

## Effects of ginsenoside Rg<sub>1</sub> on synaptic plasticity of freely moving rats and its mechanism of action<sup>1</sup>

Rg<sub>1</sub> A

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**KEY WORDS** ginseng; saponins; long-term potentiation; synapses; dentate gyrus; GAP-43 protein

### ABSTRACT

**AIM:** To investigate the effect and mechanism of ginsenoside Rg<sub>1</sub> on synaptic plasticity of freely moving rats. **METHODS:** SD rats were chronically implanted with a stimulation electrode in the perforant path (PP) of hippocampus and a recording electrode in the granule cell of dentate gyrus. After administration of ginsenoside Rg<sub>1</sub> (10, 30 mg/kg, ip) for 12 d, extracellular recording technique was used to record the population spike (PS). Mossy fiber (MF) sprouting was measured using Timm's staining, and an immunohistochemical technique was used to detect the expression of presynaptic growth-associated protein 43 (GAP-43). **RESULTS:** Rg<sub>1</sub> could significantly increase the sensitivity of evoking PS, the amplitude of PS and induce PP-DG long-term potentiation (LTP) in the dentate gyrus (DG) of freely moving rats. In the meantime, Rg<sub>1</sub> accelerated MF sprouting in CA3 cell field of hippocampus. The expression level of GAP-43 was elevated in granule cell layer and hilus of DG of Rg<sub>1</sub>-treated rats. **CONCLUSION:** The increased synaptic plasticity may attribute to the increased expression of GAP-43 in granule cell layer of DG and the onset of the sprouting of granule cell axon-MF. The MF sprouting accelerated the synaptic transmission in positive-feedback. Their interaction and the synergism is part of the mechanisms underlying the nootropic effect of Rg<sub>1</sub>.

### INTRODUCTION

*Panax ginseng* has long been used as a tonic in tra-

ditional Chinese medicine. A lot of literatures indicated that ginsenoside Rg<sub>1</sub> exhibited various pharmacological actions on the central nervous system (CNS), for example, Rg<sub>1</sub> improved acquisition, consolidation, and retrieval of memory impaired by amnesic agents and increased protein synthesis and cAMP level of brain<sup>[1-3]</sup>. In our previous studies, Rg<sub>1</sub> was shown to ameliorate spatial learning and memory impairment induced by aggregated  $\beta$ -amyloid peptide<sup>[4]</sup>. Elucidation of the physiological basis of learning is among the greatest remaining challenge of the neurosciences. To investigate further the action of ginsenoside Rg<sub>1</sub> on the CNS, the effect of crude ginseng saponins on basal synaptic transmission and long-term potentiation (LTP) evoked potential in the dentate gyrus of anesthetized rats was detected in our laboratory<sup>[5]</sup>. On the basis of the previous practice, the effect of Rg<sub>1</sub> on synaptic transmission in the hippocampal dentate gyrus of freely moving rats and its possible mechanism was studied.

### MATERIALS AND METHODS

**Rats** Male Sprague-Dawley rats (190 g  $\pm$  10 g), from the Center of Experimental Animals, Chinese Academy of Medical Sciences (Grade II, Certificate No SCXK 11-00-0006) were fed lab chow and water *ad libitum* and housed (20-24 °C) under a 12-h light/dark cycle.

**Drugs and reagent** Rg<sub>1</sub> supplied by Department of Basal Organic Chemistry, Norman Bethune Medical University was dissolved with 0.9 % NaCl solution; the ingredients of Timm's staining solution; 50 % gum arabic (60 mL, w/v), 2.55 g citric acid in 25 mL aqueous solution, 2.35 g sodium citrate, and 0.85 g hydroquinone; 0.12 g silver lactate in 15 mL aqueous solution was added in darkness just before use.

Anti-GAP-43 antibody (sc-7457, Santa Cruz Biotechnology, Inc, USA) was diluted 1:75 with 0.9 % NaCl solution (4 °C) just before use.

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**Surgical procedure** The rats were anaesthetized with urethane carbamate (1.5 g/kg, ip) in all experiment, and mounted in a stereotaxic frame. The scalp was incised and retracted, and the head position was adjusted to place bregma and lambda in the same horizontal plane. Two small burr holes (1.5 mm diameter) were drilled in the skull for the placement of a monopolar recording electrode and a bipolar stimulating electrode. They were sequentially made 3.8 mm and 7.5 mm posterior to bregma, and 2.5 mm and 4.2 mm lateral to the mid-line, respectively. The recording and stimulating electrode were made from teflon coated stainless steel wires (0.1 mm diameter). The bipolar stimulating electrode was stereotaxically placed in the left entorhinal cortex to stimulate the perforant path, and the evoked potential was extracellularly recorded from the dentate gyrus granule cell layer of ipsilateral. Electrodes were slowly lowered to a depth of 2.5 mm, the final depths were adjusted until maximal extra-cellular population spike were obtained, then fixed to the bone with acrylic dental cement. After these steps, the leads of the electrodes were connected to a head stage that was permanently affixed to the rat's skull.

