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Ginsenosides Rb1 and Rg1 Decrease Proliferation but Increase Neuronal Differentiation of Hippocampal Neural Progenitor Cells

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진세노사이드 Rb1과 Rg1에 의한 해마 신경전구세포의 분화 증가

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ABSTRACT : Ginseng is the best known and most popular herbal medicine used worldwide. In spite of reported beneficial effects of ginseng on the CNS, there is few scientific evidences established at the cellular level. Among more than 30 ginsenosides, Rb1 and Rg1, the active ingredients of ginseng, are regarded as the main compounds responsible for many pharmaceutical actions of ginseng. Daily treatment with Rb1 or Rg1 for 3 d significantly decreased the number of bromodeoxyuridine(BrdU)(+) cells in primary neural progenitor cells(NPCs) isolated from hippocampi at embryonic day 16.5(E16.5). In contrast, treatment with Rb1 or Rg1 greatly increased the number of microtubule associated protein(MAP2) (+) cells. In addition, the transcription factors, *Ngn*1 and *Hes*1, proneural members of the basic helix-loop-helix(bHLH) family, significantly increased in Rb1 or Rg1 treated-NPCs. Based on these results, we suggest for the first time that ginsenosides Rb1 and Rg1 decrease proliferation but promote neuronal differentiation of hippocampal NPCs.

Key words : Rat, Hippocampus, Ginsenosides, Rb1, Rg.

요 약 : 전세계적으로 널리 복용되고 있음에도 불구하고 인삼이 중추신경계에 미치는 효과에 대한 세포수준에서의 증거는 별로 없다. 따라서 본 연구자들은 지금까지 보고된 30여종 이상의 ginsenosides 중에서 인삼 효과의 주된 활성성분으로 알려져 있는 Rb1과 Rg1을 이용해 해마신경전구세포의 분화와 증식에 미치는 효과를 연구하였다. 증식실험결과 Rb1과 Rg1을 3일 동안 해마신경전구세포에 처리하면 대조군에 비해 BrdU(+)세포수가 상당히 감소한 반면에 분화조건에서 Rb1과 Rg1을 처리했을 때는 신경세포특이적인 MAP2 단백질을 발현하는 세포수가 증가하였다. 전구세포의 신경세포로의 분화 결정에 관여한다고 잘 알려져 있는 proneural 전사인자인 *Ngn*1과 *Hes*1유전자의 발현양상도 대조군에 비해 증가하였다. 따라서 본 연구 결과, ginsenoside Rb1과 Rg1은 해마신경전구세포의 증식을 감소시키고 대신 신경세포로의 분화를 촉진시킴으로써 해마신경발생에 관여함을 보여준다.

INTRODUCTION

Ginseng, the root of *Panax ginseng* C. A. Meyer(Araliaceae), is a well-known and popular herbal medicine which is used worldwide. Among more than 30 ginsenosides reported so far, ginsenosides Rb1 and Rg1 are regarded as the main compounds responsible for many pharmaceutical actions of ginseng(Radad *et al.*, 2004). Rg1 and Rb1 have similar effects, but show some differences in pharmacological activities and action mechanisms(Leung *et al.*, 2006a, 2006b; Xue *et al.*, 2006a, 2006b). These differences may be attributed to their different chemical structure. Rg1 is a panaxtriol with two sugars, while Rb1 is a panaxtriol with four sugars.

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Effects of ginseng have been demonstrated in the central nervous system; it is, for example, known to improve learning and memory(Bao *et al.*, 2005) and to enhance nerve growth(Rudakewich *et al.*, 2001). Rb1 plays an important role in protecting neuronal cells from overexcitotoxic damage by glutamate(Kim *et al.*, 1998). However, the effect of ginsenosides on memory and learning is still contradictory. Some earlier studies showed that ginseng extracts caused learning deficits rather than improvements(Bao *et al.*, 2005). In this context, we wanted to assess whether ginsenoids enhance hippocampal neurogenesis or not. Our results indicate that Rg1 decrease the proliferating ability of NPCs and that Rb1 and Rg1 enhance neural differentiation. These results would improve our understandings about the effects of Rb1 and Rg1 on humans who consume ginseng.

MATERIALS AND METHODS

1. Ginsenosides

Ginsenosides Rg1 and Rb1 with purity greater than 98% were kindly provided by Korea Tobacco & Ginseng Central Research Institute(Daejeon, Korea). Rg1 and Rb1 were dissolved in DMSO at the concentration of 100 mM as stock solution and were diluted with cell culture media before use.

