Glehnia littoralis Extract Promotes Neurogenesis in the Hippocampal Dentate Gyrus of the Adult Mouse through Increasing Expressions of Brain-Derived Neurotrophic Factor and Tropomyosin-Related Kinase B

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Abstract

Background: Glehnia littoralis has been used for traditional Asian medicine, which has diverse therapeutic activities. However, studies regarding neurogenic effects of G. littoralis have not yet been considered. Therefore, in this study, we examined effects of G. littoralis extract on cell proliferation, neuroblast differentiation, and the maturation of newborn neurons in the hippocampus of adult mice.

Methods: A total of 39 male ICR mice (12 weeks old) were randomly assigned to vehicle-treated and 100 and 200 mg/kg G. littoralis extract-treated groups (n = 13 in each group). Vehicle and G. littoralis extract were orally administrated for 28 days. To examine neurogenic effects of G. littoralis extract, we performed immunohistochemistry for 5-bromo-2-deoxyuridine (BrdU, an indicator for cell proliferation) and doublecortin (DCX, an immature neuronal marker) and double immunofluorescence staining for BrdU and neuronal nuclear antigen (NeuN, a mature neuronal marker). In addition, we examined expression changes of brain-derived neurotrophic factor (BDNF) and its major receptor tropomyosin-related kinase B (TrkB) using Western blotting analysis.

Results: Treatment with 200 mg/kg, not 100 mg/kg, significantly increased number of BrdU-immunoreactive (+) and DCX+ cells (48.0 ± 3.1 and 72.0 ± 3.8 cells/section, respectively) in the subgranular zone (SGZ) of the dentate gyrus (DG) and BrdU+/NeuN+ cells (17.0 ± 1.5 cells/section) in the granule cell layer as well as in the SGZ. In addition, protein levels of BDNF and TrkB (about 232% and 244% of the vehicle-treated group, respectively) were significantly increased in the DG of the mice treated with 200 mg/kg of G. littoralis extract.

Conclusion: G. littoralis extract promotes cell proliferation, neuroblast differentiation, and neuronal maturation in the hippocampal DG, and neurogenic effects might be closely related to increases of BDNF and TrkB proteins by G. littoralis extract treatment.

Key words: Brain-Derived Neurotrophic Factor; Cell Proliferation; Glehnia littoralis; Neuroblast Differentiation; Tropomyosin-Related Kinase B

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**INTRODUCTION**

Neurogenesis in the adult brain is a normal process to generate new neurons and occurs throughout life in restricted brain regions, which is called neurogenic regions, such as the subgranular zone (SGZ) of the dentate gyrus (DG) of the hippocampus and the subventricular zone of the lateral ventricle in mammals. Neural progenitor cells within the SGZ of the DG proliferate and differentiate into mature neurons in the granule cell layer, and newly generated neurons in the DG integrate into functional hippocampal network that may be crucial for learning and memory. It has been demonstrated that neurogenesis in the DG is decreased with age and diverse neurodegenerative diseases including Alzheimer’s and Parkinson’s diseases, which result in cognitive impairment. Thus, neurogenesis in the DG has received great clinical attention as a potential therapeutic target for the treatment of neurodegenerative diseases.

Traditional medicinal plants and their components possess diverse biological properties and have been widely used as attractive resources for the prevention or treatment of neurodegenerative diseases. Glehnia littoralis, a perennial member of the Glehnia genus belonging to the family Umbelliferae, is distributed in Korea, China, and Japan and has been used in traditional oriental medicine as diaphoretics, antipyretics, analgesics, and expectorant. It has been reported that G. littoralis has antioxidative constituents such as quercetin, isoquercetin, rutin, chlorogenic acid, and caffeic acid. Recent studies have proven that G. littoralis has a broad spectrum of biological properties such as antibacterial, antifungal, antioxidant, and anti-inflammatory effects. In addition, it has been recently reported that G. littoralis displays beneficial effects against transient global cerebral ischemia.

