Research article

Gintonin influences the morphology and motility of adult brain neurons via LPA receptors

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ABSTRACT

Background: Gintonin is an exogenous ginseng-derived G-protein-coupled lysophosphatidic acid (LPA) receptor ligand. LPA induces *in vitro* morphological changes and migration through neuronal LPA1 receptor. Recently, we reported that systemic administration of gintonin increases blood-brain barrier (BBB) permeability via the paracellular pathway and its binding to brain neurons. However, little is known about the influences of gintonin on *in vivo* neuron morphology and migration in the brain.
Materials and methods: We examined the effects of gintonin on *in vitro* migration and morphology using primary hippocampal neural precursor cells (hNPC) and *in vivo* effects of gintonin on adult brain neurons using real time microscopic analysis and immunohistochemical analysis to observe the morphological and locational changes induced by gintonin treatment.
Results: We found that treating hNPCs with gintonin induced morphological changes with a cell rounding following cell aggregation and return to individual neurons with time relapses. However, the *in vitro* effects of gintonin on hNPCs were blocked by the LPA1/3 receptor antagonist, Ki16425, and Rho kinase inhibitor, Y27632. We also examined the *in vivo* effects of gintonin on the morphological changes and migration of neurons in adult mouse brains using anti-NeuN and -neurofilament H antibodies. We found that acute intravenous administration of gintonin induced morphological and migrational changes in brain neurons. Gintonin induced some migrations of neurons with shortened neurofilament H in the cortex. The *in vivo* effects of gintonin were also blocked by Ki16425.
Conclusion: The present report raises the possibility that gintonin could enter the brain and exert its influences on the migration and morphology of adult mouse brain neurons and possibly explains the therapeutic effects of neurological diseases behind the gintonin administration.

1. Introduction

Lysophosphatidic acid (LPA) is a simple lysophospholipid that exhibits a potent biological effects in animal systems [1,2]. LPA is present in diverse body fluids and organs, and there is an especially high amount of LPA in the adult brain [3]. Although the LPA1 receptor was the first cloned and the primary receptor for LPA in the developing brain, six LPA receptor subtypes are currently known to be widely expressed in animal organs, including developing and mature brains [1,2]. One of the dramatic cellular effects of LPA is to induce morphological changes in neuronal cells [1]. LPA treatment of *in vitro* neuron cultures causes rapid neurite retraction and induces cell rounding via LPA1/4 receptors, Ga12/13 protein, and Rho kinase activation pathway. Although the exact role of LPA-induced morphological change in neurons remains unknown, it is currently assumed that LPA-induced morphological changes might be associated with cell proliferation, migration and/or motility, and survival in the remodeling of neurons for guidance and synaptic plasticity [1,4].

Gintonin was identified as an exogenous LPA receptor ligand originating from ginseng. Gintonin consists of a complex of LPAs, fatty acids, phospholipids, and ginseng proteins [5,6]. The primary action of gintonin is to elicit $[Ca^{2+}]_i$ transients via LPA receptor activation in neuronal cells, to exert its physiological and pharmacological actions from neurotransmitter release to cognitive functions in central nervous systems [5,6]. In addition to gintonin-induced $[Ca^{2+}]_i$ transients, gintonin treatment of PC12 cell induces neurite retraction and transient cell aggregation or clustering in hippocampal neural progenitor cells (hNPC), similar to the effects of LPA [7]. Recently, we also reported that gintonin can bind to human brain microvascular endothelial cells (HBMECs), which form the blood brain barrier (BBB) and highly express LPA receptor 1-3 subtypes, and induce morphological changes, resulting in the opening of gap junctions via paracellular pathways [8]. Gintonin further increases BBB permeability via LPA receptor signaling, and also facilitates the delivery of dopamine, 10-kDa fluorescein isothiocyanate (FITC)-dextran, and 34-kDa erythropoietin to the brain. In addition, we observed that intravenous administration of fluorescent gintonin binds to neurons and glia in the brain [8]. Although previous studies have reported that gintonin transiently enhances the delivery of small to large molecules to the brain and directly binds to brain cells, little is known about whether exogenous LPAs, such as gintonin, can induce any changes in morphology and migration in adult brain neurons *in vivo*, similar to that induced *in vitro* by gintonin or LPA [2,9].

