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Ginseng enhances contextual fear conditioning and neurogenesis in rats

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Abstract

Panax Ginseng is a commonly used galenical known to have an enhancing effect on learning. Neurogenesis in the hippocampus has been shown to be necessary for hippocampus/amygdala-dependent learning tasks. To investigate the role of Ginseng in neurogenesis and learning of rats, we administered both Ginseng and BrdU for five consecutive days. As a result, Ginseng increased the number of BrdU-positive cells in the dentate gyrus in a dose-dependent manner. Further, we administered one dose of BrdU after Ginseng treatment for five consecutive days, and the number of BrdU-positive cells did not increase significantly. However, when one dose of BrdU was given 1 day before the following five consecutive days of Ginseng treatment, the number of BrdU-positive cells markedly increased in the hippocampus. Therefore, it is likely that Ginseng enhances not proliferation but survival of newly generated neurons in the hippocampus. Second, we administered both Ginseng and BrdU to rats for five consecutive days. One day after the last Ginseng and BrdU co-administration, contextual fear conditioning (CFC) was conducted. Ginseng in a dose-dependent manner increased the % freezing time and the number of BrdU-positive cells in the dentate gyrus of rats that received CFC. Thus, an increase in CFC-related neurogenesis may be one mechanism of Ginseng's properties to enhance learning ability.

Keywords: Ginseng; Neurogenesis; Learning; Hippocampus; Dentate gyrus; Contextual fear conditioning

1. Introduction

The Ginseng root (Panax Ginseng) is a common constituent of a large number of traditional oriental medicines. Among its diverse effects on the central nervous system, Ginseng is known to improve learning and memory. Although some of the early studies reported that Ginseng extracts caused learning impairment rather than improvement (Saito et al., 1977, 1979), subsequent studies showed that Ginseng extracts improve performance in active and passive avoidance learning tasks (Lasarova et al., 1987; Petkov et al., 1990, 1992, 1993). This discrepancy may be due to the sedative effect of Ginseng (Koo, 1999), which is observed with acute administration of Ginseng. Those that reported memory impairment by Ginseng examined the acute, but not chronic effects of Ginseng.

Chronic administration of Ginseng extracts or some of its fractions is known to improve learning and memory in several different hippocampus/amygdala-dependent behavioral tasks (Chang et al., 1998; Jaenicke et al., 1991; Jin et al., 1999; Lyubimov et al., 1997; Ni et al., 1993; Nitta et al., 1995a, 1995b; Watanabe et al., 1990; Wen et al., 1996; Yoshimura et al., 1998; Zhao and McDaniel, 1998; Zhong et al., 1998). However, the molecular and cellular mechanisms by which these agents exert behavioral effects remain to be explored.

In recent years, neurogenesis in the subgranular layer of the hippocampus (Gould et al., 1999b) and subsequent

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enhancement of neurotract connections (Nakagawa et al., 2002b) have drawn attention as one of the molecular biological mechanisms of hippocampus/amygdala-dependent learning. The hippocampus is one of few brain regions where production of neurons occurs throughout the lifetime of animals, including humans. Gould et al. (1999a) reported that improvement of a hippocampus-dependent task, such as a spatial learning test, was associated with increase in survival of bromodeoxyuridine (BrdU)-positive cells in rats.

To clarify the mechanism of learning enhancement by Ginseng, we investigated the effects of Ginseng on contextual fear conditioning (CFC), a hippocampus/amygdala-dependent learning task, and on neurogenesis in the hippocampus of rats. Prior to this investigation, we examined the effect of Ginseng on the baseline number of BrdU-positive cells in the hippocampus. For analysis of the phenotype of BrdU-positive cells, double staining with BrdU, a thymidine analog that labels dividing cells in S-phase, and NeuN, a neuronal marker, was used.

2. Materials and methods

2.1. Animals and Ginseng treatment

All experiments were conducted using 60 adult male Wister/ST rats (SLC Japan, 9 weeks old, weighing 270–290 g). They were group-housed (5 rats/group, 12 h light/dark cycle) with ad libitum access to food and water. The animals were treated in accordance with the Guidelines for Animal Experimentation (of the Ethics Review Committee) of the Faculty of Medicine, University of Yamanashi.