Rats were housed individually on a 12-h light/dark cycle with food and water available *ad libitum*, and tested during the light cycle.

**Electrophysiological recording**<sup>[6,7]</sup> The rats were allowed 5 d to recover from surgery before experiments. From d 6, responses were evoked by stimulating at low frequency (0.33 Hz, duration 200  $\mu$ s), ten evoked responses were averaged as the efficacy of synaptic transmission every day. The baseline population spike (PS) amplitude was monitored daily during a 25 – 40 min period for at least 3 d or until the responses were stable, besides, the PS thresholds were recorded every time. Ginsenoside Rg<sub>1</sub> (10, 30 mg/kg, ip) or 0.9 % saline solution was administrated to rats for 12 d beginning from d 9 after the surgery, PS amplitudes and thresholds were recorded daily as mentioned above till rats were withdrew Rg<sub>1</sub> for 3 d.

**Timm's staining**<sup>[8]</sup> After treatment with ginsenoside Rg<sub>1</sub> (10 and 30 mg/kg, ip), and 0.9 % saline solution for 12 d. SD rats were anesthetized with urethane carbamate (1.5 g/kg, ip), and perfused with a sodium sulphide solution (0.1 % Na<sub>2</sub>S in phosphate buffered saline 0.1 mol/L, pH 7.4) at a flow rate of 35 mL/min for 3 min, and then perfused with 3 % glutaraldehyde for

250 mL, after decapitation, the brains were taken out and immediately put into 3 % glutaraldehyde for 1 h, then transferred into 30 % sucrose solution, when the tissue has sunk to the bottom of the container, it was frozen at -20 °C. Coronal sections (15  $\mu$ m) were cut on a cryostat and thawed onto APES-coated glass slides. After drying, the sections were immersed in a 26 °C Timm's solution (avoid light). After stained for 70 min, the sections were rinsed in 40 °C tap water for 20 min, then lightly counterstained with neutral red and coverlipped.

**Immunohistochemistry**<sup>[9,10]</sup> SD rats were treated with ginsenoside Rg<sub>1</sub> (10 and 30 mg/kg, ip) as mentioned above, and perfused with PBS 100 mol/L (pH 7.4) followed by 4.0 % paraformaldehyde in PBS, then the brains were taken out and postfixed an additional 24 h in 4.0 % paraformaldehyde/PBS, coronal paraffin-embedded sections of thickness 15  $\mu$ m were cut and mounted on APES-coated slides. Paraffin sections were processed for immunohistochemistry of GAP-43 according to standard procedures. Endogenous peroxidase was blocked by 20 min incubation in 3 % hydrogen peroxide. Free-floating tissue sections were preblocked with normal rabbit serum for 10 min, then the sections were incubated with anti-GAP-43 antibody for 16 h at 4 °C. Primary antibody was detected with the ABC procedure. The sections were incubated in biotinylated secondary antibody for 30 min, and after washing in PBS, sections were incubated in avidin-biotin-peroxidase complex solution for 30 min, finally, incubated in DBA (3,3'-diaminobenzidine tetrahydrochloride) for 15 min. The reactions was stopped by washing in an excess of PBS, then dehydrated in a graded ethanol series, and delipidated in xylene and coverslipped using gelatin.

**Data collection and statistical analysis** Every day, 10 records of evoked responses were averaged. The values for the amplitude of PS after administration of Rg<sub>1</sub> were expressed as % deviation from the baseline (100 %) and  $x \pm s$ . Data were analyzed by conducting within-animal comparisons using the paired two-tailed *t*-test for average values, and  $P < 0.05$  were considered to be statistically significant.

The greyscale values of GAP-43 staining and Timm's staining were detected by image analysis. Greyscale values of control and ginsenoside Rg<sub>1</sub>-treated groups were expressed as  $x \pm s$ . Data were determined by *t*-test, and  $P < 0.05$  were considered to be statistically significant.

## RESULTS

The rats were healthy after surgery and 4 d later appeared to suffer no discomfort from carrying the extra objects on their heads. The evoked potential waveforms showed that stimulating and recording electrodes were in the correct position.

