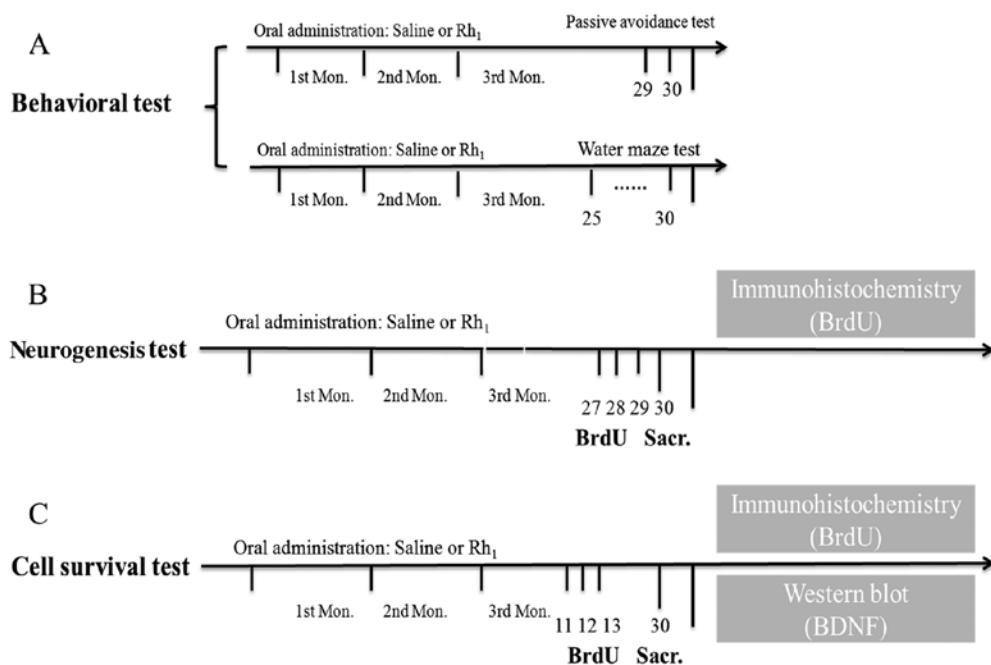


Figure 1. Overview of the experimental protocol. (A) Scheme for behavioral tests, including the passive avoidance and Morris water maze test. (B) For the analysis of neurogenesis, the animals were sacrificed 24 h after the final bromodeoxyuridine (BrdU) injection. (C) For cell survival analysis, BrdU was administered to the animals during the 3rd month and the animals were then sacrificed 20 days after the final injection. Mon., months; BDNF, brain-brain-derived neurotrophic factor.

the SGZ. Cells that were located more than two cells away from the SGZ were classified as hilar. The total number of BrdU-labeled cells per section was determined and multiplied by 6 to obtain the total number of cells per dentate gyrus.

**Western blot analysis.** The mice were anesthetized and decapitated after 3 months of treatment. The hippocampus dissected from each animal was homogenized ultrasonically in protein extraction buffer (PRO-PREP™ 17081; iNtRON Biotechnology, Seongnam, Korea). The supernatant was collected after centrifugation at 15,000 rpm for 30 min at 4°C. Following quantification, the samples (20 µg protein per lane) were subjected to preparative sodium dodecyl sulfate-polyacrylamide gel electrophoresis in a 15% gel and electrophoretically transferred onto PVDF membranes (Millipore, Billerica, MA, USA) using a trans-blot device (Bio-Rad, Hercules, CA, USA) at a 15 V constant current overnight at 4°C. The PVDF membranes were soaked in 5% skim milk in PBS solution for 2 h at room temperature to block non-specific binding, rinsed in PBST, and incubated with a rabbit polyclonal anti-SNAP-25 antibody (diluted 1:300 in 5% skim milk in TBST; Santa Cruz Biotechnology, Inc.) overnight at 4°C. The membranes were then washed 3 times for 10 min each in PBST and incubated for 2 h with a secondary antibody, goat anti-rabbit IgG (1:10,000; Santa Cruz Biotechnology, Inc.). After washing twice for 15 min in PBST, the signal was detected using an ECL system. Western blot analysis for β-actin was performed using the same procedure using a goat polyclonal anti-actin antibody (1:1,000; Santa Cruz Biotechnology, Inc.) as the primary antibody. The blots were quantified using image analysis software (ImageJ). Bond intensity values were expressed as a percentage of the control average.