In the present study, we first prepared hNPCs. Next, we examined whether gintonin can induce *in vitro* morphological changes and migration by acting on LPA receptors. We found that gintonin leads to morphological changes and migration via LPA receptor signaling. In addition, we found that intravenous administration of gintonin also induced morphological changes and migration of neurons in the adult mouse brain regions, including the cortex, hippocampus, and cerebrum, by using immunohistochemical study with antibodies against NeuN and neurofilament H (NFH). Gintonin-induced morphological changes of neurons in the adult brain were blocked by Ki16425, an antagonist of the LPA1/3 receptor. Our results demonstrate for the first time that gintonin, an exogenous LPA, can induce morphological changes and migration in adult mouse brain neurons. Finally, we discuss the neurophysiological and neuropharmacological roles of gintonin-induced *in vivo* morphological changes of brain neurons.

2. Materials and methods

2.1. Preparation of gintonin

Gintonin was prepared from *Panax ginseng* according to a previously reported method [10]. Briefly, four-year-old dry Korean ginseng roots that were purchased from local ginseng market (voucher number: NIBRVP0000730014) were stored at the herbarium of the National Institute of Biological Resources for 1—2 years (Herbarium Code: KB, http://sweetgum.nybg.org/science/ih/herbarium-details/?irn=138656). Ginseng-enriched fraction (GEF) prepared according to the description from previous publication. 350 g of extract was acquired by refluxing 1 kg of ginseng 8 times with 70% ethanol for 8 h at 80°C. Then it was dissolved in distilled cold water at the ratio of 1:10 and it was stored at 4°C for 24—96 h. The supernatant and precipitate from the water fractionation was separated by centrifuging them at 3,000 rpm for 20 min. Then the precipitate was lyophilized. Main active chemical ingredients of gintonin enriched fractions are as follows that are based on the LC-MS/MS. GEF contains 7.5% linoleic acid ($C_{18:2}$), 2.8% palmitic acid ($C_{16:0}$), and 1.5% oleic acid ($C_{18:1}$). GEF contains 0.2% LPA $C_{18:2}$, 0.06% LPA $C_{16:0}$, and 0.02% LPA $C_{18:1}$. GEF contains 0.08% lysophosphatidylcholine (LPC) 0.03% lysophosphatidylethanolamine, and 0.13% lysophosphatidylinositols. GEF contains about 1% phosphatidic acid (PA) 16:0-18:2, 0.5% PA 18:2-18:2, and 0.2% PA 16:0-18:1.

For the *in vitro* study, gintonin was dissolved in dimethyl sulfoxide (DMSO) and then diluted before use with Dulbecco's
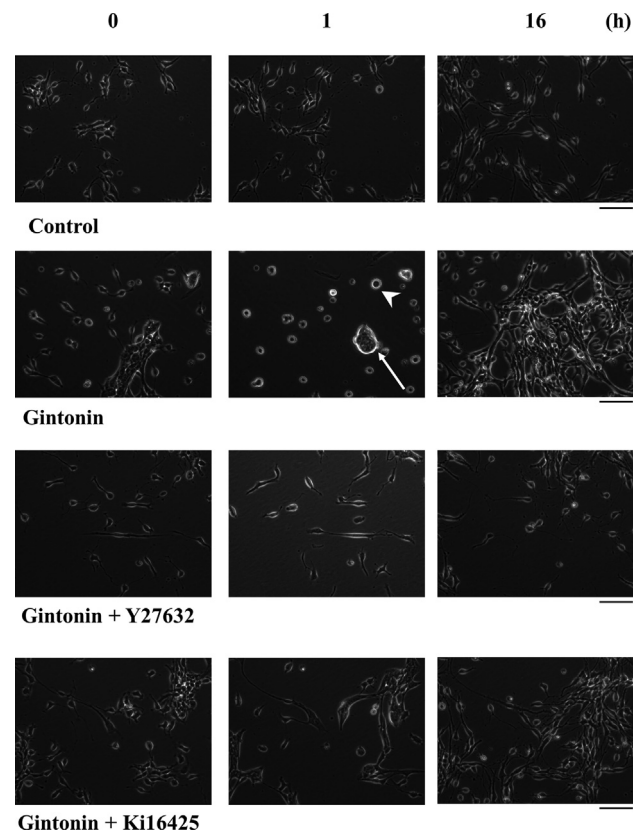
Fig. 1. Effect of gintonin on the morphology of the neuronal cells mediated by LPA receptor-specific signaling. Hippocampal neuronal progenitor cells were cultured on a plate and were treated with gintonin along with an appropriate inhibitor for ROCK (Y27632) or LPA receptor (Ki16425). Arrow and arrow head indicate where large and small aggregation of neuronal cells are observed by gintonin treatment, respectively. Images were acquired using time-lapse microscopy over the course of up to 16 h. Scale bar indicates 20 μm.