To the best of our knowledge, few studies regarding effects of G. littoralis on neurogenesis in the adult brain have been conducted. Herein, we assessed effects of G. littoralis on adult neurogenesis in the DG of the adult mouse hippocampus using 5-bromo-2-deoxyuridine (BrdU, an indicator for cell proliferation) labeling and immunohistochemistry for doublecortin (DCX, an immature neuronal marker), which has been commonly used to investigate the proliferation of neuroblast. In addition, we examined expression changes of brain-derived neurotrophic factor (BDNF) and its major receptor tropomyosin-related kinase B (TrkB), which are well known to be involved in the neurogenic process.

**METHODS**

**Experimental animals**

Adult male ICR mice (body weight 25–30 g, 12 weeks of age) were purchased from Orient Bio Inc. (Seongnam, South Korea), and they were handled by NIH Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85–23, 1985, revised 1996). The protocol used in this experiment was reviewed and approved by the Kangwon National University-Institutional Animal Care and Use Committee (Approval No.: KW-160802-3) based on ethical procedures and scientific care. All of the experiments were conducted to minimize the number of animals used and the suffering caused by the procedures used in the present study.

**Extraction of plant material**

G. littoralis was collected in Kangwon Province in October 2015 by Dr. Jong Dai Kim. For the preparation of the ethanol extract of G. littoralis (GLe), roots and rhizomes of G. littoralis were washed with distilled water, air-dried at 60°C, and ground into fine powder by a grinder (IKA M20, IKA, Staufen, Germany). The powder of the GLe was refluxed with 10 vol (v/w) of 70% ethanol at 70°C for 24 h. The extraction procedure was repeated three times. The extract was filtered through Whatman No. 1 filter paper (Whatman Ltd., Maidstone, Kent, UK), concentrated with a vacuum evaporator, and completely dried with a freeze-drier. The extraction yield was 9.24%.

**Treatment with ethanol extract of Glehnia littoralis and 5-bromo-2-deoxyuridine**

Mice were assigned to three groups (n = 13 in each group): (1) vehicle-treated group, which was treated with sterile saline (0.9% sodium chloride), (2 and 3) 100 and 200 mg/kg GLe-treated groups, which were treated with 100 and 200 mg/kg of GLe, respectively. GLe was dissolved in sterile saline. GLe or saline was fed using a feeding needle once daily for 28 days before sacrifice because it has been reported that DCX is expressed by immature newborn cells up to 28 days of cell age.

To examine newly generated neurons, BrdU (Sigma, USA) was dissolved in saline just before injection. Mice were intraperitoneally injected with BrdU (50 mg/kg) solution on day 8, 15, 22, and 27 according to our published procedure. All animals were weighed once per week during the experimental period. There were no significant differences in body weight between the experimental groups (data not shown).

**Tissue processing for histology**

To conduct histological analysis, mice (n = 7 in each group) were anesthetized with 30 mg/kg of Zoletil 50 (Virbac, Carros, France) and perfused by the aorta with 4% paraformaldehyde in 0.1 mol/L phosphate buffered (PB, pH 7.4). Their brains were removed and postfixed in the same fixative for 6 h. The brain tissues were cryoprotected by infiltration with 30% sucrose for 12 h and serially cut into 30 µm thickness of coronal sections in a cryostat (Leica, Wetzlar, Germany). The sections were kept in 6-well plates containing PB saline (PBS, pH 7.4) for the next process.

**Immunohistochemistry**

To examine neurogenic effects of GLe in the DG, the prepared sections were carefully processed under the same conditions. Six sections per animal were selected with 150 µm interval according to anatomical landmarks corresponding to −1.46 and −2.46 mm posterior to the bregma with a reference to the mouse brain atlas. As previously described, in short,
the sections were treated with 10% normal donkey serum (in 0.05 mol/L PBS) for 30 min and incubated with rat anti-BrdU (1:200, BioSource International, Camarillo, CA, USA) and goat anti-DCX (1:150, Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 12 h at 4°C. The reacted sections were exposed to biotinylated goat anti-rat or rabbit anti-goat immunoglobulin G (IgG, 1:200, Vector, Burlingame, CA, USA) and streptavidin-peroxidase complex (1:200, Vector) for 2 h at room temperature. Finally, the reacted sections were visualized with 3,3′-diaminobenzidine tetrahydrochloride (in 0.1 mol/L Tris-hydrochloric acid buffer, pH 7.2). For reference, DNA denaturation was required for BrdU immunohistochemistry. The DNA denaturation was conducted by incubating the sections in 50% formamide/2X SSC (0.3 mol/L sodium chloride and 0.03 mol/L sodium citrate) and then incubated in 2 mol/L hydrochloric acid and in 0.1 mol/L boric acid. To examine the stained sections, the sections were dehydrated and mounted in Canada balsam (Kanto Chemical, Japan).

