



## Original Article

## Effects of gintonin-enriched fraction on hippocampal gene expressions



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## ARTICLE INFO

## Article history:

Received 30 March 2020

Received in revised form 2 July 2020

Accepted 6 July 2020

Available online 15 July 2020

## Keywords:

Ginseng

Gintonin

Hippocampus gene

NGS analysis

Cognition

## ABSTRACT

**Background:** Recently, gintonin and gintonin-enriched fraction (GEF) have been isolated from ginseng, a herbal medicine. Gintonin induces  $[Ca^{2+}]_i$  transition in cultured hippocampal neurons and stimulates acetylcholine release through LPA receptor activation. Oral administration of GEF is linked to hippocampus-dependent cognitive enhancement and other neuroprotective effects; however, effects of its long-term administration on hippocampal gene expression remains unknown. Here, we used next-generation sequence (NGS) analysis to examine changes in hippocampal gene expressions after long-term oral administration of GEF.

**Methods:** C57BL/6 mice were divided into three groups: control group, GEF50 (GEF 50 mg/kg, *p.o.*), and GEF100 (GEF 100 mg/kg, *p.o.*). After 22 days, total RNA was extracted from mouse hippocampal tissues. NGS was used for gene expression profiling; quantitative-real-time PCR and western blot were performed to quantify the changes in specific genes and to confirm the protein expression levels in treatment groups. **Results:** NGS analysis screened a total of 23,282 genes, analyzing 11-related categories. We focused on the neurogenesis category, which includes four genes for candidate markers: choline acetyltransferase (ChAT) gene,  $\beta_3$ -adrenergic receptor (Adrb3) gene, and corticotrophin-releasing hormone (Crh) gene, and tryptophan 2,3-dioxygenase (Tdo2) gene. Real-time PCR showed a marked overexpression of ChAT, Adrb3, and Crh genes, while reduced expression of Tdo2. Western blot analysis also confirmed increased ChAT and decreased Tdo2 protein levels.

**Conclusion:** We found that GEF affects mouse hippocampal gene expressions, associated with memory, cognitive, anti-stress and anti-anxiety functions, and neurodegeneration at differential degree, that might explain the genetic bases of GEF-mediated neuroprotective effects.

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## Introduction

Ginseng (*Panax ginseng* C.A. Meyer) has been recognized as a traditional herbal medicine for well-being and longevity for thousands of years in Asian countries.<sup>1</sup> In animal and clinical studies, ginseng has been proved to improve immunity, prevent the aging

process, and strengthen physical performance.<sup>2,3</sup> The pharmacological effects of ginseng are limited to act not only as antioxidants but also as immune enhancers and attenuators of metabolic diseases. In addition, ginseng has been widely studied in regard to cognitive enhancement and the treatment of neurodegenerative diseases.<sup>4,5</sup> The active ingredients of ginseng have also been studied extensively and classified as saponin and non-saponin fractions.

Recently, gintonin was isolated as one of the non-saponin components from ginseng through multiple steps using crude ginseng total saponin fraction. A gintonin-enriched fraction (GEF) was later obtained from ethanol extraction of ginseng through water frac-

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**Table 1**  
Sequences of the primers for real-time polymerase chain reaction.

Gene name	Forward primer sequence	Reverse primer sequence	Size (bp)
Chat	AGGGCAGCCTCTCTGTATGA	ATCCTCGTTGGACGCCATTT	241
Tdo2	GGTGCTGCTCTGCTTGTGTTG	TCGAGGCTCTCCCTGTAAA	124
Adrb3	ATCACTCTGTCTCCAGGCTCT	TGCCTATTGTGAGAGATGGTCC	139
Crh	CAACCTCAGCCGTTCTGAT	GGAAAAAGTTAGCCGCAGCC	160

tionation producing a higher yield than gintonin.<sup>6</sup> Gintonin acts as a novel G protein-coupled lysophosphatidic acid (LPA) receptor ligand from outside of cells, since its active ingredients are LPAs.<sup>7</sup> Thus, gintonin is a non-saponin and non-acidic polysaccharide component isolated from ginseng.<sup>6</sup> The functional studies have shown that gintonin transiently increases the intracellular calcium level in neuronal cells through LPA receptor signal transduction pathways and elicits calcium-dependent diverse physiological activity.<sup>8</sup> In *in vivo* studies, gintonin-mediated activation of LPA-LPA receptor axis induces many beneficial brain functions; the increased hippocampal synaptic transmission and long-term potentiation (LTP), brain-derived neurotrophic factor (BDNF) expression, attenuation of hippocampal aging, enhancement of hippocampal dependent-cognitive functions, and finally prevention and attenuation of neurodegenerative diseases.<sup>9–11</sup>

Relatively little is known on the relationships between gintonin-mediated beneficial effects on hippocampal functions and hippocampal gene expression regulations after gintonin administration. In the present study, we used next-generation sequencing (NGS) analysis to identify gintonin-mediated hippocampal gene regulations after long-term oral administration of GEF.<sup>13,14</sup> We further confirmed the changes in them through quantitative real-time polymerase chain reaction (qRT-PCR) and western blotting and tried to elucidate the co-relationships between gintonin-mediated regulations of hippocampal gene expression and its beneficial effects on brain functions.

## Methods

### Gintonin-enriched fraction preparation

Gintonin-enriched fraction (GEF) was prepared from *Panax ginseng* as per previously reported method.<sup>15</sup> Briefly, one kilogram of four-year-old Korean white ginseng (Korea Ginseng Cooperation, Daejeon, Korea) was locally purchased. The ginseng was cut into small pieces (>3 mm), heated in 70% ethanol at 80 °C for 8 h, and repeated 8 times for extraction. The extracts (344 g) were dissolved in distilled cold water in a ratio of 1:10 and stored at 4 °C for 24 h. Next, the extracts were passed through water fractionation and centrifugation (3000 rpm, 20 min) to obtain a precipitate before lyophilization. The lyophilized sample was designated as GEF. For *in vivo* studies, the GEF stock was prepared with saline. Thus, dosage of GEF (50 or 100 mg/kg) was dissolved in saline before oral administration with gavage.