modified Eagle's medium (DMEM) for hNPCs. The final DMSO concentration was less than 0.01%. For the *in vivo* studies, the gintonin-enriched fraction was dissolved in saline before use [11]. All other reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Animals

Male BL6 mice (six weeks old, 18−20g) were purchased from Orient Bio Inc., (Chuncheon, Korea) and maintained according to Institutional Animal Care and Use guidelines of Konkuk University. All the experiments were conducted in a manner that minimized the number of animals used and their suffering. Animals were housed in plastic cages under controlled conditions: $50 \pm 5\%$ humidity, 12/12-h light−dark cycle, and free access to standard food and water. This study was performed in accordance with the National Institutes of Health Guide of the Care and Use of Laboratory Animals, and all experimental procedures were approved by the Institutional Animal Care and Use Committee of the Biomedical Research Institute at Konkuk University (Permit number: 16-206).

2.3. Animal experimental design

Mice underwent intravenous retro-orbital injection with 10 mg/kg of gintonin for different time points [12] and were sacrificed after ketamine/xylazine-induced anesthesia. The dose of gintonin was determined based on previous experiments from previous publications [8,12]. For the LPA receptor blockade experiment, mice were pretreated with Ki16425 (30 mg/kg, *i.p.*) for 30 min and then treated with gintonin. To obtain brain sections, the mice (n = 3−4 per group) were anesthetized with ketamine (100 mg/kg) and

xylazine (10 mg/kg) intraperitonially and perfused with ice-cold PBS. The brain was dissected out, placed in optimum cutting temperature (OCT) solution, snap-frozen with liquid nitrogen, and saved at −80°C until analysis. The brain was sectioned with a cryostat (thickness, 8 μm) and fixed with 4% paraformaldehyde (PFA) for 15 min. For thicker brain sections, the perfused mouse brain was post fixed with 4% PFA overnight and sectioned using a vibratome at a thickness of 100 μm. For thin sections, sections were blocked with 5% goat serum for 20 min at room temperature, incubated with primary antibodies overnight at 4°C, and washed twice with PBS. The sections were stained with fluorochrome-conjugated secondary antibody for 1 h at room temperature and washed twice with PBS. Finally, the slides were coverslipped with Vectashield containing DAPI. Images were captured using a Zeiss Axio Imager M1 microscope. For thicker sections, sections were blocked with 5% goat serum for 1 h and incubated with primary antibody in 1% TritonX/PBS/1% BSA at room temperature overnight. Sections were washed three times with 0.2% TritonX/PBS for 30 min each time and further incubated with secondary antibody in 1% TritonX/PBS/1% BSA for 8 h at room temperature. Sections were transferred to a glass slide and coverslipped with Vectashield containing DAPI.