**PS threshold** The freely moving rats's PS thresholds were ( $226 \pm 70$ )  $\mu\text{A}$ , which was significantly decrease ( $P < 0.01$ ) comparing with anesthetized rats's ( $453 \pm 100$ )  $\mu\text{A}$ . After treatment with ginsenoside  $\text{Rg}_1$  for 4 d, the PS thresholds decreased to ( $134 \pm 40$ )  $\mu\text{A}$ , ( $P < 0.05$ ), and decreased further to ( $69 \pm 10$ )  $\mu\text{A}$  ( $P < 0.01$ ) at the final day. However, the PS thresholds of control group remained unaffected during the experiment. The result showed that  $\text{Rg}_1$  could significantly increase the sensitivity of evoking PS in the dentate gyrus of freely moving rats.

**PS amplitude** Evoked responses recorded in the granule cell of DG were shown in Fig 1.  $\text{Rg}_1$ -treated rats began to show a significantly increase in the PS amplitude of freely moving rats at d 5 (10 mg/kg) and d 4 (30 mg/kg), the peak of PS amplitudes were kept in the steady level at d 7 and d 8 respectively, when  $\text{Rg}_1$  was withdrawn 3 d later, the PS amplitudes were not significantly decreased.

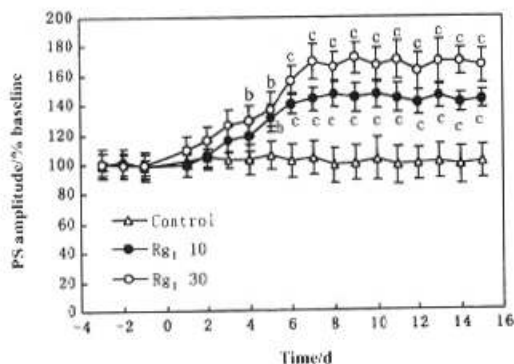


Fig 1. The effect of ginsenoside  $\text{Rg}_1$  on the basic PS amplitude evoked in the dentate gyrus of freely moving rats. Sprague-Dawley rats were treated with  $\text{Rg}_1$  (mg/kg, ip) or 0.9 % saline solution for 12 d.  $n = 5$ .  $x \pm s$ .  $^b P < 0.05$ ,  $^c P < 0.01$  vs baseline.

The result was consistent with our earlier studies on effect of ginsenoside  $\text{Rg}_1$  on synaptic transmission of anesthetized rats, ie,  $\text{Rg}_1$  could induce LTP in granule cell of dentate gyrus of freely moving rats either. The

PS amplitude of control group remain unaffected during the period.

**GAP-43 immunoreactivity** In rat hippocampus, GAP-43/B-50, an axonal growth-associated protein, is highly expressed in pyramidal cells, but is absent in granule cells. As shown in Fig 2, there were little GAP-43 immunoreactivity in the granule cell of dentate gyrus of control group, that was coincidence with the literature. But after administration of ginsenoside  $\text{Rg}_1$  for 12 d, the deep stained granule cell layer and the increased density were seen in granule field of DG.

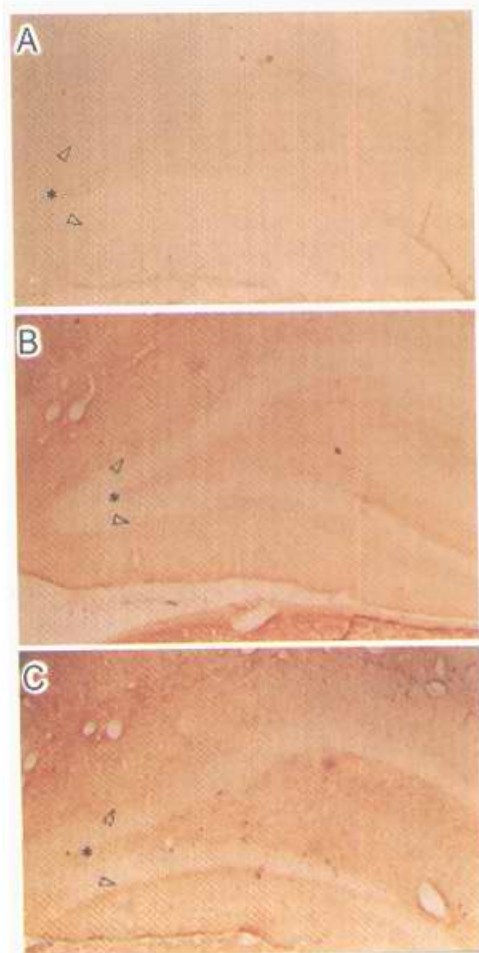


Fig 2. GAP-43 immunoreactivity in the granule cell and the hilus of dentate gyrus in 0.9 % saline solution (A) and ginsenoside  $\text{Rg}_1$  10 mg/kg (B) and 30 mg/kg (C) groups after a 12-d treatment. The arrows indicate the GAP-43 expression at granule cell, and the asterisks indicate the expression of GAP-43 at hilus of DG.  $\times 60$ .