2. Hippocampal NPC Culture

The present study employed the conditions used by Kim *et al.*(2006) with slight modifications, and Sprague Dawley adult pregnant female rats(Harlan Sprague Dawley, Indianapolis, IN, USA) were used. Briefly, hippocampi were dissected from E16.5 rat embryos in calcium -and magnesium- fee Hank's balanced salt solution(HBSS). Cells were plated on 10-cm-diameter dishes coated with 15 μg/mL poly-L-ornithine and 1 μg/mL fibronectin(Invitrogen, Carlsbad, CA) at 2.5×10^4 cells/cm^2 in N2 media and incubated at 37℃ in 95% air/5% CO_2 gas. Basic fibroblast growth factor(bFGF, 20 ng/mL, R&D system, Minneapolis, MN) was added daily in order to expand the population of proliferative progenitors and medium was changed every 2 days as described previously(Kim *et al.*, 2006). For high-density cultures, the cells on 80% confluency were subcultured at a density of 6×10^4 cells/cm^2 in a 24-well plate in N2 medium in the presence of bFGF and these subcultured cells were designated as passaged once(P1). All experiments were carried out using the P1 neural progenitors. The neural progenitors were induced to differentiate by withdrawing bFGF and kept in a differentiation media(Neurobasal medium: NB) for 3~5 d in the presence or absence of Rb1/Rg1. Control cultures were treated with the same concentration of DMSO.

3. BrdU Incorporation and Cell Death Assay

5-bromo-2-deoxyuridine(BrdU)(10 μM; Sigma, St. Louis, MO, USA) was added to the cultures at indicated time points, and the cultures were further incubated for 1 h or 24 h. For proliferation studies, BrdU was briefly washed out with PBS, followed by fixation. For differentiation studies, BrdU was briefly washed out with NB medium and then the medium was changed to new NB medium in the absence of bFGF. For apoptosis assays, a TUNEL assay was performed using *in situ* cell death detection POD kit(Roche Molecular Biochemicals, Basel, Switzerland). Cells were mounted using VECTASHIELD® with DAPI (Vector Laboratories, Burlingame, CA, USA) in mounting medium.

4. Immunocytochemistry

Cells were fixed in 4% paraformaldehyde/0.15% picric acid in PBS for 20 min. The following primary antibodies were used: for characterization of undifferentiated NPCs; for differentiation, monoclonal anti-MAP2 at 1:200(Sigma-Aldrich, St. Louis, MO), monoclonal anti-GFAP at 1:100 (ICN Biochemicals, Costa Mesa, CA) and polyclonal anti-GFAP at 1:400(Dako, Glostrup, Denmark); for neuronal subtype differentiation; and for BrdU staining, rat-anti BrdU at 1:100(Accurate Chemical, Westbury, NY, USA). For detection of primary antibodies, cells were then incubated in PBS containing Cy3-(Jackson Immunoresearch; 1:100), Cy2-(Jackson ImmunoResearch; 1:200) or FITC-labeled(Molecular Probes; 1:500) secondary antibodies for 1 h at room temperature. Cells were mounted in Vectashield mounting medium for fluorescence and photogra-

phed with a fluorescence microscope(Nikon).

5. Real-Time RT-PCR and Quantification of Gene Expression

Reactions were performed in a 50 μL volume reaction mixture. The PCR was started with 94℃ for 2 min and then continued with 45 cycles of 40 s at 94℃, 40 s at 60℃, 40 s at 72℃, and 15 s at 80℃ for SYBR green detection(Molecular Probes, Eugene, OR, USA) on the iCycler iQ Multi-Color Real Time PCR Detection System(Bio-Rad Laboratories, Hercules, CA). A melting curve was obtained for each PCR product after each run to confirm that the signal corresponded to a unique amplicon of the predicted size. The expression value of each gene was normalized to the amount of GAPDH in that sample to calculate the relative amount of transcript present for each gene. The normalized expression values for all control and treated samples was averaged, and average fold changes were determined. A Student's *t* test was conducted between the normalized relative expression values for each individual control and treated samples to determine statistical relevance.

6. Cell Counting and Statistical Analysis

Statistical significance was determined using Student's *t* test. All statistical analyses were conducted with Sigma plot 2000 software.