**Statistical analysis.** Data are expressed as the means ± SEM. Statistical differences were assessed by one-way analysis of variance (ANOVA) using repeated measures where appropriate. The post-hoc Duncan's test was carried out where appropriate. The level for a statistically significant difference was set at  $P<0.05$ .

## Results

**Behavioral test.** For the Morris water maze, repeated ANOVA (time x group) revealed a significant effect decrease in escape latency (in days) following treatment with Rh<sub>1</sub> [5 mg/kg, F(2,24)=3.72, P=0.067; 10 mg/kg, F(3,42)=11.65, P<0.0001].

In fact, all groups, including the control group showed a general decrease in overall latency throughout the acquisition phase (Fig. 2A).

In the probe tests, the group treated with 10 mg/kg ginsenoside Rh<sub>1</sub> showed a significant increase in the number of crosses (number of times mouse crosses the platform location) in the target quadrant ( $P<0.05$ ), whereas the group treated with 5 mg/kg ginsenoside Rh<sub>1</sub>, did not show statistically significant results ( $P>0.48$ ) compared with the control group (Fig. 2B).

ANOVA for the time spent in the platform quadrant yielded significant results for the groups treated with Rh<sub>1</sub> [5 mg/kg, F(1,14)=13.62, P=0.071; 10 mg/kg, F(1,14)=17.85, P<0.002]. Post-hoc comparisons revealed that a general increase in the time spent in the target quadrant throughout the acquisition phase (Fig. 2C).

For the passive avoidance tests, no differences were observed among all the groups in the step-through latency during the acquisition trials (Fig. 3). ANOVA for the step-through latency during the retention trials revealed significant differences