GAP-43 immunoreactivity in the dentate gyrus was quantified by image analysis, the quantitative analysis was shown in Fig 3 that the greyscale values of GAP-43 protein expression in dentate gyrus were significantly increased. The immunoreactivity in the hilus of dentate gyrus was also significantly increased (as asterisk indicated).

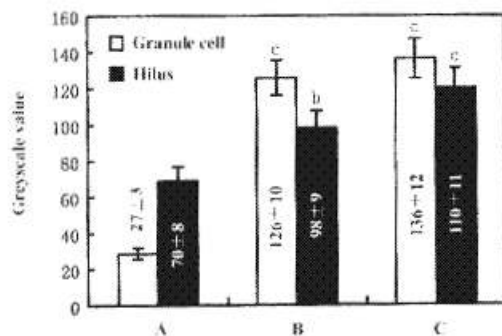


Fig 3. Effect of ginsenoside Rg<sub>1</sub> on the expression of GAP-43 in the dentate granule cell layer and hilus of dentate gyrus. A: Control group. B: Rg<sub>1</sub> 10 mg/kg. C: Rg<sub>1</sub> 30 mg/kg.  $n = 6$ .  $\bar{x} \pm s$ . \* $P < 0.05$ , \*\* $P < 0.01$  vs control.

**MF sprouting** MF from dentate granule cells contains zinc and therefore it is stained black in Timm's sulphide silver preparations. After rats administrated Rg<sub>1</sub> for 12 d, mossy fiber sprouting in the CA3 area of hippocampus was visualized clearly using Timm's staining. As Fig 4 indicated, comparing with control group, Rg<sub>1</sub> could dose-dependently induce the sprouting. Timm stained MF were distributed a dense band observed in CA3 area of Rg<sub>1</sub> (30 mg/kg)-treated rats, and relatively fewer staining was observed in Rg<sub>1</sub> (10 mg/kg)-treated rats. As Fig 5 indicated, the quantitative analysis revealed that greyscale values of Rg<sub>1</sub> (10, 30 mg/kg)-treated rats were significantly increased comparing with control group.

## DISCUSSION

LTP has been studying at several different levels, behavioral, cellular, and molecular, but the detailed mechanisms underlying LTP remain unclear up to now. The growth-associated protein GAP-43 (also being known as B-50, neuromodulin, F1, etc) is a presynaptic protein. Its expression is largely restricted to the nervous system. The high expression of protein GAP-43 is associated with axonal growth, regeneration, and establish-

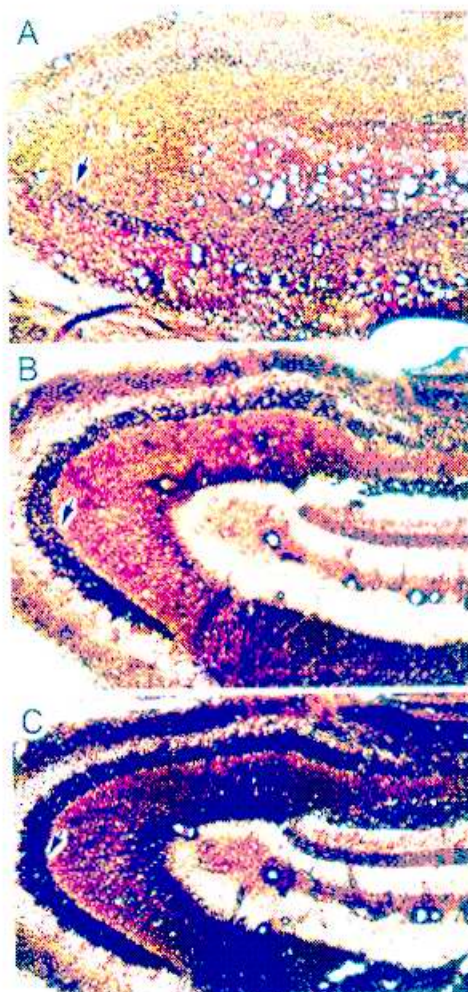


Fig 4. Timm stained coronal sections of rats hippocampus in 0.9% saline solution (A) and ginsenoside Rg<sub>1</sub> 10 mg/kg (B) and 30 mg/kg (C) groups after a 12-d treatment. The arrows indicate the mossy fiber sprouting at CA3 of hippocampus.  $\times 60$ .

ment of synaptic contacts<sup>[1]</sup>. In rat hippocampus, GAP-43 is highly expressed in pyramidal cells, but is absent in granule cells. In the present study, there was no GAP-43 immunoreactivity observed in the granule cell of dentate gyrus of control group, the result was consistent with the literature. After administration of ginsenoside Rg<sub>1</sub> for 12 d, the deep stained granule cell and the increased density indicated that Rg<sub>1</sub> could significantly induce the expression of GAP-43 in granule cell layer of DG.