### Animals and experimental protocols

8-week-old C57BL/6 mice males (18–20 g) were purchased from OrientBio (Chuncheon, Korea) and used for all experiments. Animals were kept at a humidity level of 50 ± 5% with a 12 h/12 h light-dark cycle and were fed with *ad libitum*. All experiments were performed in accordance with the Guidelines for the Care and Use of the Institute for Laboratory Animal Research (ILAR, 2010). This protocol was approved by the Institutional Animal Care and Use Committee of Konkuk University (No. KU17109). Mice were randomly divided into three groups (n = 6/group): control group, gintonin-enriched fraction (GEF) at 50 mg/kg group (GEF50 group),

and GEF at 100 mg/kg (GEF100 group). The control group was treated only with saline, while the GEF50 and GEF100 groups were given GEF at 50 mg/kg or 100 mg/kg, respectively, after dissolving in saline daily for 22 days. After drug administration, mice were anesthetized with diethyl ether and hippocampal tissue samples were removed and frozen for further experiment.

### RNA sample preparation

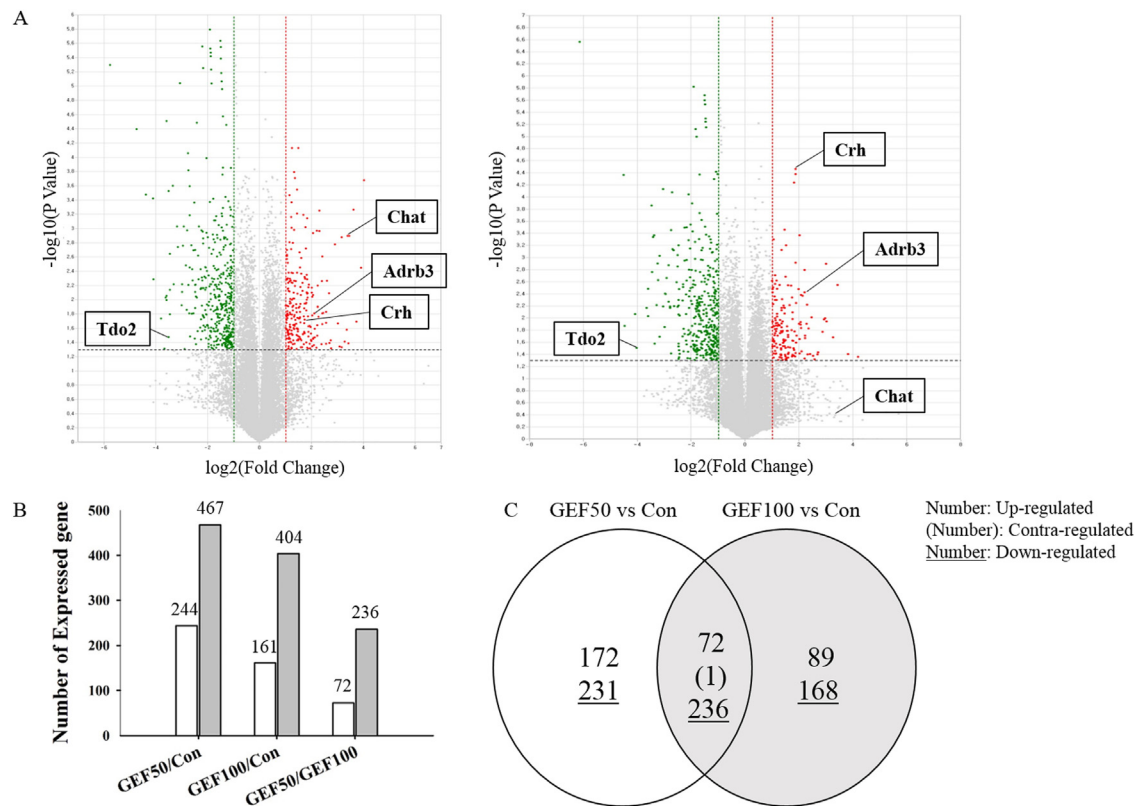
Total RNA from mouse hippocampus was isolated using TRIzol reagent (Invitrogen, Waltham, MA, USA) according to the user's manual with minor modification. The RNA concentration and purity were analyzed *via* spectrophotometer at 260/280 nm (NanoDrop ND1000 3.8.1 System, Thermo Fisher Scientific, Waltham, MA, USA), and the cDNA was synthesized using the total RNA. Briefly, the cDNA reaction mixture was prepared in advance and synthesized from 1 µg of total RNA using a High Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific, Waltham, MA, USA), according to the user's manual. After the PCR conditions were confirmed, real-time PCR was performed with reverse-transcribed cDNA. All the experiments were conducted on ice, and the remaining RNA and synthesized cDNA was stored at –70 °C for further experimental processes.

### Quantitative real-time polymerase chain reaction (qRT-PCR) and primer design

Quantitative real-time PCR analysis was performed as follows: the mRNA levels of each target gene were normalized to that of β-actin mRNA and fold-change was calculated using the 2<sup>–ΔΔCT</sup> method as previously described.<sup>16</sup> All qRT-PCR primer pairs were designed within 250 bp, and the sequences are shown in Table 1. The reaction buffer contained 10 pmol/µL of each primer set and template (1st strand cDNA), iQ SYBR Green Supermix (Bio-Rad, USA, California) according to the manufacturer's protocol. After qRT-PCR was performed in the CFX96 Touch Real-Time PCR Detection System (Bio-Rad, CA, USA), the quantitative data were analyzed using CFX Maestro software using β-actin as a reference gene. The qRT-PCR amplification conditions were as follows: pre-denaturation at 95 °C for 3 min, 44 repeats of the denaturation step at 95 °C for 10 s, and at 60–62 °C for 30 s. To detect the presence of non-specific products, a melt curve was set to 75–95 °C at 0.5 °C increments. All samples were analyzed in duplicate.