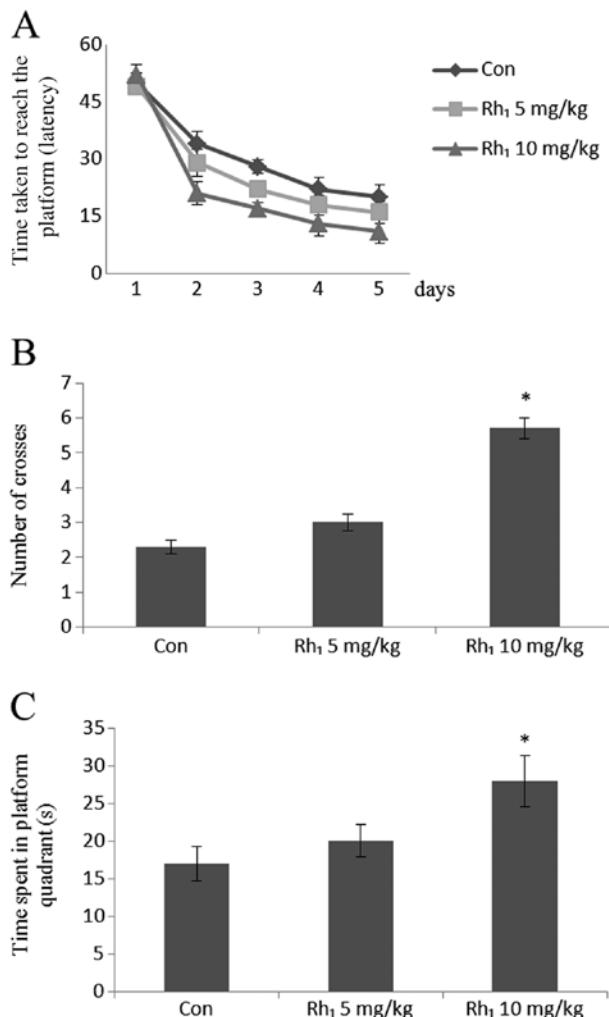


Figure 2. Three parameters in which the ginsenoside Rh<sub>1</sub>-treated mice differed from the control group in the Morris water maze test. (A) Escape latency. (B) Number of crosses (number of times mouse crosses the platform location). (C) Time spent in platform quadrant. On day 1, there was no significant difference between the ginsenoside treated groups and the control group, indicating an equal baseline. At the other time points, significant differences were observed between the control group and the group treated with 10 mg/kg ginsenoside Rh<sub>1</sub> ( $P<0.05$ ) but with the group treated with 5 mg/kg Rh<sub>1</sub> ( $P>0.05$ ), and similar statistical results were observed in the number of crosses and the time spent in the platform quadrant. Data are expressed as the means  $\pm$  SEM (\* $P<0.05$  vs. control group). Con, control.

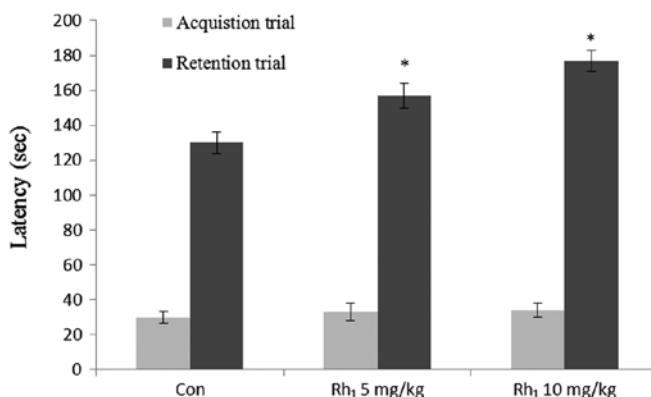


Figure 3. Differences were observed between the ginsenoside Rh<sub>1</sub>-treated mice and the control group in the passive avoidance test. Data are expressed as the means  $\pm$  SEM (\* $P<0.05$  vs. normal control group). Con, control.

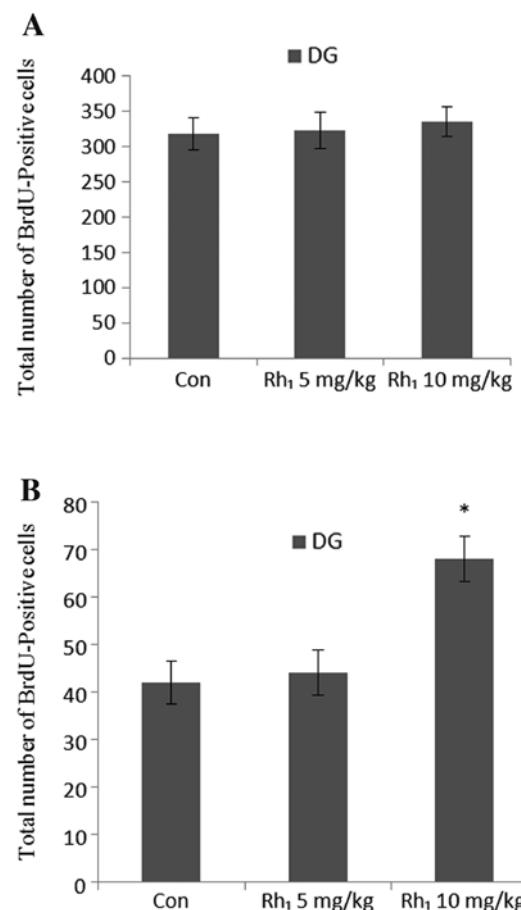


Figure 4. Effects of long-term administration of ginsenoside Rh<sub>1</sub> on neurogenesis. (A) Cell proliferation. Mice were sacrificed 2 h after the final bromodeoxyuridine (BrdU) injection. The number of BrdU-positive cells was determined. There was no statistical difference in observed in the proliferation of cells in the dentate gyrus (DG) of the hippocampus. (B) Cell survival. Mice were sacrificed 20 days after the final BrdU injection. Significant differences were observed in cell survival in the DG of the hippocampus following treatment with ginsenoside Rh<sub>1</sub>. \* $P<0.05$  vs. control group. Con, control.

among the groups treated with Rh<sub>1</sub> [5 mg/kg,  $F(1,16)=0.59$ ,  $P<0.031$ ; 10 mg/kg,  $F(1,16)=0.40$ ,  $P<0.01$ ].