For the first experiment, Ginseng powder (supplied by Tsumura Pharmaceutical Co., Tokyo, Japan) was administered orally at doses of 0, 100, and 200 mg/kg/day for five successive days via a gastric tube. Dried Ginseng powder was suspended in tap water just before the use. The group denoted as 0 mg/kg/day received water only. Although in some previous reports Ginseng was administered by intraperitoneal (Mook-Jung et al., 2001) or intracerebroventricular injection (Kim et al., 1998), we considered that oral administration of Ginseng was crucial, because in traditional medicine Ginseng has always been taken orally. In addition, no data are available regarding the disposition of Ginseng in the body, such as absorption and metabolism, and more importantly, biologically active forms are not well identified. Some herbs are known to be metabolized into active forms by intestinal bacteria and then absorbed to exert pharmacological actions (Hasegawa et al., 1996). Thus, non-oral administration of Ginseng may result in non-pharmacological artifacts.

2.2. Contextual fear conditioning

CFC was conducted according to the method of Silva et al. (1998). The CFC task was performed in a conditioning chamber housed in a sound-attenuating box during the light phase of the cycle. The conditioning chamber (28 cm (width) × 21 cm (height) × 22 cm (diameter)) was constructed of clear Plexiglas. The floor of the chamber was lined with 18 stainless steel bars (4 mm in diameter; 1.5 cm spacing), which formed a foot shock grid to deliver scrambled shocks produced by a stimulator. The foot shock was 2 s direct current of 0.75 mA and served as the aversive unconditioned stimulus. The sound-attenuating box (48 cm (width) × 48 cm (height) × 48 cm (diameter)) was provided with a 20 W houselight, and a ventilation fan supplying background white noise (74 dB) was located on the top of the box. A discrete tone conditioned stimulus (CS) was given as a general contextual stimulus. The tone cue (800 Hz, 20 s duration, 80 dB) was delivered by two speakers located in the lower corner of the sound-attenuating box.

Prior to the conditioning, all rats received 3 days of habituation, in which they were placed in the conditioning chamber for 1 min and returned to their home cages once a day. On the day of conditioning, the rats were placed in the conditioning chamber and allowed to explore for 3 min. A foot shock was delivered 18 s after the tone CS. The rats were then allowed to recover for 30 s in the conditioning chamber and returned to their home cages. Two hours later, the rats were again introduced into the conditioning chamber and were tested for a 5 min period, during which no tone CS was delivered. Behavior was evaluated in terms of total freezing time during a 5 min (% freezing time) stay in the conditioning chamber. Freezing behavior was defined as cessation of all but respiratory movement. The rats were sacrificed immediately after % freezing time was measured.

Rats that experienced footshock were allocated to the CFC group and rats that did not experience footshock to the no-CFC group. There were three subgroups in the CFC and no-CFC groups, each of which was treated with 0, 100, or 200 mg/kg/day Ginseng powder. There were five rats in each subgroup.

2.3. Open field locomotion test

To test whether % freezing time was influenced by the sedative effect of Ginseng or not, the open field locomotion test was performed at the end of the treatment period on the sixth day. This test was performed on the rats that were administered 0 or 200 mg/kg/day of Ginseng.

The open field is a 750 mm× 750 mm wooden arena, with 300 mm high walls surrounding the field, painted black on all inner surfaces. Thin white stripes are painted across the floor, dividing it into 25 quadratic blocks. The open field instrument was cleaned after each individual test session to prevent the next rat from being influenced by the odors deposited in the urine and feces of the previous rat.

The rat was placed in the area with its head pointing to a corner. An observer manually quantified the rat's spontaneous ambulatory locomotion in the horizontal plane by scoring the number of squares entered (crossing into a different adjacent section with all four extremities), during 5 min.