### Western blot analysis

15 µg of total proteins from lysed mouse hippocampus were used to detect ChAT and Tdo2. The cells were lysed with modified Radio immunoprecipitation assay buffer (RIPA buffer) with a protease inhibitor cocktail. Choline acetyltransferase (ChAT) and tryptophan 2,3-dioxygenase (Tdo2) were detected by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and blotted to 0.45 µm PVDF membrane using either rabbit anti-ChAT polyclonal antibody(1:1000) or rabbit anti-Tdo2 polyclonal antibody (1:1000). The blotted membrane was stripped and reprobed with mouse anti-β actin monoclonal antibody conjugated with HRP (1:25,000). Images were visualized using Clarity



**Fig. 1.** Transcriptomic analysis of mouse hippocampus after oral administration of GEF. A. Visualization of gene expression through the Volcano map. These data show the gene expression distributions between the control and GEF treated groups. These figures show a broad range of expression patterns for the selection of genes with specific brain-related functions. The results were analyzed based on more than 2.0-fold change and factors with  $p$ -value  $< 0.05$ . The x-axis represents the fold-change and the y-axis represents the  $p$ -value. The red dot in the right shows the distribution chart of up-regulated genes, and the green dot shows the distribution chart of down-regulated genes. It represents the overall expression of genes and identifies distribution of selected candidate genes associated with specific brain functions. The left panel represents the GEF50 group and the right panel represents the GEF100 group. B. The number of genes expressed between control and GEF treatment groups with different dosages. The results were analyzed using the criteria of more than 2.0-fold change and factors. The white bar shows the up-regulated genes and the grey bar shows the down-regulation genes. C. Venn diagram showing the comparison of genes expressed differently between the groups treated with GEF at different dosages. The results were analyzed using the criteria of more than 2.0-fold change; the left side of the Venn diagram (hatched) represents the GEF50 group and the right side represents the GEF100 group. The top of the numbers in each Venn diagram denotes the number of up-regulated genes and an underline denotes the number of down-regulated genes.

Western ECL Substrate (Bio-Rad, Hercules, CA, USA) using the iBright CL1000 (Thermo Fischer Scientific, MA, United States). Chemi-documentation was conducted for further documentation preservation followed by densitometry analysis of the blots using the ImageJ program.

#### NGS (RNA-sequencing) analysis

The animals of each treatment group were anesthetized and the hippocampus was isolated from the mouse brain. The hippocampus was immediately stored in liquid nitrogen. After total RNA samples were isolated, RNA quality check (500 pg–2  $\mu$ g total RNA for RNA-seq), fluorescence labeling, hybridization, and scanning were performed according to the manufacturer's protocol (E-biogen, Seoul, Korea). All data normalization and selection of fold-change of genes were analyzed using ExDEGA (Excel-based Differentially Expressed Gene Analysis tool, E-biogen Technology). Fold changes were analyzed as the ratio between the GEF50 or GEF100 group normalized to the control group resulting in  $\log_2$  gene expression values.

#### Statistical analysis

All values are presented as either mean  $\pm$  S.E.M. or % of control. A  $p$ -value under 0.05 was considered to be statistically significant.

Differences between the means of the control and treatment groups were compared using unpaired Student's  $t$ -test or one-way ANOVA.

## Results

#### Visualization of overall gene expression patterns and selection of candidate genes

After NGS analysis using different GEF treatments, we first obtained volcanic plots, a type of scatter plot, which shows a relative difference in the negative base-10 log and base-2 log fold-changes, respectively. The overall mouse hippocampal gene expression aspects after GEF50 or GEF100 administrations were visually confirmed with fold change  $> 2$  (x-axis) and  $p$ -value  $< 0.05$  (y-axis) compared with the control vehicle group using volcanic plots (Fig. 1A). Red dots represent up-regulated genes and green dots represent down-regulated genes in the hippocampus. This allows for immediate identification of gene expression and significant genes in this volcano map.

As shown in Fig. 1B, in terms of the number of genes expressed, the GEF50 group as compared to the control group, expressed a total of 712 genes, including 244 up-regulated genes and 467 down-regulated genes (Fig. 1B). GEF100 group showed gene expression changes for a total of 566 genes, including 162 up-regulated genes and 404 down-regulated genes (Fig. 1B). The Venn diagrams show the number of expressed genes either up/down in common for the

control and GEF groups (Fig. 1C). The Venn diagram results were analyzed under the condition that the fold change value was at least 2.00 and the  $p$ -value was  $<0.05$ . Compared to the control group, 72 genes were up-regulated, 236 genes were down-regulated, and 1 was contra-regulated in common for the GEF50 and GEF100 treatment groups (Fig. 1C).