2.4. Hippocampal neural progenitor cells (hNPC) culture

hNPC cultures were prepared according to the procedure described by Kim et al. [12]. Briefly, embryos on embryonic day 14.5 (E14.5) were dissected out of BL/6 adult pregnant female mice. The hippocampal region of the embryonic brain was isolated in calcium/magnesium-free Hanks' Balanced Salt Solution (HBSS). Cells were plated, 2.5×10^4 cells/cm^2 on 10-cm-diameter plates
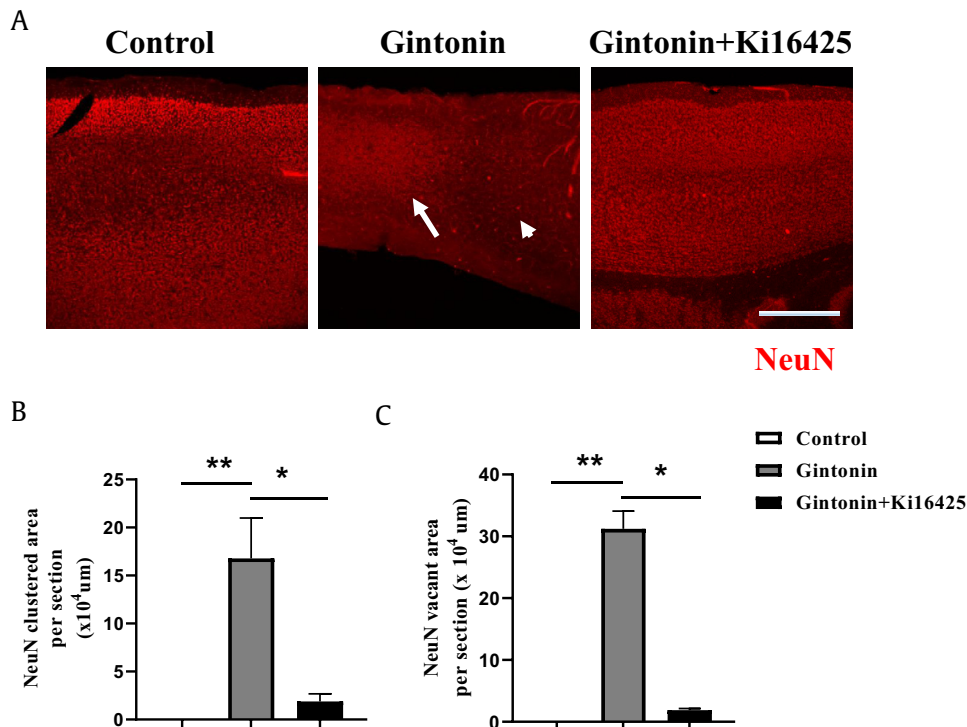

Fig. 2. Changes in segregation and migration of neuronal cells in the cortical regions of gintonin-treated mice. (A) Mice were treated with 10 mg/kg of gintonin or gintonin and Ki16425, an antagonist for the LPA receptor. Brain sections were stained with NeuN (Red), a marker for neuronal nuclei. Arrowhead indicates where the NeuN signal is absent, whereas the arrow indicates where cells are aggregated. Scale bar indicates 500 μm. (B and C) Quantification of cortical area where neuronal cell aggregation was observed (B) and absence of cells (C) were observed in the brain section (** indicates where $p < 0.01$ and * indicates where $p < 0.05$, two way ANOVA with Bonferroni multiple comparison test, images three different section, n = 3). Scale bar indicates 500 μm.

coated with 15 μg/ml poly-l-ornithine and 1 μg/ml fibronectin (Invitrogen, Carlsbad, CA, USA). Cells were placed in N2 medium supplemented with B27 (Invitrogen) at 37°C in a 95% air/5% CO_2 gas incubator. Basic fibroblast growth factor (bFGF, 20 ng/ml, R&D Systems, Minneapolis, MN, USA) and epidermal growth factor (EGF, 20 ng/ml, R&D Systems) were added daily in order to increase the hNPC population, and the medium was changed every other day. Cells at 80% confluency were sub-cultured and maintained at 6×10^4 cells/cm² in B27-supplemented N2 medium containing bFGF and EGF. Differentiation of these sub-cultured cells was induced *via* withdrawal of bFGF and EGF, and they were maintained in differentiation medium (Neurobasal medium supplemented with B27) for 3—5 days.

2.5. Gintonin-induced morphological changes and proliferation of hNPCs

hNPCs were divided into two groups; one group was treated with gintonin and the other served as a control group (N2 media without gintonin). We observed the morphological changes and proliferation of cells for 16 h using a real-time Cell Observer® (Carl Zeiss, Germany), which enabled the real-time visualization of dynamic processes in living cells with nanoscale resolution.

2.6. Statistical analysis

All statistical analysis was carried out using GraphPad 8.0 software. Statistical significances were assessed using two-way analysis of variance (ANOVA) with Bonferroni multiple comparison test. p values less than 0.05 were considered to be statistically significant.