**Effects of long-term ginsenoside Rh<sub>1</sub> administration on cell proliferation in the mouse hippocampus.** The animals were administered ginsenoside Rh<sub>1</sub> for 3 months and sacrificed after the final BrdU injection. Analysis of the number of BrdU-labeled cells demonstrated that the long-term administration of ginsenoside Rh<sub>1</sub> had no statistically significant effect on the number of BrdU-positive cells in the dentate gyrus (5 mg/kg,  $P>0.30$ ; 10 mg/kg,  $P>0.47$ ) (Fig. 4A).

**Effects of long-term ginsenoside Rh<sub>1</sub> administration on cell survival in the mouse hippocampus.** To specially determine the effects of long-term ginsenoside Rh<sub>1</sub> administration on cell survival, a BrdU injection was administered on the 1st day of the 3rd month. ANOVA revealed an overall significant effect in the number of BrdU-positive cells [ $F(2,18)=51.87$ ,  $P<0.0003$ ], and post-hoc tests revealed that treatment with 10 mg/kg Rh<sub>1</sub> yielded significant results ( $P<0.05$ ) compared with the control group (Fig. 4B). The results of BrdU immunohistochemistry

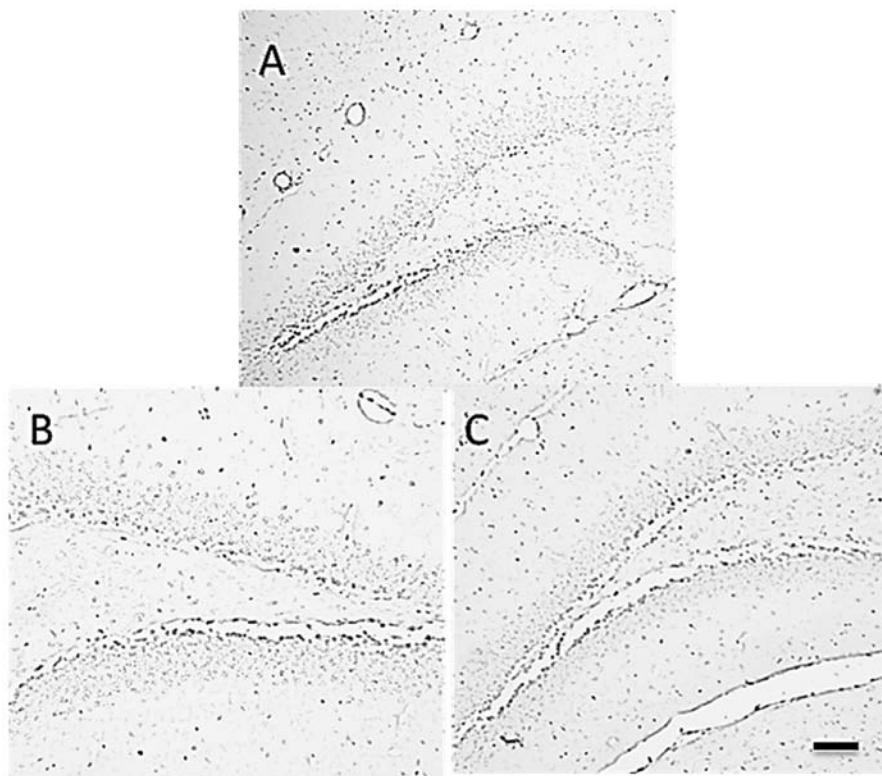


Figure 5. Bromodeoxyuridine (BrdU) immunohistochemistry for cell survival. BrdU-positive cells in the dentate gyrus (DG). (A) control; (B) Rh<sub>1</sub> 5 mg/kg; (C) Rh<sub>1</sub> 10 mg/kg. Scale bar, 100  $\mu$ m.