#### Differentially expressed genes in mouse hippocampus by hierarchical clustering (HCL) method

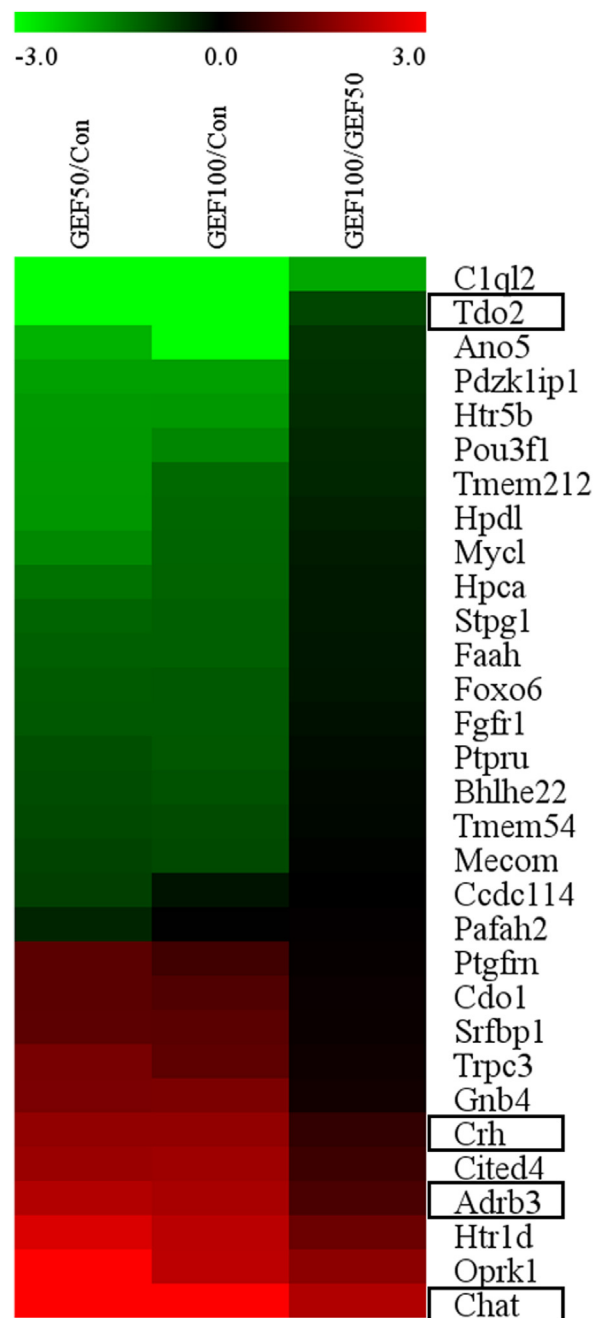
Gene expression changes in the mouse hippocampus of control vehicle, GEF50 or GEF100 group NGS analysis (RNA-seq) was also screened. Multi Experimental Viewer (MeV) program was used to generate hierarchical clustering (HCL) based on the expression patterns of the differentially expressed gene candidates. The resulting gene list with expression pattern levels was clustered together, showing the up-regulated genes with red color and the down-regulated genes with a green color marker (Fig. 2). The up/down-regulated genes were classified as  $>4.0$ -fold change after oral administration of GEF50 or GEF100 as compared to the control group. These data confirmed the 14 most up-regulated genes and the gene list according to the expression pattern (Fig. 2).

#### Identification of genetic changes after GEF administration

The differentially up/down-regulated genes between control and GEF treatment groups were classified into the following 11 functional groups: aging, angiogenesis, apoptotic, cell differentiation, cell migration, cell proliferation, extracellular matrix, immune response, inflammatory response, neurogenesis, and secretion. This classification comes from differentially expressed genes (DEGs) analysis, which is defined based on Quickgo (<https://www.ebi.ac.uk/QuickGO/>). Gene expression of GEF-related functions in mouse hippocampus was identified (Table 2 and Supplementary Tables 1, 2, 3, 4, 5, 6, 7, 8, and 9), and the most significantly regulated genes in the classification analysis category were neurogenesis. As shown in Fig. 1A, we selected candidates such as hippocampal choline acetyltransferase (ChAT),  $\beta_3$ -adrenergic receptor (Adrb3), corticotropin-releasing hormone (Crh), and tryptophan 2,3-dioxygenase (Tdo2) genes (Fig. 1A).

#### Confirmation of changes in hippocampal gene expression by qRT-PCR

To confirm the reliability of the NGS (RNA-seq) data, the relative expression analysis of four selected genes was performed by quantitative real-time PCR (qRT-PCR) (Fig. 3). The expression of the ChAT gene was significantly increased in GEF50 compared to the control group (Fig. 3A). Interestingly, the ChAT gene expression level with GEF50 were higher than that of GEF100, showing that ChAT gene expression was not dose-dependent on GEF and low dose of GEF seems more effective for the increase of brain ChAT gene expression. The expression of the Tdo2 gene was found to be gradually decreased in both dosages of GEF treatment groups compared to the control group (Fig. 3B). The expression of Adrb3 showed a significant increase only in GEF100, even though it showed a slight increase in the GEF50 group (Fig. 3C). Crh gene expression was significantly increased in both GEF50 and GEF100 groups when compared to the control group (Fig. 3D). The consistency of qRT-PCR results with the NGS (RNA-seq) data confirmed the influence of GEF administration on the expression of the selected genes. Thus, results were consistent with NGS analysis data, even at the mRNA level of the genes.



**Fig. 2.** Hierarchical clustering heatmap analysis of mouse hippocampus after oral administration of GEF. Log<sub>2</sub> fold-change vs.  $p$ -value plot of next-generation sequencing (NGS) data analysis of gene expression in isolated mouse hippocampus after oral administration of GEF over saline-treated mice ( $n = 6$ ), highlighting down-regulated genes (green  $<4$ -fold) and up-regulated genes (red  $>4$ -fold). Hierarchical clustering heatmap shows the result of hierarchical clustering (HCL) through MeV programs. Top 11 upregulated genes and the top 20 downregulated genes are shown in GEF vs Saline-treated mice. Black color represents the baseline, up-regulated genes are denoted by red color, and down-regulated genes by green color.