3. Results

3.1. Gintonin induces in vitro morphological changes and migration of hNPCs via the LPA receptor and ROCK kinase

We first examined the effects of gintonin on *in vitro* morphological changes and migration. For this experiment, we used hippocampal NPCs. Cells were treated with gintonin (1 μg/ml) and morphological changes over the course of 16 h were observed. The control group showed slight morphological changes, whereas gintonin treatment rapidly and dramatically induced morphological changes, neurite retraction, and neuronal cell rounding. Over time, gintonin caused some cells to form aggregates of different sizes and finally the cells returned to their original state. Gintonin treatment also induced migration of cells. In addition to morphological changes and migration after gintonin treatment, gintonin also induced cell proliferation (Fig. 1). The gintonin-induced morphological changes and migration were blocked by the LPA1/3 receptor antagonist, Ki16425, and ROCK inhibitor, Y27632, indicating that these effects are mediated by the LPA receptor-ROCK pathways (Fig. 1). Overall, these results suggest that morphological changes observed hNPC by gintonin is specific for LPA receptor activation and its resultant activation of Rho-GTPase pathway.

3.2. Gintonin induces in vivo morphological changes and migration in cortex, cerebellum and hippocampal neurons in adult brain

It has been reported that LPA receptor activation is limited in the early life span in the mouse brain and it was hypothesized that LPA receptor mediated signaling pathway may be suppressed in adult brain [1,2]. However, in our previous studies, we have shown that treatment of gintonin can alleviate symptoms of neurodegenerative diseases, and opening of the BBB in adult brain which was specific

for the LPA receptor signaling and somewhat different from previous hypothesis [3]. To address this question, we treated mouse with 10 mg/kg of gintonin retro-orbitally to test if LPA receptor activation in the adult mouse brain can indeed induce neuronal morphological changes in the mouse brain. We observed that NeuN signals were observed to be sequestered in a certain regions of the cortex whereas it is vacant in a specific regions of the brain that was not observed in the control animal (Fig. 2A—C). These sequestration patterns was not also observed in the Ki16425 pre-treated group, suggesting that such changes in the neuronal location in the brain by gintonin treatment is mediated by LPA receptor specific manner (Fig. 2A—C). These were very similar to the patterns that were observed in our *in vitro* hNPC with gintonin stimulation which was specific for the LPA receptor signaling.

Based on these results, we hypothesized that gintonin can activate the LPA receptor signaling in the neuronal cells in adult brain which can indeed induce the reorganization or migration of these cells. To further address these questions, we have decided to determine the changes in the distribution of neuronal layers of
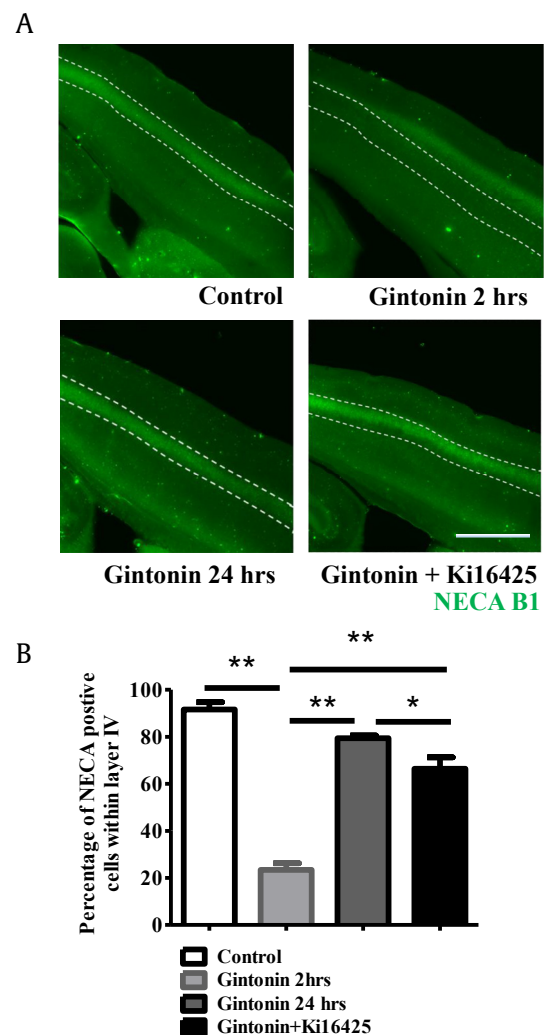