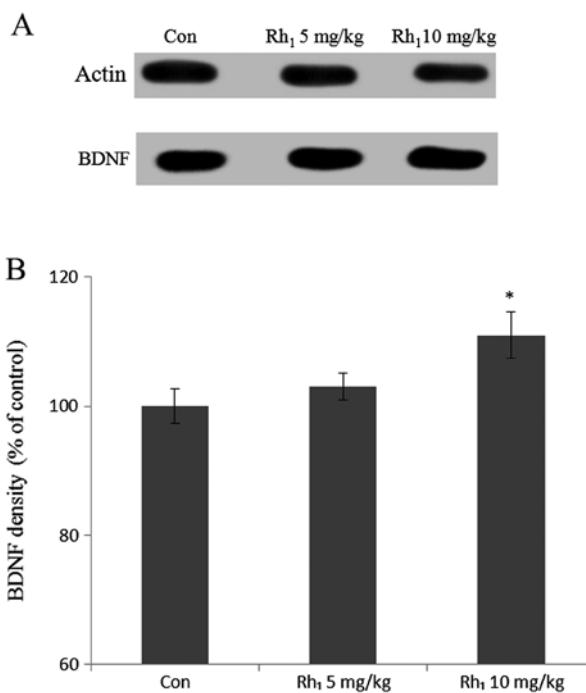


Figure 6. Effects of ginsenoside Rh<sub>1</sub> on brain-derived neurotrophic factor (BDNF) expression levels. (A) Western blot analysis of hippocampal tissue using polyclonal antibody against BDNF. (B) Data showing BDNF densities in the hippocampus (% of the control). Bars represent the means  $\pm$  SEM. \*P<0.05 vs. normal control group. Con, control.

are presented in Fig. 5. These results suggest that the long-term administration of Rh<sub>1</sub> increased cell survival in the hippocampus.

*Effects of long-term ginsenoside Rh<sub>1</sub> administration on BDNF expression levels.* The mice were sacrificed and BDNF protein expression was quantified by western blot analysis. BDNF density was measured in the hippocampus (Fig. 6). BDNF density in the control group was 100 $\pm$ 2.8% and in the groups treated with 5 mg/kg and 10 mg/kg Rh<sub>1</sub> was 103.2 $\pm$ 2.4 and 112 $\pm$ 3.7% of the control, respectively. Treatment with 10 mg/kg Rh<sub>1</sub> yielded statistically significant results compared with the control group (P<0.05).

## Discussion

In this study, we report that the long-term administration of ginsenoside Rh<sub>1</sub> enhances spatial recognition memory, as shown by a Morris water maze test and a passive avoidance test. Both of these tasks require the involvement of the hippocampus; thus, we observed a significant increase in hippocampal cell survival in the treated animals, as shown by the increase in the number of BrdU-labeled cells. These findings are consistent with those from a previous study, demonstrating that the long-term administration of ginsenoside Rb1 enhanced spatial learning and memory (21). To the best of our knowledge, only a few studies have reported the pharmacological functions of ginsenoside Rh<sub>1</sub> and PPT (22) in learning and memory. In the present study, we investigated the potential pharmacological effects of ginsenoside Rh<sub>1</sub> (Fig. 7).

In fact, a relatively higher dose of Rh<sub>1</sub> is required for optimal memory and learning in a water maze task. However, we observed a trend for the enhancing effects on memory to be more pronounced in the group administered the higher dose vs. the group administered the lower dose, although there was