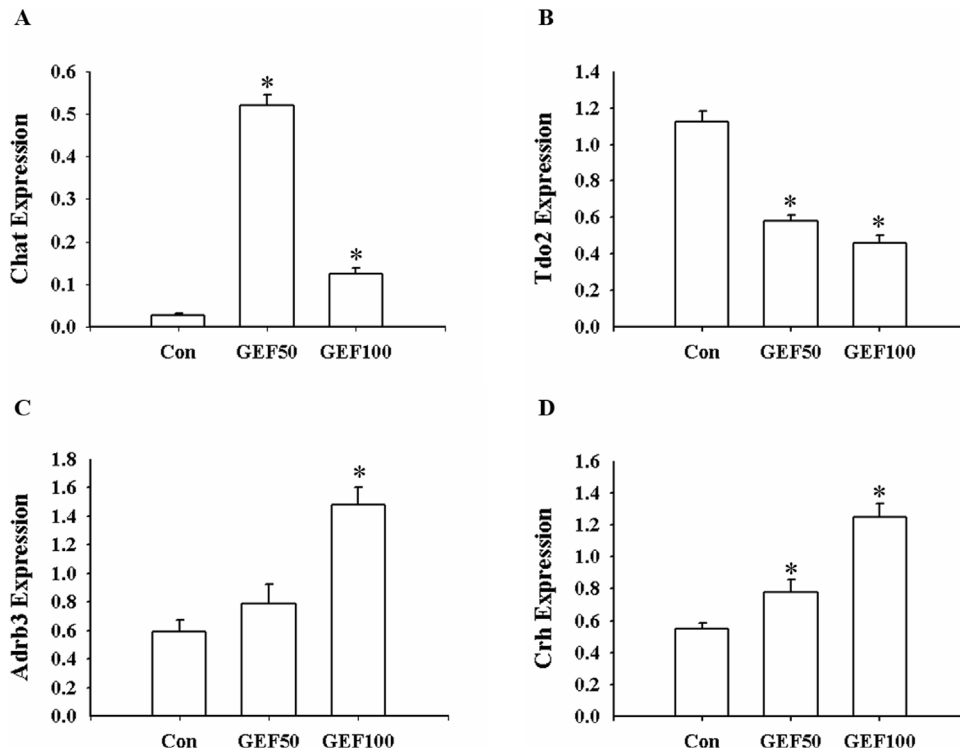
#### Confirmation of changes in protein levels by western blotting

In addition, western blotting was performed to analyze the two selected enzymes that were related to the functions of neurotransmitter synthesis (Fig. 4). GEF administration also increased ChAT expression at protein levels, and the rise was almost similar in both GEF50 and GEF100 groups (Fig. 4A). Next, the Tdo2 protein expression level was observed and the overall expression in both dosages was reduced compared to the control group (Fig. 4B). Western blot-

**Table 2**

List of expressed genes by GEF: Aging group.

Gene symbol	GEF50/Control	GEF100 /Control	transcript.id	Description
Plxnd1	3.396	2.421	NM.026376	plexin D1
Itgb3	2.932	2.219	NM.016780	integrin beta 3
Kdr	0.347	0.482	NM.010612	kinase insert domain protein receptor
Nrp1	0.335	0.458	NM.008737	neuropilin 1
Sema5a	0.417	0.453	NM.009154	sema domain, seven thrombospondin repeats (type 1 and type 1-like), transmembrane domain (TM) and short cytoplasmic domain, (semaphorin) 5A
E2f7	0.384	0.443	NM.178609	E2F transcription factor 7
Jun	0.474	0.396	NM.010591	jun proto-oncogene
Ccbe1	0.239	0.222	NM.178793	collagen and calcium binding EGF domains 1



**Fig. 3.** Quantitative real-time polymerase chain reaction (qRT-PCR) analysis of gene expression change. 50 mg/kg (GEF50) and 100 mg/kg (GEF100) were orally administered to mice and the total RNA from the hippocampus was extracted. A. ChAT expression mRNA changes. ChAT expression is overexpressed in both GEF50 and GEF100 compared to controls. B. Tdo2 expression shows gradual down-regulation in GEF treated groups compared to control as GEF dose increases. C. Adrb3 expression significantly increases in GEF50 group compared to controls. D. In both GEF50 and GEF100 groups, Crh expression is significantly increased.  $\beta$ -actin was used as a comparative quantitation control. All values are expressed as the mean  $\pm$  SEM of the independent experiment (n = 4) in triplicate. \* $p$  < 0.05.

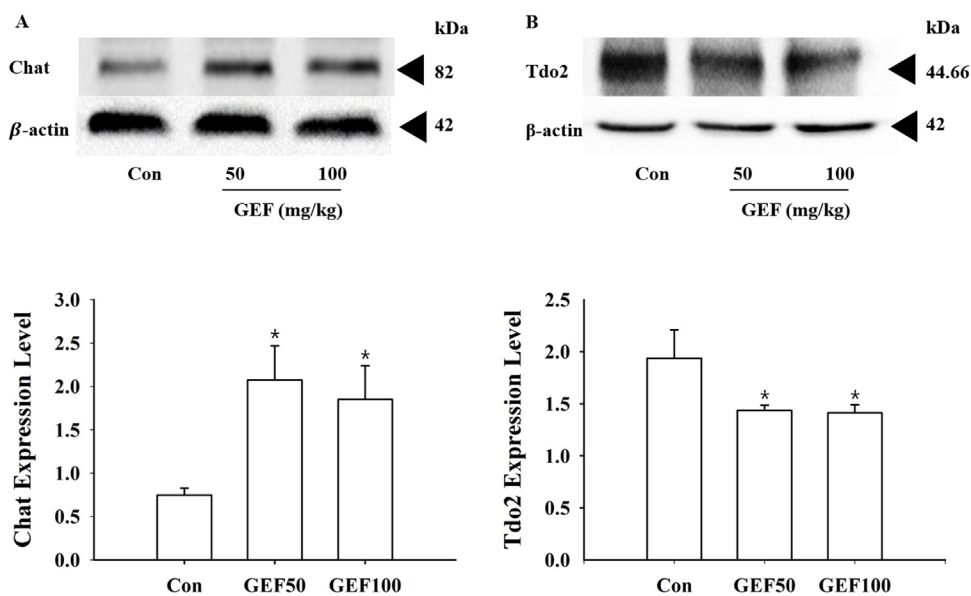
ting results for the ChAT and the Tdo2 protein expression analysis were well consistent with the NGS data and qRT-PCR data.