2.4. Immunostaining

On the sixth day, immediately after CFC performance, the rats were deeply anesthetized with sodium pentobarbital (50 mg/kg ip) and perfused transcardially with 350 ml of 4% paraformaldehyde in 0.1 M phosphate buffer. The rat brain was quickly removed, and then post-fixed for 24 h in paraformaldehyde. Then, 40 μm-thick frontal sections were cut on a cryostat and collected in PBS (0.1 M; pH 7.4). In accordance with the rat brain map of Paxinos and Watson (1986), 40 μm-thick free-floating sections were prepared, and 12 sections were collected at 160 μm intervals for staining. DNA denaturation was conducted by incubation for 30 min in 50% formamide/2 × SSC at 65 °C followed by several rinses in 2 × SSC. Sections were then incubated for 30 min in 2N HCl and 10 min in boric acid. After washing in PBS, sections were incubated in 3% H_2O_2 to block endogenous peroxidase for 10 min. After blocking with 10% normal goat serum (NGS), sections were incubated with anti-BrdU (1:1000 Haralan Sera Lab. OBT0030) for 24 h at 4 °C. Sections were then incubated for 1 h with secondary antibody (biotinylated goat, anti-rat IgG; Vector BA9400) followed by amplification with an avidin–biotin complex, before developing the color using DAB.

The same number of free-floating sections from the control and the Ginseng (200 mg/kg/day for 5 days)-treated animals ($n = 6$) surviving 4 weeks after administration of one dose of BrdU before Ginseng treatment was used for analysis of phenotypes. Double immunostaining procedures with fluorescent chromogens were used to evaluate the co-expression of BrdU with neuronal nuclear protein NeuN. In the double-labeling experiment, BrdU was visualized with Streptavidin-Texas Red (Amersham Pharmacia Biotech, 1:100), while the neuronal marker NeuN (Chemicon International Inc., MAB377; 1:500) was visualized with FITC (anti-mouse Ig, fluorescein-linked whole antibody 1:20). Fluorescent signals were viewed using a TCS4D confocal laser-scanning microscope. The emission signals of Texas Red and FITC were assigned to red and green, respectively.

2.5. Quantification of BrdU labeling

Every fourth section throughout the hippocampus was processed for BrdU immunohistochemical study. All BrdU-labeled cells in the dentate gyrus (granular cell layer) and the hilus were counted in each section. To distinguish single cells within clusters, all counts were performed at 400× and 1000× magnification under a light microscope (Olympus BX-60), omitting cells in the outermost focal plane.

A cell was counted as being in the subgranular zone (SGZ) of the dentate gyrus if it was touching or in the SGZ. Cells that were located more than two cells away from the SGZ were classified as hilar cells. The cell number was divided by the area of the dentate gyrus, and then the mean positive cell number per square millimeter was counted.

2.6. Protocol of BrdU administration

To investigate the overall effects of Ginseng on neurogenesis in the dentate gyrus of rats and its association with CFC performance, rats were orally administered both Ginseng (0, 100, 200 mg/kg/day) and BrdU (200 mg/kg/day) simultaneously for five consecutive days. On the sixth day, the animals were subjected to CFC test and then sacrificed for immunohistochemical study as described above (Fig. 1A).

BrdU is taken up into cells that are in the S-phase of DNA synthesis. The S-phase lasts for approximately 2 h (Packard et al., 1973). When one dose of BrdU is given, it is incorporated only into cells in the S-phase. At least one cell cycle is completed in 24 h by cells in the S-phase after the time of BrdU injection (Nowakowski et al., 1989). Therefore, if an animal is given one dose of BrdU and decapitated 2 h later for immunostaining, cells that have newly proliferated are observed. On the contrary, if an animal is given one dose of BrdU and is decapitated a few days later, cells that were produced a few days ago and are still surviving at the time of decapitation are observed. Accordingly, it is possible to differentiate newly proliferated cells from surviving cells by varying the time interval between BrdU administration and decapitation.

In our study, to determine the effects of Ginseng on cell survival, BrdU was given once to drug-naive rats, and Ginseng was given for the following 5 days. Then, the rats were sacrificed for immunohistochemical study (Fig. 1B). On the other hand, to determine the effects of Ginseng on cell proliferation, BrdU was given once on the sixth day, after Ginseng treatment for five consecutive days, and sacrificed 2 h after BrdU labeling (Fig. 1C).