RESULTS

Culture conditions for hippocampal NPCs were adapted from previous studies(Amoureux *et al.*, 2000), and the resulting cultures were characterized. To investigate whether Rb1 and Rg1 affected the proliferation of hippocampal NPCs, the cells were pulsed with BrdU(10 μM), a marker for cell proliferation, during expansion. When pulsed with BrdU for 1 h 4 days *in vitro*(DIV) after passage, an average 48.3+5.0% of NPCs were BrdU-positive in the presence of bFGF(Fig. 1A and B). When Rb1 was added daily to the cultures in the presence of bFGF, the fraction of BrdU(+) cells, revealed by co-staining with DAPI nuclear stain, was significantly decreased in a dose-dependent manner, reaching a minimum of 9.2+2.0% at a dose of 100 μM, compared with control(Fig. 1A and B; n=5 Rb1 50 μM, 21.8+2.1%, $p<0.001$; Rb1 100 μM, 9.2+2.0%, $p<0.001$). Rg1, at the same concentrations(50 and 100 μM), also led to a reduced BrdU incorporation(50 μM: 26.8+4.0%, $p<0.001$ and $p<0.05$, compared to control, respectively; 100 μM: 11.3+2.2%, $p<0.001$ compared to control). However, treatment with DMSO(0.001%) did not alter the number of BrdU(+) cells either in the presence or absence of bFGF (data not shown), indicating that the effect of ginsenosides on the number of BrdU(+) cells was specifically caused by ginsenosides, and not by solvent.

A possible explanation for the decrease of BrdU(+) cell number by Rb1 and Rg1 might be that Rb1 and Rg1 increased cell death or decreased survival of newly divided progenitors. To prove such possibilities, TUNEL assay and Western blotting analysis for Bcl-2, an anti-apoptotic protein, were performed. Rb1 or Rg1 was added to the cultures in N2 for 4 d in the presence of bFGF. At 100 μM, the percentage of TUNEL(+) cells was not significantly different from the controls(untreated, 5.3+0.9%; Rb1-treated, 4.5+1.8 %, $p>0.05$; Rg1-treated, 4.0+0.3%, $p>0.05$) (Fig. 1C). In addition, Rb1 or Rg1 did not upregulate Bcl-2 protein level(Fig. 1D). To rule out the possibility that the decrease in the number of BrdU(+) cells was due to damage of cells by stimuli, we compared total cell numbers between control cultures and cultures stimulated with Rb1 or Rg1. Three days after stimulation, there was no significant difference in the number of cells in control and stimulated cultures(per well: control, 15750±60; stimulated, 14800±57; n=9; $p>0.05$). Therefore, it appears that cell death-related activity does not contribute to the reduced yield of BrdU (+) progenitors following Rb1 or Rg1 treatment.

1. Rb1 and Rg1 Promotes the Differentiation of Rat Hippocampal NPCs

Next, we hypothesized that Rb1 and Rg1 may influence the balance between the proliferation and the differentiation of NPCs. To address this possibility, P1 progenitors expanded in N2 in the presence of bFGF were rinsed free of bFGF and then, treated with Rb1 or Rg1 for 3 d