GAP-43 plays a key role in synaptic plasticity and

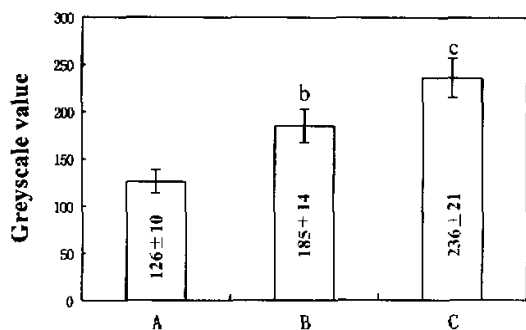


Fig 5. Effect of ginsenoside Rg<sub>1</sub> on the mossy fiber sprouting in CA3 field of hippocampus. A: Control group. B: Rg<sub>1</sub> 10 mg/kg. C: Rg<sub>1</sub> 30 mg/kg. n = 6, x ± s. <sup>b</sup>P < 0.05, <sup>c</sup>P < 0.01 vs control.

membrane extension, it can be membrane associated and binds CaM under ambient Ca<sup>2+</sup>-concentration. GAP-43 has been implicated the regulation of neurotransmitter release. Therefore, the effect of Rg<sub>1</sub> on increasing the expression level of GAP-43 could trigger a series of reaction simultaneously.

The present results indicate that Rg<sub>1</sub> can increase the MF sprouting, the newly sprouted MF may form a local excitatory feedback circuit on the granule cells and improve the synaptic transmission. This may be one of the mechanisms of Rg<sub>1</sub> in enhancing synaptic plasticity. On the other hand, several lines of evidence support the idea that GAP-43 might be involved in the regulation of MF sprouting. Rapid kindling stimulations and kainic acid induced seizures give rise to both GAP-43 mRNA expression and mossy fiber sprouting in adult animals. It is believed that the induced expression of GAP-43 in granule cells was well linked to MF sprouting.

In conclusion, the present findings showed that Rg<sub>1</sub> induced LTP in dentate gyrus of freely moving rats, and accelerated expression of GAP-43 and sprouting of mossy fibers in parallel. The elevated level of GAP-43 expression contributes to MF sprouting. These data can be considered as the basis for the elucidation of the nootropic effect of Rg<sub>1</sub>.

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## 人参皂甙 Rg<sub>1</sub> 对自由活动大鼠突触可塑性的影响及其作用机制<sup>1</sup>

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**关键词** 人参; 皂甙类; 长时程增强; 突触; 齿状回; GAP-43 蛋白质

**目的:** 研究人参皂甙 Rg<sub>1</sub> 对自由活动大鼠突触功能可塑性的影响及作用机制。 **方法:** 应用细胞外微电极记录技术, 大鼠埋植电极后 d 6 予以 Rg<sub>1</sub> (10, 30 mg/kg, ip) 12 d, 记录(直至停药后 d 3)其齿状回群体峰电位(PS)。大鼠给予 Rg<sub>1</sub> (10, 30 mg/kg, ip) 12 d, 据 Timm 染色法观察海马 CA3 区苔藓纤维出

芽情况。以免疫组化技术检测齿状回颗粒细胞层 GAP-43 表达水平。结果: 1) R<sub>g1</sub> 可显著降低诱发 PS 的阈值, 提高清醒自由活动大鼠的突触传递效能, 诱导 LTP 形成, 停药 3 d 后, LTP 仍可维持。2) R<sub>g1</sub> 组大鼠齿状回颗粒细胞层及齿状回门区 GAP-43 表达显著增加。3) Timm 染色显示海马 CA3 区苔藓纤维出芽增加。结论: R<sub>g1</sub> 可使自由活动大鼠

PP-DG 突触传递效能发生以 LTP 为主的可塑性变化, 其机制为齿状回颗粒细胞 GAP-43 表达增加, 其投射靶区海马 CA3 苔藓纤维明显出芽增加, 这呈正反馈性增强了突触传递效能。

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