2.7. Statistical analysis

All the results are presented as mean ± S.E.M. One-way analysis of variance (ANOVA) was used to examine the effects of CFC and Ginseng administration on % freezing time because of their interaction, since there was interaction between the two groups (Fig. 6). Two-way analysis of variance (ANOVA) was used to examine the effect of CFC and Ginseng on number of BrdU-positive cells in rats subjected to CFC (Fig. 2). As shown in Fig. 5, Student's *t*-test was used to determine whether the effect of Ginseng treatment on the number of BrdU-positive cells is related to cell proliferation or cell survival. Differences in the values of locomotion measured in the open field test were examined by Student's *t*-test.

A P-value < 0.05 was considered to indicate a statistically significant difference.

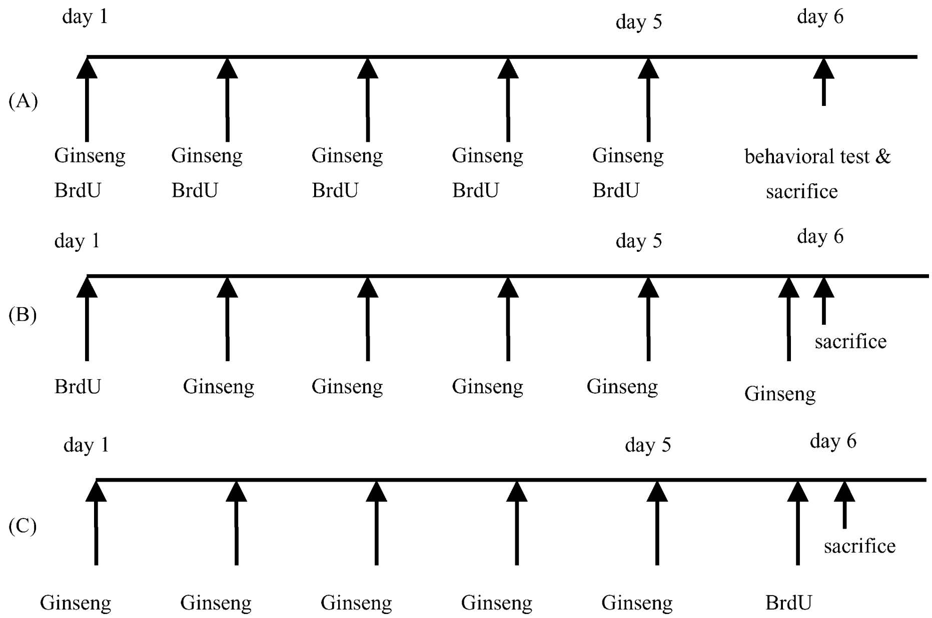

Fig. 1. Experimental procedures to examine effects of Ginseng on CFC-associated neurogenesis, cell survival and cell proliferation in rat hippocampus. Rats were orally administered both Ginseng (0, 100, 200 mg/kg/day) and BrdU (200 mg/kg/day) for five consecutive days. On the sixth day, the animals were subjected to CFC test (A). To determine the effects of Ginseng on cell survival, BrdU was given once to drug-naive rats, and Ginseng was given for the following 5 days. (B) To determine the effects of Ginseng on cell proliferation, BrdU was given once on the sixth day, after Ginseng treatment for five consecutive days, and sacrificed 2 h after BrdU labeling (C).

3. Results

3.1. Effect of Ginseng on baseline number of BrdU-positive cells

To test the effect of Ginseng on neurogenesis in rats, we counted the number of BrdU-positive cells in the dentate gyrus. The number of BrdU-positive cells increased in a dose-dependent manner in the Ginseng treatment group (Figs. 2A and 3).