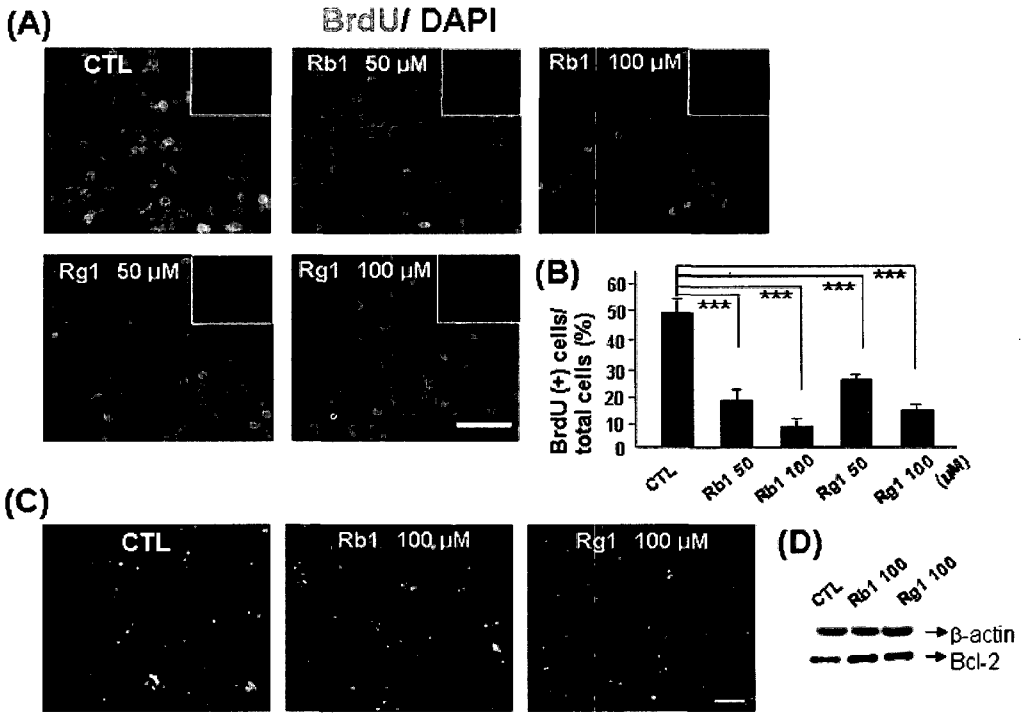

Fig. 1. BrdU incorporation decreases following repetitive pulses of Rb1 or Rg1. A-B: BrdU incorporation.(green) after repetitive treatment with Rb1 or Rg1 for 3 consecutive days in the presence of bFGF. 6 DIV cultures were exposed daily to Rb1 or Rg1(50 and 100 μM) for 3 d, followed by a 1 h pulse of BrdU(50 μM), and fixed on 9 DIV. B: Effects of different doses of Rb1 or Rg1 on proliferation. C: Apoptosis assay: green fluorescent nuclei correspond to apoptotic cells in which the terminal transferase has incorporated fluorescent dUTP(Bodipy-dUTP) into the fragmented DNA. TUNEL(+) cells are shown as a fraction of the total cell number defined by DAPI staining of nuclei. D: Western blotting for Bcl-2 shows that Rb1(100 μM) or Rg1(100 μM) did not significantly upregulate the protein level of Bcl-2. CTL: control; Student's *t*-test: *** $p<0.001$, ** $p<0.01$, * $p<0.05$. Scale bar: 100 μm.

in NB medium to allow differentiation. We applied Rb1 or Rg1 at two concentrations(50 and 100 μM), followed by double immunocytochemistry for MAP2/GFAP(Fig. 2A). Cells of a neuronal lineage were identified by MAP2 expression, and cells of an astrocyte lineage were identified by GFAP expression. When Rb1 or Rg1 was added to rat hippocampal NPCs at a concentration of 100 μM, a maximal increase of MAP2(+) cells was observed compared to untreated control cells(Fig. 2B, untreated, 14.8+1.7%; Rb1-treated, 27.4+4.7%, $p<0.001$; Rg1-treated, 34.6+3.9%, $p<0.001$). Rb1 or Rg1 did not significantly affect the fraction of astrocytes in the rat hippocampal NPC cultures(Fig. 2A).

2. Altered Expression of Several Genes Including bHLH Transcription Factors during Differentiation of Rb1- and Rg1-treated NPCs

To elucidate the possibility that Rb1 or Rg1 promotes neuronal differentiation via regulating the expression of transcription factors, we investigated the expression of several transcriptional factors in these Rb1 or Rg1-treated cells: Basic helix-loop-helix(bHLH) transcription factors, such as *Ngn*1, are thought to positively regulate neuronal development at the level of commitment and postmitotic differentiation, whereas *Hes*1 negatively regulates the positive bHLH genes(Lee, 1997). In addition, homeodomain transcription factor *Emx*2, has been shown to play a role in the genetic regulation of cortical arealization and to be expressed in the developing hippocampus(Bishop *et al.*, 2002). Semiquantitative reverse transcription-PCR was used to assess differential expression of genes regulated by ginsenosides.