## Discussion

As previously reported, treatment of gintonin or GEF to neuronal cells induces  $[Ca^{2+}]_i$  transient through LPA receptor activation, which is involved in intracellular and intercellular communications and in neurotransmitter and hormone release regulations that enhance synaptic transmission and further improve cognitive functions.<sup>3</sup> We further showed that *in vitro* gintonin-mediated cellular effects *via* LPA receptors are coupled to beneficial effects in animal models of neurodegenerative diseases such as Alzheimer's disease (AD) and Parkinson's disease (PD).<sup>8,10,17,18</sup> Although previous *in vitro* and *in vivo* studies demonstrated GEF-mediated actions on normal brain and neurodegenerative diseases after oral administration, the effects of GEF on a broad spectrum of genetic expressions in animal brain, especially in the hippocampus was not

fully understood. In the present study, we used NGS analysis (RNA-seq) to elucidate GEF-mediated gene expression regulations.<sup>12</sup> Here, we screened mouse hippocampal gene expression changes using NGS analysis, quantitative real-time PCR, and western blot analysis to confirm differentially expressed genes after oral administration of GEF.

In this study, we performed next-generation sequencing (NGS) analysis to determine the changes in hippocampal gene expressions after GEF administration. The volcano map, a type of scatter plot which shows the level of gene expression between the control group and the treatment groups, showed the overall gene expression patterns and we selected 4 candidate genes that were related to the brain, specifically those associated with cognitive functions, emotion, and stress memory such as ChAT, Adrb3, Crh, and Tdo2 (Fig. 1A). Genetic plots were used to observe the expression patterns of each group (Supplementary Fig. 5). The plot showed a group expression of the ChAT gene (Supplementary Fig. 5A). The expression of ChAT gene was significantly increased in the GEF50



**Fig. 4.** Expression of ChAT and Tdo2 proteins detected by western blot analysis. A. A significant increase in ChAT expression was observed in both groups with GEF administration. Either saline, GEF 50 mg/kg or GEF 100 mg/kg was orally administered to mice and the protein from the hippocampus was extracted.  $\beta$ -actin was used as a control. B. Tdo2 expression change was observed using an anti-Tdo2 antibody showing a significant increase in either GEF50 or GEF100 vs control group. All values are represented as the mean  $\pm$  SEM of three independent experiments in triplicate ( $n=3$ ). \* $p < 0.05$ .

group as compared to the control group. The expression patterns of the *Adrb3* gene (Supplementary Fig. 5C) and the *Crh* gene (Supplementary Fig. 5D) showed up-regulation in both GEF groups (GEF50 and GEF100) compared to the control group. The *Tdo2* gene expression exhibited overall down-regulation, showing a gradual decrease in GEF50 and GEF100. Thus, the oral administration of GEF down-regulated the *Tdo2* gene in the mouse hippocampus (Supplementary Fig. 5B). This suggests that after GEF administration, ChAT, *Adrb3*, *Crh* genes were up-regulated, whereas the *Tdo2* gene was down-regulated. In addition, gene and protein expressions were confirmed by real-time PCR and western blot (Fig. 3 and Fig. 4). Thus, GEF administration regulates 4 genes in a differential manner. In western blotting analysis, we especially chose ChAT and *Tdo2*, since two enzymes are closely related with the syntheses of neurotransmitters such as acetylcholine and 5-HT in brain.

ChAT is a gene involved in the synthesis of acetylcholine.<sup>19</sup> Acetylcholine acts on two types of receptors (muscarinic and nicotinic) in the central nervous system and the muscarinic action of acetylcholine in the central nervous system (CNS) is associated with learning and memory.<sup>20</sup> In AD, the ChAT gene is down-regulated in the hippocampus and acetylcholine decreases<sup>19</sup> and ChAT is a biological candidate marker gene for AD.<sup>21</sup> Therefore, the up-regulation of the hippocampal ChAT gene activity is an important factor in the prevention of mild cognitive dysfunction.<sup>22</sup> In this study, we found ChAT gene up-regulation in both GEF administered groups. Thus, up-regulated expressions of the ChAT gene after administration of GEF suggest that GEF may help to improve cognitive function and to relieve AD by increasing acetylcholine synthesis in brain.<sup>19,22</sup> These results are well consistent with the previous report that gintonin administrations enhance ChAT protein expression in hippocampal neural progenitor cells and AD animal model.<sup>23</sup>

$\beta_3$ -adrenergic receptor (*Adrb3*) increases Trp content upon receptor activation, thereby increasing 5-HT synthesis.<sup>24</sup> The adrenergic receptor expression is also affected by stress.<sup>25</sup> During stress, the *Adrb3* gene is down-regulated and increases anxiety.<sup>25</sup> However, the up-regulation of *Adrb3* reduces anxiety by increasing 5-HT levels in stress situations.<sup>24,25</sup> *Adrb3* activation also increases firing rate of noradrenergic neurons in the rat locus coeruleus.<sup>24</sup>