3.2. Phenotype of BrdU-positive cells

The phenotype of the BrdU-positive cells in the granule cell layer was examined in the Ginseng group 4 weeks after BrdU labeling. We performed immunostaining for BrdU as

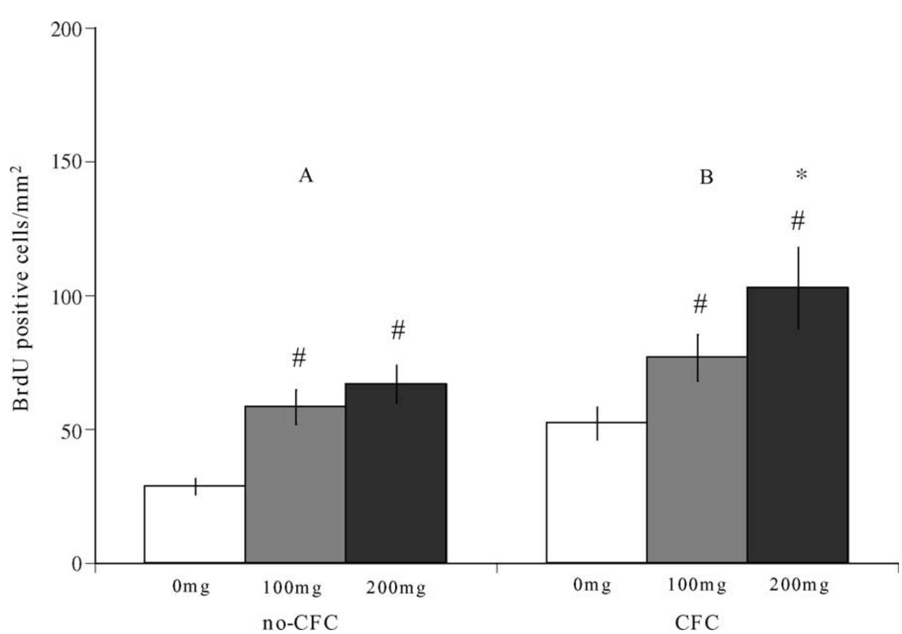

Fig. 2. Change in BrdU-positive cells in rat hippocampus after CFC performance. The number of BrdU-positive cells was higher in the CFC groups than in the no-CFC groups ($P = 0.0113$, $F_{1,24} = 13.718$). The number of BrdU-positive cells was significantly increased in a dose-dependent manner by Ginseng treatment, both in the no-CFC groups (A) and the CFC groups (B) ($P = 0.0001$, $F_{2,24} = 13.538$) (mean ± S.E.M.). (#) $P < 0.05$ vs. 0 mg; (*) $P < 0.05$ for no-CFC vs. CFC.

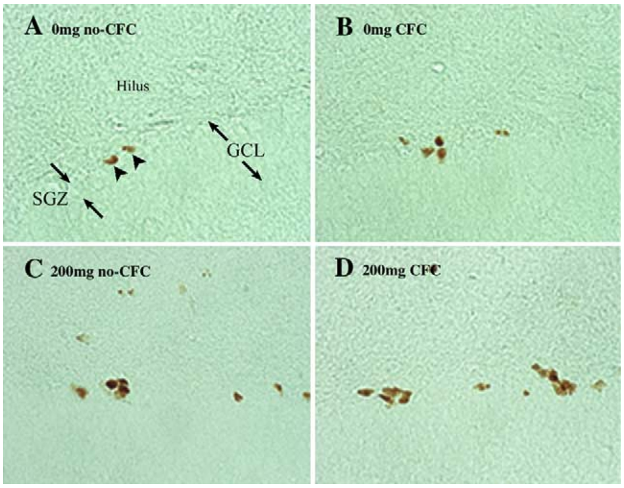

Fig. 3. Optical photomicrographs of changes in number of BrdU-positive cells in the hippocampus after CFC (40× magnification) BrdU-positive cells (arrowhead) were increased in the CFC groups (B and D) compared to the no-CFC groups (A and C). BrdU-positive cells were increased in a dose-dependent manner by Ginseng, in both the CFC groups and no-CFC groups. (A) Ginseng 0 mg and no-CFC; (B) Ginseng 0 mg and CFC; (C) Ginseng 200 mg and no-CFC; (D) Ginseng 200 mg and CFC. The majority of BrdU-positive cells were located in the subgranular zone (SGZ) of the hippocampus—the region between the granular cells layer (GCL) and hilus.

well as for NeuN, a marker for neurons (Fig. 4). It was found that ~80% of BrdU-positive cells expressed NeuN, and no significant difference existed in % NeuN-positive cells regardless of whether Ginseng was administered or not (data not shown).