Fig. 3. Effect of gintonin treatment on layer IV neuron localization in the mouse brain. (A) Migration of neuronal cells was tracked using staining with a layer IV marker, NECAB1 (Green). Layer IV is marked with a dotted line. Scale bar indicates 200 μm. (B) NECAB1-positive cells from Layer IV were counted in different regions of the cortex were counted and are depicted in a graph. (** indicates where p < 0.01 and * indicates where p < 0.05, two-way ANOVA with Bonferroni multiple comparison test, five different areas, n = 3). Scale bar indicates 1500 μm.

which the structure is constantly maintained in the brain. For this, mouse were treated with gintonin along with its control and the brain sections were stained with layer IV specific marker NECA B1. Compared to its control, dislocalization of layer IV was more prominently observed in gintonin treated group (Fig. 3A and B). However, these changes were inhibited by the treatment of Ki16425 suggesting such changes were induced by LPA receptor specific manner (Fig. 3A and B). Also, such migratory changes of NECA B1 positive neurons were decreased in 24 h treatment group suggesting that these migratory changes is transient in vivo (Fig. 3A and B).

3.3. Gintonin induces in vivo changes in the neurofilament organization in the mouse brain

We questioned whether neurofilament re-organization may be observed in the mouse brain if such dramatic neuronal migration is observed following gintonin treatment. To answer this question, we stained the mouse brain for NFH, a marker for thick axons in the brain. We found that the cortical area stained for NFH in the gintonin-treated brain was less than that in the PBS control-treated brain (Fig. 4A—C). This implies that gintonin can induce not only neuronal migration but also modifies axonal morphology. This is very similar to our in vitro observation that showed dramatic clustering of neuronal cells, which accompanied the general

shrinkage of neuronal cells that may have induced cytoskeletal reorganization (Fig. 4A—C). Similar to the results in the neuronal reorganization studies, we observed that this NFH reorganization is inhibited by Ki16425 pre-treatment, indicating that this is mediated by an LPA receptor-specific mechanism (Fig. 4A—C).

3.4. Gintonin induces in vivo morphological changes and migration in cortical, cerebellar, and hippocampal neurons in the adult brain

Because we found morphological changes, neuronal layer changes, and changes in the organization of neurofilament in the mouse brain, we further questioned if gintonin treatment can induce signs of regeneration in neurons in the brain. LPA receptor signaling in the early postnatal period is critical in neural development and can be detected by increased tubulin beta III (Tub B III) signaling [13]. We wondered if similar findings would be observed following gintonin treatment. Tub B III signaling, a marker for neuroprogenitor cells, was increased in the treatment group in a time-dependent manner up to 2 h, suggesting that LPA receptor signaling may induce the activation of the neuroprogenitor cell population in both the cortex and hippocampus (Fig. 5A and B). Notably, similar Tub B III signals were also observed in the cortical plate, which is a phenomenon observed in the developmental stage where there is vertical migration of neuronal cells to the meninges (Fig. 5A). This is very similar to the findings from neuronal
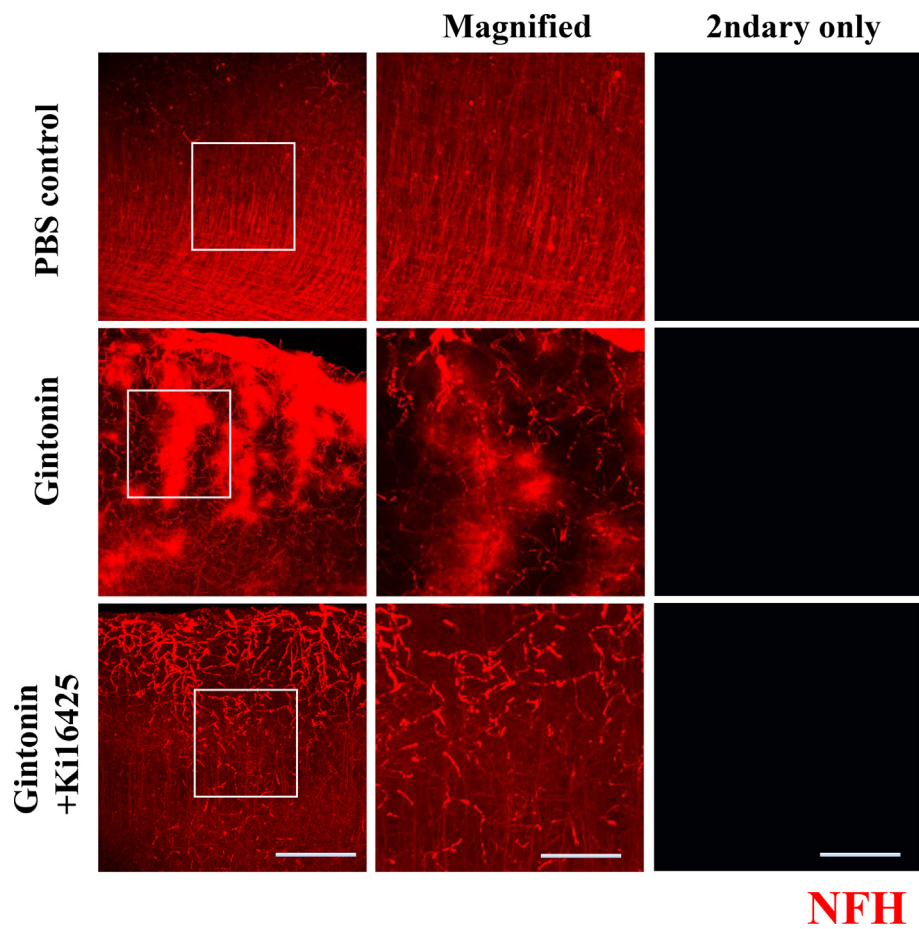