Corticotropin-releasing hormone (*Crh*), also known as corticotropin-releasing factor, is a gene that is a major regulator of the hypothalamic-pituitary-adrenal axis, which is an important neuroendocrine system that mediates the stress response.<sup>26</sup> The *Crh* gene regulates cortisol secretion, which is associated with lethargy and anxiety, increases cognitive concentration, and enables quick judgment under stress.<sup>27</sup>

In this study, we found the upregulation of *Adrb3* and *Crh* genes after GEF administration. Thus, the up-regulation of these two genes may increase 5-HT levels, which may help to alleviate anxiety-related behaviors and to increase attention under stress followed by a rapid decision-making process.<sup>25,26</sup> Overall, GEF administration may help to reduce stress and anxiety through the regulation of *Adrb3* and *Crh* genes. In a previous report, we showed that gintonin administration increases plasma 5-HT concentration derived from enterochromaffin cells and attenuates depression-like behavior in mice.<sup>28</sup> These results indicate that GEF administration might exhibit anti-depression effects through central and peripheral pathways.

Tryptophan 2,3-dioxygenase (*Tdo2*) is a gene that plays a central role in the physiological regulation of tryptophan flux in the human body as part of the entire biological process of tryptophan (Trp) metabolism and is involved in neurotoxic kynurenine pathway.<sup>29,30</sup> It is known that an overexpression of *Tdo2* increases the metabolism of kynurenine and induces neurodegenerative diseases, and that *Tdo2* protein expression levels are significantly increased in AD patients.<sup>29</sup> In addition, anxiety and depression get worse when Trp, a precursor of 5-HT, and 5-HT concentrations are lowered. However, Trp and 5-HT concentrations increase when *Tdo2* genes are suppressed, thereby relieving anxiety-related behavior and enhancing cognitive function.<sup>31</sup> In this study, we found the down-regulation of the *Tdo2* gene after the oral administration of GEF. Thus, decreased kynurenine metabolites and increased Trp and 5-HT concentrations can lead to a reduction of anxiety in the hippocampus and midbrain for improving search activity and cognitive ability.<sup>32</sup>

In summary, oral administration of GEF affects genetic changes in the mouse hippocampus. Among the plethora of genes influenced, 4 candidate genes were selected. GEF effects on these

selected genes suggest that oral intake of GEF might be beneficial for mitigation of cognition-related AD symptoms through upregulation of ChAT and for the prevention of neurodegenerative diseases through down-regulation of Tdo2. The analyses also indicate that genes related to depression, anxiety, and stress can lower anxiety-like behavior and depression in a stressful environment through up-regulation of ADRB3 and CRH. Finally, long-term GEF administration might be beneficial for cognitive functions, anxiety and depression, and neurodegeneration, although further clinical studies are required to prove them.

### Author contributions

Conceptualization: S-YN; Methodology: RL and N-EL; Software: RL; Validation: S-YN and RL; Formal Analysis: RL and N-EL; Investigation: RL and N-EL; Resources: HR and I-HC; Data Curation: RL, N-EL; Writing - Original Draft: RL and N-EL; Writing - Review & Editing: S-YN and RL; Visualization: S-HC, SMN and H-CK; Supervision: S-YN; Project Administration: S-HH; Funding Acquisition: S-YN.

### Conflict of interest

The authors declare no conflict of interest.

### Funding

This work was supported by the Basic Science Research Program and the Brain Research Program through the National Research Foundation of Korea (NRF), funded by the Ministry of Science, ICT, and Future Planning (NRF 2020R1F1A1058460 and NRF2016M3C7A1913894). This paper was supported by Konkuk University Researcher Fund in 2019.

### Ethical statement

This work was approved by the Institutional Animal Care and Use Committee of Konkuk University (No. KU17109).

### Data availability

The data will be made available upon reasonable request.

### Supplementary material

Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.imr.2020.100475>.