3.3. Effect of Ginseng on cell survival

The increased neurogenesis may have been due to an increase in cell survival and/or cell proliferation. To investigate the effect of Ginseng specifically on cell survival, one dose of BrdU was given orally to the rats, followed by 5 days' administration of Ginseng (200 mg/kg/day). As a result, Ginseng did significantly increase the number of BrdU-positive cells. This finding showed that Ginseng enhanced the survival rate of newly generated cells in the hippocampus (see Fig. 5A).

3.4. Effect of Ginseng on cell proliferation

On the other hand, in order to investigate the effect of Ginseng on cell proliferation, Ginseng (200 mg/kg/day) was given for 5 days, and then one dose of BrdU was given to the rats 2 h before they were sacrificed for immunostaining. With this administration protocol, the number of BrdU-positive cells did not increase significantly in the dentate gyrus. This result suggested that the Ginseng employed in our experiment did not induce a significant change in cell proliferation in the hippocampus (see Fig. 5B).

3.5. Effect of Ginseng on % freezing time in CFC test and CFC-associated increase in BrdU-positive cells

As shown in Fig. 6A, in the rats that were not given foot shocks in the conditioning chamber (no-CFC group), Ginseng administration did not change % freezing time at any dose. CFC itself increased % freezing time. In the rat groups that received CFC (CFC groups), Ginseng treatment increased % freezing time in a dose-dependent manner (see Fig. 6B). Ginseng at 200 mg/kg significantly increased % freezing time as compared to Ginseng at 0 or 100 mg/kg. This result shows that Ginseng significantly enhances the performance of rats in the CFC, a hippocampus/amygdala-dependent learning task.

When Ginseng and BrdU were co-administered for five consecutive days, the number of BrdU-positive cells increased in a dose-dependent manner in the CFC groups, and the increase was significant at a dose of Ginseng of 200 mg/kg as compared to doses of 0 and 100 mg/kg (Figs. 2B and 3). CFC also increased the number of BrdU-positive cells as compared to the no-CFC groups, even when saline was co-administered with BrdU for 5 days (Figs. 2 and 3).

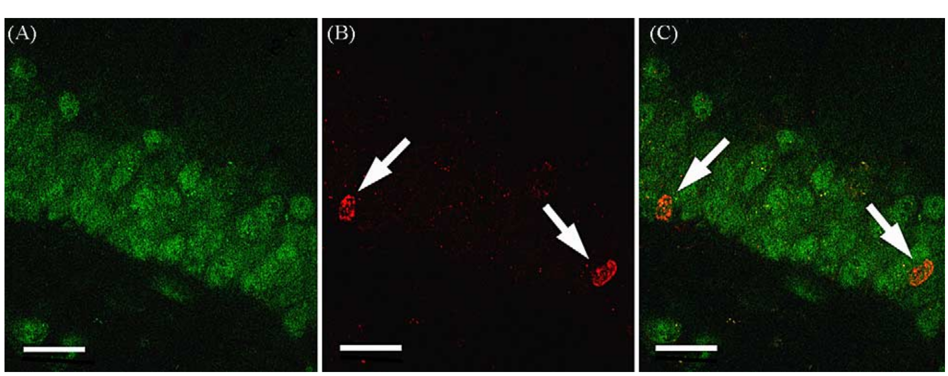

Fig. 4. Double immunolabeling of NeuN and BrdU in adult dentate gyrus of Ginseng-treated rats. Double immunolabeling of NeuN (green, A), a marker of mature neurons, and BrdU (red, B) in the adult dentate gyrus of Ginseng-treated rats surviving 4 weeks after BrdU administration. Confocal images (630× magnification) show NeuN immunoreactivity in BrdU-labeled cells (arrows, C) in both Ginseng-treated and non-treated animals. Scale bar: 20 μm.