Fig. 4. Changes in the staining pattern of NeuN and Neurofilament H, markers for neuronal nuclei and axonal fibers, respectively, following gintonin treatment. (A and B) Mice were intravenously treated with 10 mg/kg of gintonin or gintonin with Ki16425, an LPA receptor antagonist. Brain sections were stained with Neurofilament H (Red), markers for axonal fibers. Images from left panel (rectangular area) are presented as magnified images in the middle panel. Secondary antibody controls omitting primary antibody are presented on the right panel. Scale bar indicates 200 μm (left) and 75 μm (middle and right), respectively. Intensities of signal from magnified images from Gintonin injected mouse brain (middle, right) was reduced to enhance the visuality of the structure of neurofilaments.
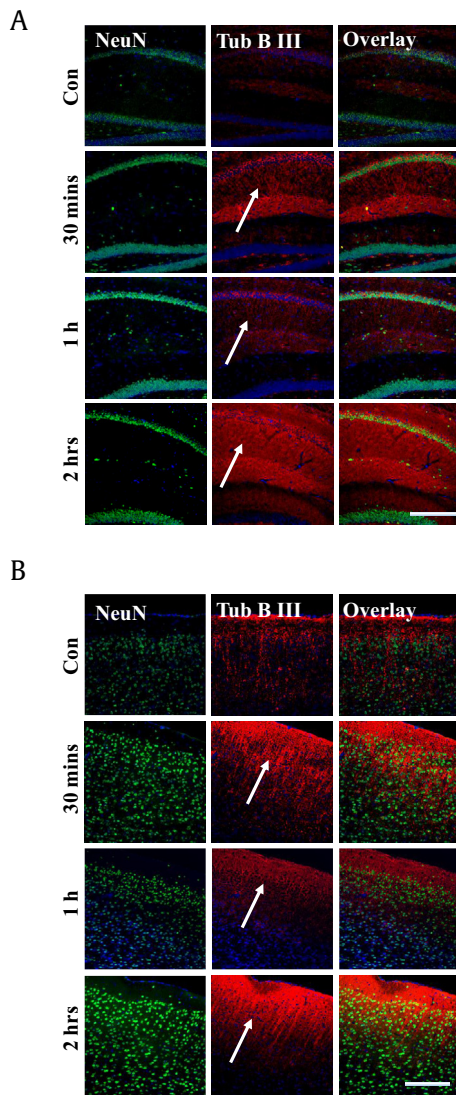
A

B

Fig. 5. Effect of gintonin on the expression of Tubulin beta III, a marker for neuro-progenitor cells, in the hippocampus, cortex, and cerebellum. Mice were intravenously treated with 10 mg/kg of gintonin for different time periods. Brain sections were stained with NeuN (Green) and Tubulin beta III (Red). Images show different areas of the brain, hippocampus (A), cortex (B). Sections were counter stained with DAPI (blue). Arrows indicate where increased intensity of Tubulin beta III are observed in the hippocampus and cortical plates with treatment of gintonin. Scale bar indicates 200 μm.

progenitor cells in terms of its segregation and expansion. It is a very intriguing result since it has been suggested by various re-searchers that the effect of the LPA receptor is present only in the embryonic stage and/or in early postnatal period.