Negative control tests were conducted using preimmune serum instead of primary antibodies for establishing the specificity of the immunostaining. Negative controls resulted in no immunoreactivity in the tissues conducted (data not shown).

**Double immunofluorescence**

To examine the differentiation of newly generated cells to mature neurons, five sections per animal were chosen with 150 µm interval, and the sections were stained by double immunofluorescence staining with BrdU and neuronal nuclear antigen (NeuN, a mature neuronal marker) according to a published procedure.[22] Briefly, DNA denaturation was conducted like the above-mentioned method. The denatured sections were incubated in a mixture of rat anti-BrdU (1:100, BioSource International, Camarillo, CA, USA) and rabbit anti-NeuN (1:500, Chemicon, International Temecula, USA) 12 h at 4°C. They were then incubated in a mixture of both FITC-conjugated anti-rat IgG (1:200; Jackson ImmunoResearch, West Grove, PA, USA) and Cy3-conjugated anti-rabbit IgG (1:500; Jackson ImmunoResearch) for 3 h at room temperature.

**Western blotting analysis**

To observe changes in expression levels of BDNF and TrkB proteins in the DG, six mice from each group were anesthetized with Zoletil 50® (30 mg/kg) and sacrificed by cervical dislocation. Western blotting analysis was conducted by a published protocol.[23] Briefly, brains of the mice were removed and serially cut into coronal sections of 400 µm thickness by a vibratome (Leica Camera AG, Wetzlar, Germany). Hippocampal tissues containing the DG were dissected with a surgical blade. The tissues were homogenized in 50 mmol/L PBS (pH 7.4) containing ethylene glycol tetraacetic acid (pH 8.0), 0.2% Nonidet P-40, 10 mmol/L ethylenediaminetetraacetic acid (pH 8.0), 15 mmol/L sodium pyrophosphate, 100 mmol/L β-glycerophosphate, 50 mmol/L sodium fluoride, 150 mmol/L sodium chloride, 2 mmol/L sodium orthovanadate, 1 mmol/L phenylmethylsulfonyl fluoride, and 1 mmol/L dithiothreitol (DTT). The homogenized tissues were centrifuged at 15,000 × g for 25 min at 4°C. Protein levels in the supernatants were determined using a Micro Bicinchoninic Acid Protein Assay Kit with bovine serum albumin as a standard (Pierce Chemical, Rockford, IL, USA). The aliquots containing 50 µg total protein were boiled in the loading buffer that contained 250 mmol/L Tris (pH 6.8), 10 mmol/L DTT, 10% sodium dodecyl sulfate, 0.5% bromophenol blue, and 50% Glycerol, and they were subsequently loaded onto a 10% polyacrylamide gel (SigmaAldrich). After the electrophoresis, the gels were transferred onto nitrocellulose membranes (Pall Corp., Pittsburgh, PA, USA). The membranes were incubated with 5% non-fat dry milk in TBS (pH 7.4) containing 0.1% Tween 20 for 50 min to reduce background staining. The membranes were incubated with rabbit anti-BDNF (1:500, Abcam), rabbit anti-TrkB (1:500, Santa Cruz Biotechnology, Inc.), and rabbit anti-β-actin (1:5,000, Sigma-Aldrich, MO, USA) 12 h at 4°C, and they were exposed to peroxidase-conjugated goat anti-rabbit IgG (1:4,000, Santa Cruz Biotechnology, Inc.) and an enhanced chemiluminescence kit (GE Healthcare Life Sciences, Chalfont, UK).

**Data analysis**

The quantitative analysis of numbers of BrdU-immunoreactive (+) and DCX+ cells was conducted by our published procedure.[23] Briefly, images of all BrdU+ and DCX+ structures were captured from immunostained sections of the DG using a light microscope (BX53, Olympus, Germany), which was equipped with a digital camera (DP72, Olympus) that was connected to a PC monitor. A total number of BrdU+ or DCX+ cells were counted in six sections/each mouse using an image analyzing system equipped with a computer-based CCD camera (Optimas 6.5, CyberMetrics, Scottsdale, AZ, USA). Each cell count was conducted by averaging the total cell numbers of each mouse. In addition, for calculating a number of BrdU+NeuN+ cells in the DG, the double immunoreaction of BrdU/NeuN was observed using a confocal microscope (LSM 510 META NLO; Carl Zeiss, Jena, Germany) and the cell count was conducted as described above.