### References

- Choi KT. Botanical characteristics, pharmacological effects and medicinal components of Korean Panax ginseng C A Meyer. *Acta Pharmacol Sin* 2008;29:1109–18.
- Zhang YC, Li G, Jiang C, Yang B, Yang HJ, Xu HY, et al. Tissue-specific distribution of ginsenosides in different aged ginseng and antioxidant activity of ginseng leaf. *Molecules* 2014;19:17381–99.
- Nah SY. Ginseng ginsenoside pharmacology in the nervous system: Involvement in the regulation of ion channels and receptors. *Front Physiol* 2014;5:98.
- Kim HJ, Kim JY, Lee BH, Choi SH, Rhim H, Kim HC, et al. Gintonin, an exogenous ginseng-derived LPA receptor ligand, promotes corneal wound healing. *J Vet Sci* 2017;18:387–97.
- Lee E, Kim S, Chung KC, Choo MK, Kim DH, Nam G, et al. 20(S)-ginsenoside Rh2, a newly identified active ingredient of ginseng, inhibits NMDA receptors in cultured rat hippocampal neurons. *Eur J Pharmacol* 2006;536:69–77.
- Pyo MK, Choi SH, Shin TJ, Hwang SH, Lee BH, Kang J, et al. A simple method for the preparation of crude gintonin from ginseng root, stem, and leaf. *J Ginseng Res* 2011;35:209–18.
- Choi SH, Jung SW, Lee BH, Kim HJ, Hwang SH, Kim HK, et al. Ginseng pharmacology: A new paradigm based on gintonin-lysophosphatidic acid receptor interactions. *Front Pharmacol* 2015;6:245.
- Hwang SH, Shin TJ, Choi SH, Cho HJ, Lee BH, Pyo MK, et al. Gintonin, newly identified compounds from ginseng, is novel lysophosphatidic acids-protein complexes and activates G protein-coupled lysophosphatidic acid receptors with high affinity. *Mol Cells* 2012;33:151–62.
- Choi JH, Jang M, Oh S, Nah SY, Cho IH. Multi-target protective effects of Gintonin in 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine-Mediated model of parkinson's disease via lysophosphatidic acid receptors. *Front Pharmacol* 2018;9:515.
- Choi SH, Kim HJ, Cho HJ, Park SD, Lee NE, Hwang SH, et al. Gintonin-mediated release of astrocytic vascular endothelial growth factor protects cortical astrocytes from hypoxia-induced cell damages. *J Ginseng Res* 2019;43:305–11.
- Kim S, Kim MS, Park K, Kim HJ, Jung SW, Nah SY, et al. Hippocampus-dependent cognitive enhancement induced by systemic gintonin administration. *J Ginseng Res* 2016;40:55–61.
- Bruchova H, Borovanova T, Klamova H, Brdicka R. Gene expression profiling in chronic myeloid leukemia patients treated with hydroxyurea. *Leuk Lymphoma* 2002;43:1289–95.
- Tenedini E, Roncaglia E, Ferrari F, Orlandi C, Bianchi E, Biciat S, et al. Integrated analysis of microRNA and mRNA expression profiles in physiological myelopoiesis: Role of hsa-mir-299-5p in CD34+ progenitor cells commitment. *Cell Death Dis* 2010;1:e28.
- Sampson J, Jacobs K, Yeager M, Chanock S, Chatterjee N. Efficient study design for next generation sequencing. *Genet Epidemiol* 2011;35:269–77.
- Choi SH, Jung SW, Kim HS, Kim HJ, Lee BH, Kim JY, et al. A brief method for preparation of gintonin-enriched fraction from ginseng. *J Ginseng Res* 2015;39:398–405.
- Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. *Methods* 2001;25:402–8.
- Jo MG, Ikram M, Jo MH, Yoo L, Chung KC, Nah SY, et al. Gintonin mitigates MPTP-induced loss of nigrostriatal dopaminergic neurons and accumulation of alpha-synuclein via the Nrf2/HO-1 pathway. *Mol Neurobiol* 2019;56:39–55.
- Moon J, Choi SH, Shim JY, Park HJ, Oh MJ, Kim M, et al. Gintonin administration is safe and potentially beneficial in cognitively impaired elderly. *Alzheimer Dis Assoc Disord* 2018;32:85–7.
- Wilcock GK, Esiri MM, Bowen DM, Smith CC. Alzheimer's disease. Correlation of cortical choline acetyltransferase activity with the severity of dementia and histological abnormalities. *J Neurol Sci* 1982;57:407–17.
- Oda Y. Choline acetyltransferase: The structure, distribution and pathologic changes in the central nervous system. *Pathol Int* 1999;49:921–37.
- Hampel H, Mesulam MM, Cuello AC, Farlow MR, Giacobini E, Grossberg GT, et al. The cholinergic system in the pathophysiology and treatment of Alzheimer's disease. *Brain* 2018;141:1917–33.
- DeKosky ST, Ikonomic MD, Styren SD, Beckett L, Wisniewski S, Bennett DA, et al. Upregulation of choline acetyltransferase activity in hippocampus and frontal cortex of elderly subjects with mild cognitive impairment. *Ann Neurol* 2002;51:145–55.
- Kim HJ, Shin EJ, Lee BH, Choi SH, Jung SW, Cho IH, et al. Oral administration of gintonin attenuates cholinergic impairments by scopolamine, Amyloid-β protein, and mouse model of Alzheimer's disease. *Mol Cells* 2015;38:796–805.
- Claustre Y, Leonetti M, Santucci V, Bougault I, Desvignes C, Rouquier L, et al. Effects of the beta3-adrenoceptor (Adrb3) agonist SR58611A (amibegron) on serotonergic and noradrenergic transmission in the rodent: Relevance to its antidepressant/anxiolytic-like profile. *Neuroscience* 2008;156:353–64.
- Li L, Feng X, Zhou Z, Zhang H, Shi Q, Lei Z, et al. Stress accelerates defensive responses to looming in mice and involves a locus coeruleus-superior colliculus projection. *Curr Biol* 2018;28, 859–871 e5.
- Jiang A, Tran TT, Madison FN, Bakker A. Acute stress-induced cortisol elevation during memory consolidation enhances pattern separation. *Learn Mem* 2019;26:121–7.
- Heinrichs SC, Koob GF. Corticotropin-releasing factor in brain: A role in activation, arousal, and affect regulation. *J Pharmacol Exp Ther* 2004;311:427–40.
- Kim HJ, Park SD, Lee RM, Lee BH, Choi SH, Hwang SH, et al. Gintonin attenuates depressive-like behaviors associated with alcohol withdrawal in mice. *J Affect Disord* 2017;215:23–9.
- Breda C, Sathyasaikumar KV, Sograte Idrissi S, Notarangelo FM, Estranero JG, Moore GG, et al. Tryptophan-2,3-dioxygenase (TDO) inhibition ameliorates neurodegeneration by modulation of kynurenine pathway metabolites. *Proc Natl Acad Sci U S A* 2016;113:5435–40.
- Meng B, Wu D, Gu J, Ouyang S, Ding W, Liu ZJ. Structural and functional analyses of human tryptophan 2,3-dioxygenase. *Proteins* 2014;82:3210–6.
- Kanai M, Funakoshi H, Takahashi H, Hayakawa T, Mizuno S, Matsumoto K, et al. Tryptophan 2,3-dioxygenase is a key modulator of physiological neurogenesis and anxiety-related behavior in mice. *Mol Brain* 2009;2:8.
- Hattori S, Takao K, Funakoshi H, Miyakawa T. Comprehensive behavioral analysis of tryptophan 2,3-dioxygenase (Tdo2) knockout mice. *Neuropsychopharmacol Rep* 2018;38:52–60.