As shown in Fig. 3, quantitative mRNA analysis showed

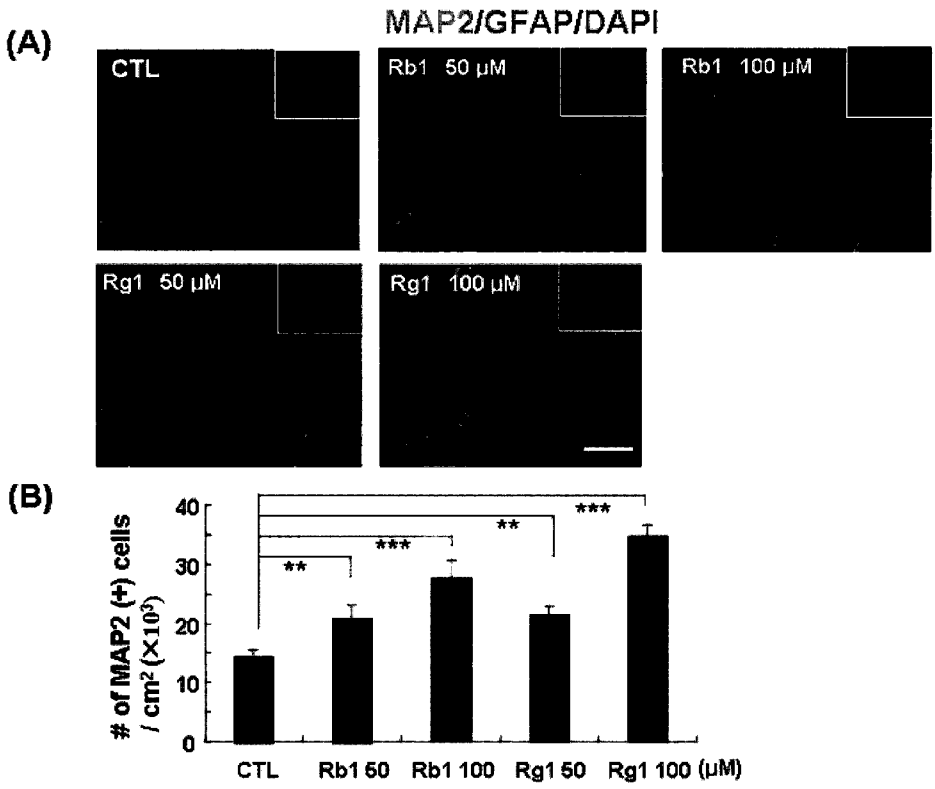

Fig. 2. Cells were induced to differentiate by withdrawal of bFGF in the presence of Rb1 or Rg1 for 3 d, followed by dual immunocytochemistry. A: Dual-label immunocytochemistry for MAP2(+)(red) and GFAP(+)(green) cells 3 d after being induced to differentiate. B: MAP2 (+) cells were counted and expressed as cell number in unit area in the four experiments. Error bars indicate the SEM. CTL: control; Student's *t*-test: *** $p<0.001$, ** $p<0.01$, * $p<0.05$. Scale bar: 100 μm.

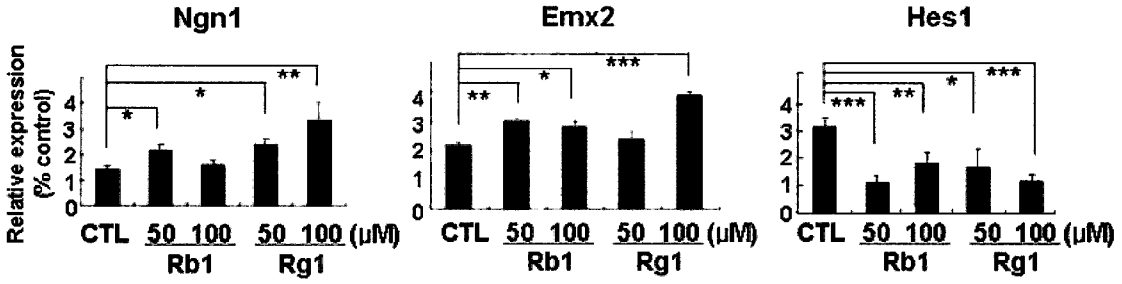

Fig. 3. Rb1 or Rg1 regulates the expression of proneural bHLH and homeogene transcription factors. Real time RT-PCR analysis shows that treatment with Rb1 or Rg1 in medium permissive of neuronal differentiation caused an increase in the expression of *Ngn*1 and *Emx*2. The expression of each gene was normalized to the amount of GAPDH to obtain the relative level of each transcript(n=5 per group) and is shown relative to the expression level in control cells. Error bars indicate the SEM. CTL: control; Student's *t*-test: *** $p<0.001$, ** $p<0.01$, * $p<0.05$.