Western blotting analysis was conducted according to our published method.[23] Shortly, bands of the Western blot were scanned, and a densitometric analysis was performed for the quantification of the bands. Scion Image 4.0.2 software (Scion Corp., Frederick, MD, USA) was used to calculate a relative optical density (ROD). Each ratio of the ROD was calibrated as a percent, and the ratio was compared with the vehicle-treated group, designated as 100%.

**Statistical analysis**

The data shown in this study represent the means ± standard deviation of mean. All statistical analyses were conducted using GraphPad Prism (version 5.0; GraphPad Software, La Jolla, CA, USA). Statistical analyses of differences between the groups were performed using one-way analysis of variance imaging.
sections, the bars indicate mean ± SEM. SEM: Standard error of the mean; BrdU: 5-bromo-2-deoxyuridine; DG: Dentate gyrus; bar = 200 (A, B, and C) and 40 (a, b, and c) µm. (D) The mean number of BrdU+ cells per section (n = 7 per group; *P < 0.05 vs. the vehicle-treated group). The bars indicate mean ± SEM.

**RESULTS**

**Cell proliferation**

In the vehicle-treated group, BrdU+ cells were mainly found in the SGZ of the DG [Figure 1A and 1a], and the mean number of BrdU+ cells in the SGZ was 16.0 ± 4.7 cells per section [Figure 1D]. In the 100 mg/kg GLe-treated group, the distribution pattern and mean number of BrdU+ cells in the DG were not significantly different from those in the vehicle-treated group [Figure 1B, 1b, and 1D]. However, in the 200 mg/kg GLe-treated group, BrdU+ cells were significantly increased in the SGZ and GCL compared with those in the vehicle-treated group [Figure 1C and 1c], and the mean number of BrdU+ cells was 48.0 ± 3.1 cells per section [Figure 1D].

**Neuroblast differentiation**

In the vehicle-treated group, DCX+ cells (29.0 ± 4.5 cells) as neuroblasts were easily found in the SGZ of the DG [Figure 2A]. Some of them revealed poorly developed processes [Figure 2a]. In the 100 mg/kg GLe-treated group, there were no significant differences in the morphology and number of DCX+ cells in the SGZ compared with those in the vehicle-treated group [Figure 2B, 2b, and 2D]. However, in the 200 mg/kg GLe-treated group, a number of DCX+ cells were significantly increased (72.0 ± 3.8 cells/section) in the SGZ, and most of them had long and thick processes compared with those in the vehicle-treated group [Figure 2C, 2c, and 2D].

**Neuronal maturation**

In the vehicle-treated group, cells co-labeled with BrdU and NeuN immunoreaction, as newly generated neurons, were mainly detected in the SGZ of the DG [Figure 3a–3c], and the mean number of BrdU+/NeuN+ cells was 7.0 ± 1.1 cells per section [Figure 3J]. In the 100 mg/kg GLe-treated group, the distribution pattern and mean number of BrdU+/NeuN+ cells were not different from the vehicle-treated group [Figures 3d-3f and 3j]. However, in the 200 mg/kg GLe-treated group, a significant increase in number of BrdU+/NeuN+ cells (17.0 ± 1.5 cells/section) was observed in the SGZ, and some of them were detected in the granule cell layer [Figures 3g–3j].

**Protein levels of brain-derived neurotrophic factor and tropomyosin-related kinase B**

Protein levels of BDNF and TrkB in the 100 mg/kg GLe-treated group were not significantly different from those in the vehicle-treated group [Figure 4]. However, protein levels of BDNF and TrkB in the 200 mg/kg GLe-treated group were significantly increased (about 232% and 244% of the vehicle-treated group, respectively) compared with the vehicle-treated group [Figure 4].