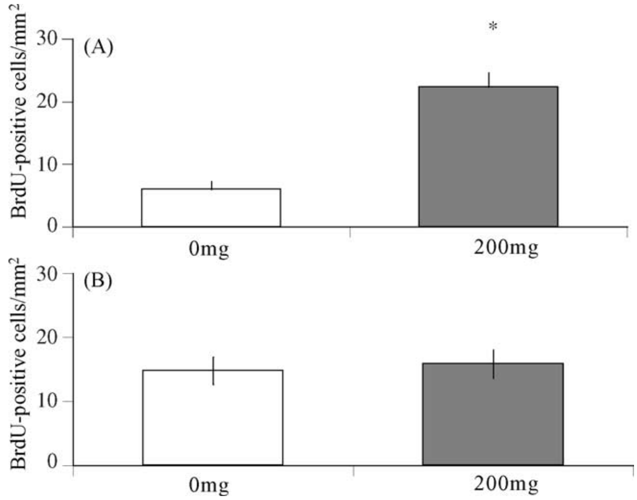

Fig. 5. Effects of Ginseng on BrdU-positive cells in rat hippocampus administration of Ginseng for five consecutive days after one treatment with BrdU significantly increased BrdU-positive cells compared to control rats ($n = 5$, $^{*}P = 0.0003$) (A). Administration of Ginseng for five consecutive days followed by one treatment with BrdU 24 h later caused no significant change in BrdU-positive cells compared to control rats ($n = 5$, $P = 0.7341$) (B).

3.6. *Effect of Ginseng on open field locomotion*

To examine whether % freezing time was influenced by the sedative effect of Ginseng, the open field test was performed. Total locomotion distance of the group treated with Ginseng 200 mg/kg/day for five consecutive days was not significantly different compared to the no Ginseng group (data not shown). There was no significant difference in grooming and rearing either (data not shown). This result indicates that Ginseng at 200 mg/kg did not cause any difference in spontaneous activity or locomotion compared with the non-treated rats.

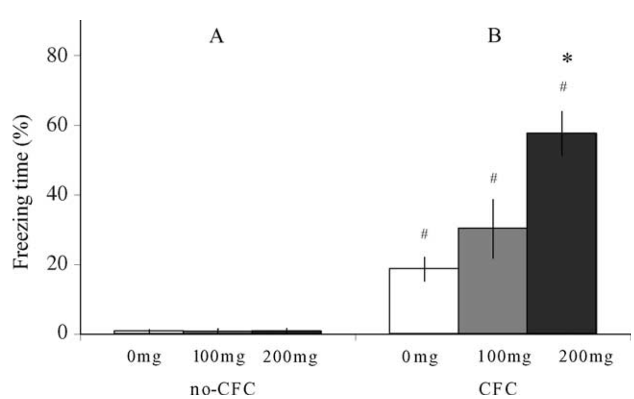

Fig. 6. Effects of Ginseng on % freezing time of rats tested in a conditioning chamber 1 day after finishing administration of Ginseng with BrdU for five consecutive days. Data are shown as mean ± S.E.M. There was a significant difference in % freezing time among the tested groups ($F_{5,24} = 25.312$, $P < 0.007$). (#) The 0, 100, and 200 mg Ginseng groups showed a significantly higher % freezing time in the CFC group than the no-CFC group, respectively. (*) The 200 mg/kg Ginseng group showed the highest % freezing time out of all the CFC groups (B). In contrast, the no-CFC groups did not show any change in % freezing time (A).

4. Discussion

Ginseng administration increased the number of BrdU-positive cells of the dentate gyrus in the no-CFC groups in a dose-dependent manner. Double staining with BrdU and NeuN suggested that the increase in BrdU-labeled cells may be mainly based on an increase in neurogenesis, and not gliogenesis.

Neurogenesis, defined as the creation of new nerve cells, consists of a series of distinct developmental steps, two of which can be examined separately; proliferation and survival/differentiation (Malberg et al., 2000). Our study suggested that the increased number of BrdU-positive cells induced by Ginseng was due to an increase in cell survival, and not cell proliferation.