4. Discussion

LPA and the LPA1 receptor play crucial roles in the developing brain [3]. The LPA1 receptor is abundantly expressed in the devel-oping brain and the knockout of this receptor causes lethal nervous system defects [1,4,14,15]. The amount of LPA in the brain is also much greater than that in other peripheral organs [1,3]. In the developing brain, LPA, via LPA1 receptor signaling pathways, in-duces neurogenesis, morphological changes, and neuronal migra-tion [13,16]. Moreover, it has important roles in establishing the neuronal polarity in the initial phase of neuronal development [17].

Interestingly, in the postnatal brain the expression of neuronal LPA1 receptor is diminished and instead glial LPA receptor expression increases, especially in oligodendrocytes, indicating that LPA and the LPA1 receptor are mainly involved in the prenatal period of brain development [18,19]. Such a switch in expression pattern suggests that the role of LPA receptors is more related to the regulation of non-neuronal cells rather than of neuronal cells [20]. A recent line of evidence demonstrated that LPA and the LPA1 re-ceptor also play important roles in a variety of adult brain functions, e.g., long-term potentiation, synaptic transmission, hippocampal neurogenesis, cognition, and psychological disorders including anxiety and mood disorders [21–24]. However, little is known about how endogenous LPA affects adult brain neurons to exhibit its diverse effects. In a previous report, we demonstrated that fluorescent gintonin not only transiently opens the BBB and in-creases brain delivery via the paracellular pathway, but also binds to brain neurons and glia [8]. These results raise the possibility that an exogenous LPA, like gintonin, might exert its effects by crossing the BBB and activate a subsequent signaling pathway by binding to the LPA receptor. Naturally, we questioned if gintonin can elicit a signaling cascade in neuronal cells. The demonstration of the LPA receptor-mediated clustering and expansion of neuro-progenitor cells suggests that the LPA receptor plays important roles in neuro-progenitor cells, which is very similar to findings from the Chun group [19]. However, the observation of neuronal clustering *in vivo* was not expected since the LPA receptor expression level was previously reported to be lower in adult neurons. This finding suggests that adult neuronal cells can be activated by LPA receptor signaling pathway and the potential neuroprotective effect elicited by gintonin might be mediated by this signaling cascade that was not reported previously.

It is still debated whether the LPA receptor functions in the adult brain. Based on our experimental data, we have observed that LPA receptor expression level is significantly lower in the adult mouse brain. However, when we injected the mouse with gintonin (or an antagonist), we observed that the expression level of LPA receptor increased dramatically, similar to the findings from our recent report on BBB permeability. Perhaps this self-positive feedback loop for LPA receptor expression may explain why low LPA receptor expression is not a hurdle for the exertion of effects mediated by LPA receptor agonists. However, it is hard to interpret how neuronal re-localization occurs in the mouse brain after gintonin treatment. However, based on our findings and those from Fukushima et al., it is possible to speculate that LPA receptor signaling induces cyto-skeletal reorganization that may induce the redistribution of neuronal cells [19,21]. It is difficult to conjecture whether this re-distribution of neuronal cells is harmful to the brain, whereas the neurogenesis induced by LPA receptor signaling can strengthen the possibility that gintonin will be beneficial for the brain.Fur-thermore, previous studies from our groups and others have shown that gintonin can enhance locomotor and cognitive functions in Alzheimer's disease animal models, and LTP supports our assumption [23]. To our best knowledge, this is the first report demonstrating the effect of LPA receptor signaling on neuronal cells in the adult brain. This provides not only good evidence suggesting that the modulation of LPA receptor signaling can be an important factor controlling adult neuronal migration and morphological changes but also that gintonin, a natural ginseng extract, can directly modulate brain physiology.

Authors' contributions

D-G Kim and S-Y Nah designed experiments, analyzed and interpreted data and wrote paper. D-G Kim, H-J Kim, S-H Choi, S-N Nam, H-C Kim, H Rhim, I-H Cho performed research and provided

productive advices on experimental designs. All authors read papers and approved the final manuscript.

Conflicts of interest

The authors declare no conflict of interest.

Acknowledgements

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