**DISCUSSION**

Until now, many researchers have been trying to find new medical plants and their components, which can promote neurogenesis in the neurogenic regions, as a potential therapeutic agent of neurodegenerative diseases and many studies have demonstrated that some plant extracts and their components have the capacity to promote neurogenesis.[26,27] Although some researchers recently showed that extracts of plants in the family Umbelliferae including Oenanthe javanica and Angelica sinensis improved cell proliferation and neuroblast differentiation in the SGZ of the hippocampal DG,[28,29] neurogenic effects of GLe in the brain have not yet been investigated. Therefore, in the present study, we examined effects of GLe treatment on cell proliferation, neuronal differentiation, and maturation of neurons to neural progenitor cells. Our results revealed that the number of BrdU+ and DCX+ cells only in the 200 mg/kg GLe-treated mice, not 100 mg/kg GLe, were significantly increased in the SGZ of the DG compared with the vehicle-treated mice; especially, increased DCX+ cells in the 200 mg/kg GLe-treated...
Figure 2: Representative images of DCX immunohistochemistry in the DG of the vehicle-treated (A and a), 100 mg/kg GLe-treated (B and b) and 200 mg/kg GLe-treated (C and c) groups. In the vehicle-treated group, DCX⁺ cells are found in the SGZ, and some of them show poorly developed processes (arrowheads). A significant increase in the number of DCX⁺ cells with well-developed processes (arrows) is observed only in the 200 mg/kg GLe-treated group. GCL: Granule cell layer; ML: Molecular layer; PL: Polymorphic layer. Scale bar = 200 (A, B, and C) and 40 (a, b, and c) µm. (D) The mean number of DCX⁺ cells per section (n = 7 per group; *P < 0.05 vs. the vehicle-treated group). The bars indicate mean ± SEM. SEM: Standard error of the mean; GLe: Ethanol extract of Glehnia littoralis; SGZ: Subgranular zone; DG: Dentate gyrus; DCX: Doublecortin.

Figure 3: Representative confocal images of cells double-labeled with BrdU (green; a, d, and g), NeuN (red; b, e, and h) and merged images (c, f, and i) in the DG of the vehicle-treated (a-c), 100 mg/kg GLe-treated (d-f), and 200 mg/kg GLe-treated (g-i) groups. In all groups, BrdU⁺/NeuN⁺ cells (arrows) are mainly distributed in the SGZ. BrdU⁺/NeuN⁺ cells are significantly increased only in the 200 mg/kg GLe-treated group. GCL: Granule cell layer; PL: Polymorphic layer. Scale bar = 40 µm. (j) The mean number of BrdU⁺/NeuN⁺ cells per section (n = 7 per group; *P < 0.05 vs. the vehicle-treated group). The bars indicate mean ± SEM. SEM: Standard error of mean; GLe: Ethanol extract of Glehnia littoralis; DG: Dentate gyrus; BrdU: 5-bromo-2-deoxyuridine; NeuN: Neuronal nuclear antigen.

mice showed a well-developed dendritic complexity, which is an important marker of neuronal development and is crucial for the functional integration of newborn neurons into hippocampal neuronal circuitry. In addition, we found a
increasing adult hippocampal neurogenesis is evidence for reduced neurogenesis in the aging. Severely impaired hippocampal neurogenesis associates with an early serotonergic deficit in a BAC α-synuclein transgenic rat model of Parkinson’s disease. Neurobiol Dis 2016;85:206-17. doi: 10.1016/j.nbd.2015.10.021.

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In summary, the results of this study revealed that treatment with GLe resulted in significant increases of cell proliferation, neuroblast differentiation, and neuronal maturation as well as BDNF and TrkB expressions in the hippocampal DG of adult mice. These results indicate that GLe can promote adult neurogenesis in the DG of the hippocampus and that increases of BDNF and TrkB expressions in the DG following GLe treatment might be closely associated with neurogenic effects of GLe. Thus, we suggest that G. littoralis can be used as a therapeutic potential candidate to prevent and treat neurodegenerative diseases, which involve the impairment of neurogenesis.

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**Conflicts of interest**

There are no conflicts of interest.

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Vanillin and 4-hydroxybenzyl alcohol promotes cell proliferation and neuroblast differentiation in the dentate gyrus of mice via the increase of brain-derived neurotrophic factor and tropomyosin-related kinase.