We also found enhancing effects of Ginseng on performance in CFC, which is hippocampus/amygdala-dependent learning, as well as on neurogenesis in the dentate gyrus in the CFC groups. The unaltered result of the open field test in the Ginseng group shows that Ginseng causes neither a stimulating nor sedating effect at the doses employed in this study, which could potentially interfere with evaluation of CFC. Another interfering factor in evaluation of CFC is that Ginseng's effect on pain sensitivity. Since there is no previous data to suggest this effect, the possibility is low, but still remains to be examined.

Ginseng increased the CFC-related increase in the number of BrdU-positive cells in a dose-dependent manner. Although in this study we did not examine whether this CFC-related increase in the number of BrdU-positive cells was due to increased cell proliferation or cell survival, a previous study suggested that learning was associated with enhancement of cell survival but not cell proliferation (Fulder, 1981; Gould et al., 1999a), although it remains to be answered whether Ginseng and CFC have an additive or interactive effect on neurogenesis. It is reasonable to conclude that the increase in CFC-related neurogenesis may be one mechanism of Ginseng's property to enhance learning ability.

What are the molecular mechanisms underlying the regulation of hippocampal neurogenesis by Ginseng? Ginseng root consists of two major constituents: crude Ginseng saponin and crude Ginseng non-saponin fractions. To date, more than 20 saponins have been isolated from Ginseng root and identified chemically (Lim et al., 1997). Ginsenosides (the saponin constituents of Ginseng root) have been reported to have a number of actions on the CNS. These include CNS stimulation or depression (Watanabe et al., 1990), anticonvulsant activity (Gupta et al., 2001), anti-psychotic activity (Yoshimura et al., 1998), anti-fatigue (Wang et al., 1983) and anti-stress activity (Fulder, 1981; Kim et al., 1998), and improvement of performance in various memory paradigms (Jin et al., 1999; Ni et al., 1993; Nitta et al., 1995a).

The beneficial effects of Ginseng on learning and memory have often been attributed to ginsenoside Rb1 and Rg1 (Mook-Jung et al., 2001). Ginsenoside Rg1 increases cAMP level and c-fos gene expression in the rat hippocampus (Liu and Zhang, 1996). The elevation of intracellular cAMP level induces c-fos expression (Vaccarino et al., 1993). The cAMP–CREB cascade could contribute to the actions of neurotransmitters and neurotrophic factors on adult neurogenesis (Nakagawa et al., 2002a). In recent reports, CREB was shown to be necessary for both steps of neurogenesis: proliferation and cell survival (Nakagawa et al., 2002b). Since in our study, Ginseng did not enhance cell proliferation, activation of the cAMP-CREB cascade by Ginseng could not solely explain our findings.

Alternatively, the ginsenosides Rb1 and Rg1 (Mook-Jung et al., 2001) are also thought to enhance learning and memory by facilitation of cholinergic function, which is apparently essential for the functional integration of learning processes. For example, Rb1 facilitated acetylcholine (ACh) release and improved passive avoidance learning (Benishin et al., 1991). Rb1 and Rg1 increased the number of ACh receptors and improved passive avoidance learning in anisodine-treated mice (Shan et al., 2002). Rg1 improved the performance of scopolamine-injected rats in an eight-arm radial maze task. Consistent with these results, Rb1 facilitated choline uptake and increased choline acetyltransferase (Salim et al., 1997). ACh is also shown to increase neurogenesis (Ma et al., 2000). Thus, the increase of neurogenesis by Ginseng may be mediated via an increase in ACh release and ACh receptors.

In conclusion, Ginseng had an enhancing effect on CFC and increased the number of BrdU-positive cells in the dentate gyrus. The increase of neurogenesis by Ginseng was due to enhancement of cell survival, and not proliferation. Future study will be required to determine the components of Ginseng that are responsible for the enhancement of CFC and neurogenesis. Elucidation of the exact components and their mechanisms will lead to novel drugs for the treatment of memory impairment.

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