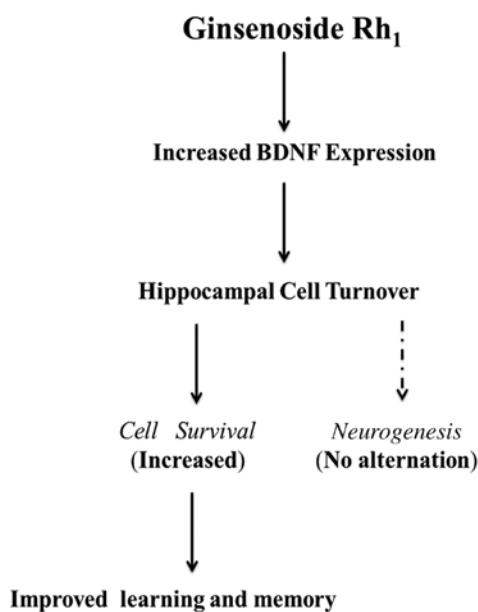


Figure 7. Schematic representation of Rh<sub>1</sub>-regulated potential mechanisms of action leading to improved learning and memory. Rh<sub>1</sub> promotes brain-derived neurotrophic factor (BDNF) expression in the hippocampus, resulting in hippocampal cell turnover in terms of increased cell survival.

no significant difference observed between the 2 Rh<sub>1</sub> treatment groups in the passive avoidance test.

The regulation of neurogenesis can occur at different stages, including cell proliferation, differentiation and survival. Several experimental methods have been conducted to investigate the role of adult hippocampal neurogenesis in learning and memory, such as low-dose brain irradiation (23,24), various types of stress (25) and methylazoxymethanol acetate treatment (8). These methods have been shown to significantly reduce neurogenesis, as well as hippocampal-dependent tasks, whereas these studies suggest some potential roles for neurogenesis in learning and memory. In addition, the decreased survival of proliferating cells in the hippocampus is associated with a decline in spatial memory, as observed in a previous study (26). Hence, to elucidate the memory enhancing effects of treatment with ginsenoside Rh<sub>1</sub>, we hypothesized that this treatment would have an impact on hippocampal neurogenesis. A significant increase in cell survival in the hippocampus was observed in the treated groups compared with the control group. By contrast, the number of BrdU-positive cells in the hippocampus did not differ between the treated groups and the control group, suggesting that cell proliferation at the time of BrdU injection was unaffected. Nonetheless, further studies are required to determine whether the treatment-induced increase in cell survival underlies the enhancement of memory and learning which was observed.

It is also possible that this increase was consistent with the mechanisms observed in previous studies, as in the central nervous system, BDNF regulates neuronal activity and is important for the positive selection and survival of functionally active neurons (27) and protects newborn neurons from death during the differentiation process from immature to mature neurons (28). In the present study, ginsenoside Rh<sub>1</sub> enhanced the survival of cells in the dentate gyrus following the increase in BDNF expression. In this perspective, this result may partly

explain the mechanisms by which ginsenoside Rh<sub>1</sub> improves the learning and memory process.

Apart from the neurogenesis factor, there are still other factors affecting the learning and memory process. A critical role for T cell-derived interleukin (IL)-4 in the regulation of learning and memory through the meningeal myeloid cell phenotype and BDNF expression has been indicated (29). Stress and corticosteroid hormones are known to affect learning and memory processes (30). Plasticity levels in the gray and white matter of the brain change during the learning process (31). The activation of cAMP-response element binding protein (CREB)-gene expression has a significant impact on memory (32).

On the other hand, Alzheimer's disease is a progressive neurologic disease that results in the irreversible loss of neurons, particularly in the cortex and hippocampus. Parkinson's disease is the second most common neurodegenerative disorder, after Alzheimer's disease. It is characterized pathologically by the loss of neurons. According to a previous study (33), ginsenoside Rg<sub>1</sub> significantly inhibits  $\beta$ -secretase activity *in vitro* and protects against A $\beta$ -induced cytotoxicity in PC12 cells. In our study, ginsenoside Rh<sub>1</sub>, a metabolite of the major ginsenoside Rg<sub>1</sub>, demonstrated great potential as a therapeutic agent by promoting cell survival.

In conclusion, the long-term administration of ginsenoside Rh<sub>1</sub> resulted in improved behavioral performance in hippocampal-dependent tasks. Although ginsenoside Rh<sub>1</sub> is able to promote cell survival in the dentate gyrus of the mouse hippocampus, it is likely that a combination of increased cell survival, as well as unknown factors, contribute to the enhanced performance induced by the long-term administration of ginsenoside Rh<sub>1</sub>. Further studies are required for the analysis of the differentiation of survived cells and other possible factors.

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