that expression of *Ngn*1 in Rb1- or Rg1-treated cultures was increased 3 d after differentiation compared with control cultures(Fig. 3: control: 1.4+0.1, Rb1 50 μM, 2.2+0.2, $p<0.05$; Rg1 100 μM, 3.1+0.8, $p<0.01$). Similar to *Ngn*1, the expression level of Em×2 mRNA was significantly increased 3 d after differentiation in Rb1- or Rg1-treated cultures compared with control cultures(Fig. 3: control: 2.1+0.1, Rb1 50 μM, 2.9+0.0, $p<0.01$; Rb1 100 μM, 2.7+0.1, $p<0.05$; Rg1 100 μM, 3.7+0.2, $p<0.001$). *Drosophila hairy* and *enhancer-of-split*(Hes) genes are widely known as

anti-neurogenic members of the bHLH gene family and have been reported to block commitment to a neuronal fate (Ishibashi *et al.*, 1995). Thus, we asked whether the expression pattern of *HES*1 was changed in Rb1 or Rg1-treated cultures. The expression level of *Hes*1 mRNA was significantly decreased by ginsenosides treatment compared with untreated control cultures(Fig. 3, control, 3.1+ 0.3, Rb1 50 μM, 1.0+0.1, $p<0.001$; Rb1 100 μM, 1.6+ 0.2, $p<0.01$; Rg1 50 μM, 1.4+0.7, $p<0.05$; Rb1 100 μM, 1.0+ 0.1, $p<0.001$). Together with the experiments on immunocytochemical staining, these results indicate that Rb1 and Rg1 increase the differentiation of progenitor cells to a neuronal lineage.

DISCUSSION

We report herein the novel observation that ginsenosides Rb1 and Rg1 with concentrations within their micromolar range facilitate differentiation of hippocampal NPCs into a neuronal cell type, as well as decrement in the number of proliferating cells. We further demonstrated that the effects of ginsenosides on neuronal differentiation also appear to involve the activation of bHLH transcriptional factors in embryo hippocampus-originated NPCs.

Independent of the effects of Rb1 or Rg1 on differentiation, Rb1 and Rg1 appear to act on proliferation, mediated perhaps by a yet unidentified signal transduction pathway. Prior to our study on the neurogenesis-promoting effect of ginseng, Shen and Zhang reported that ginseng decreased the number of BrdU-positive cells in the hippocampus(Shen and Zhang, 2004). It is essential to distinguish whether the persistent decrease in the number of BrdU(+) neurons was due to reduced proliferation, survival, or both. When we assessed the possible effects of ginsenosides on cell survival during expansion by TUNEL assay, Rb1 and Rg1 did not appear to affect survival rates *in vitro*. Previous results showed that Rg1 upregulated the proliferation of hippocampal NPCs *in vivo*(Liao *et al.*, 2002). The contradictory findings between the results obtained by Liao *et al.* and by us could be due to the ability of Rb1 or Rg1 to affect different cell types in the intact brain. In the intact brain, a systemic intake of ginsenosides may affect many more types of neurons than a local application could achieve in an *in vitro* system. Further work using a combination of *in vivo* and *in vitro* systems should shed light on the intracellular signaling pathways promoting the proliferation of immature NPCs.

Rg1 appears to affect the expression of *Ngn*1 and *Emx*2. It has been previously reported that *Ngn*1 does not only promote neurogenesis but also inhibits astrocytic differentiation of cortical progenitors(Sun *et al.*, 2001). *Emx*2 is essential for terminal differentiation of hippocampal granular cells and the formation of hippocampal connections (Savaskan *et al.*, 2002). These observations led us to propose that Rg1, via the activation of *Ngn*1, might play an instructive role which on the one hand induces neuronal differentation and on the other hand inhibits the production of glial cells. Further work needs to be done to determine how ginsenosides regulate the glial cell differentiation and the expression of bHLH genes required for neuronal differentiation of NPCs.

In view of previous studies demonstrating that ginenosides are able to promote learning and cell survival as well as neuroprotection(Wen *et al.*, 1996), and of our own study that ginenosides enhance neuronal differentiation of hippocampal NPCs, we conclude that the intake of ginenosides may play a beneficial role in their long term therapeutic actions in the CNS.

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