Research Article

Ginsenoside Rb1 exerts neuroprotective effects through regulation of Lactobacillus helveticus abundance and GABA<sub>A</sub> receptor expression

Huimin Chen<sup>1</sup>, Jiajia Shen<sup>1</sup>, Haofeng Li<sup>1</sup>, Xiao Zheng, Dian Kang, Yangfan Xu, Chong Chen, Huimin Guo, Lin Xie, Guangji Wang<sup>2</sup>, Yan Liang<sup>3</sup>

Key Lab of Drug Metabolism & Pharmacokinetics, State Key Laboratory of Natural Medicines, China Pharmaceutical University, Nanjing, China

A R T I C L E   I N F O

Article history:
Received 30 January 2018
Received in Revised form 2 June 2018
Accepted 11 September 2018
Available online 19 September 2018

Keywords:
Gamma-aminobutyric acid receptor
Ginsenoside Rb1
Lactobacillus helveticus
Microbiota
Neuroprotective effects

A B S T R A C T

Background: Ginsenoside Rb1 (Rb1), one of the most abundant protopanaxadiol-type ginsenosides, exerts excellent neuroprotective effects even though it has low intracephalic exposure.

Purpose: The present study aimed to elucidate the apparent contradiction between the pharmacokinetics and pharmacodynamics of Rb1 by studying the mechanisms underlying neuroprotective effects of Rb1 based on regulation of microflora.

Methods: A pseudo germ-free (PGF) rat model was established, and neuroprotective effects of Rb1 were compared between conventional and PGF rats. The relative abundances of common probiotics were quantified to reveal the authentic probiotics that dominate in the neuroprotection of Rb1. The expressions of the gamma-aminobutyric acid (GABA) receptors, including GABAA receptors (α2, β2, and γ2) and GABAB receptors (1b and 2), in the normal, ischemia/reperfusion (I/R), and I/R + Rb1 rat hippocampus and striatum were assessed to reveal the neuroprotective mechanism of Rb1.

Results: The results showed that microbiota plays a key role in neuroprotection of Rb1. The relative abundance of Lactobacillus helveticus (Lac.H) increased 15.26 fold after pretreatment with Rb1. I/R surgery induced effects on infarct size, neurological deficit score, and proinflammatory cytokines (IL-1β, IL-6, and TNF-α) were prevented by colonizing the rat gastrointestinal tract with Lac.H (1 × 10<sup>9</sup> CFU) by gavage 15 d before I/R surgery. Both Rb1 and Lac.H upregulated expression of GABA<sub>A</sub> receptors in I/R rats. Coadministration of a GABA<sub>A</sub> receptor antagonist significantly attenuated neuroprotective effects of Rb1 and Lac.H.

Conclusion: In sum, Rb1 exerts neuroprotective effects by regulating Lac.H and GABA receptors rather than through direct distribution to the target sites.

© 2018 The Korean Society of Ginseng. Publishing services by Elsevier B.V. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

1. Introduction

The human body is considered a super-complex ecosystem, and the human gastrointestinal tract is inhabited by 10<sup>13</sup>–10<sup>14</sup> microorganisms, which is thought to be 10 times greater than the number of cells [1]. The microbiome in the gastrointestinal tract is mainly defined by two bacterial phylotypes, namely Bacteroidetes and Firmicutes. Other bacterial phylotypes, including Actinobacteria, Fusobacteria, Proteobacteria, and Verrucomicrobia, are present in relatively low abundance [2]. Recent studies have provided compelling evidence that gut microbiota play crucial roles in shaping the metabolic and regulatory networks that define good health and a spectrum of disease states [3]. For instance, it has been found that gut microbiota can supply the host with multiple functions (e.g., by contributing to food digestion, drug biotransformation, vitamin supplementation, regulating expression of genes involved in utilization of carbohydrates and lipids, and providing defense against pathogenic strains) by interacting with the host organism through direct contact or various indirect soluble molecules [4,5].

Apart from autonomic regulation of digestion by the central nervous system (CNS) and neuroendocrine factors, increasing evidence has suggested that gut microbiota are closely involved with brain functions, including mood, cognitive function, and stress-

* Corresponding authors. Key Lab of Drug Metabolism & Pharmacokinetics, State Key Laboratory of Natural Medicines, China Pharmaceutical University, 24 Tongjia Alley, Gulou, Nanjing 210009, China.

E-mail addresses: guangjiwang@hotmail.com (G. Wang), liangyan0679@163.com (Y. Liang).

<sup>1</sup> These authors contributed equally to this work.

https://doi.org/10.1016/j.jgr.2018.09.002

p1226-8453 e2093-4947$ — see front matter © 2018 The Korean Society of Ginseng. Publishing services by Elsevier B.V. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).
associated anxiety or depression in humans [6,7]. The brain affects
the gut function; similarly, the gut can induce changes in the CNS.
Bidirectional communication between enteric microbiota and brain
function has been defined as the “gut—brain axis” which may have
profound effects on CNS development and most aspects of behavior
relevant to pathological cognitive function [8–11]. Probiotics,
defined as “live microorganisms which when administered in
adequate amounts confer a health benefit on the host,” were found
to play a crucial role in the gut—brain axis [12]. For example, germ-
free mice displayed exaggerated stress and anxiety-like behaviors
compared with conventional specific-pathogen–free mice, but the
germ-free animals exhibited a complete normalization of behavior
after being treated with Bifidobacterium (Bif.) infantis [12]. Ingestion
of some Lactobacillus (Lac.) strains was demonstrated to not only
treat certain disorders but also attenuate emotional behavior and
impairment of cognition [13,14]. To date, multiple probiotic bacteria
with psychotropic potential have been reported, including Bif. bifidum,
Bif. breve, Bif. longum (Bif.L), Bif. lactis, Lact. acidophilus, Lact.
casei, Lac. plantarum, Lac. reuteri, Lac. rhamnosus (Lac.R), Lac. sali-
varius, and Enterococcus sp. [15–17].

Gamma-aminobutyric acid (GABA), one of the main inhibitory
neurotransmitters in the adult brain, plays a central role in synaptic
plasticity by modulating the inhibitory—excitatory balance neces-
sary for proper brain function in adult brains [18]. In general, the
physiological effects of GABA are mediated by two major classes of:
ionotropic GABA_A receptors, which are formed by coas-
semble of different subunits (α, β, and γ subunits), and GABA_B re-
ceptors, which are G protein–coupled receptors, composed of two
types of subunits (1b and 2) [19–21]. GABA receptors are found in a
wide range of immune cells, such as dendritic cells, mast cells, and T
cells, and are involved in regulating various immunological pro-
cesses [4,22]. Panax notoginsenoside extract (PNE), which is an
extract from the traditional Chinese herb Panax notoginseng, has
been commonly used to treat cardio-cerebrovascular diseases for
thousands of years [23,24]. However, cerebral exposure levels to
extract from the traditional Chinese herb Panax notoginseng (PNE), which is an
class of different subunits (1b and 2 subunits) and anti-GABAB (1b and 2 subunits) antibodies were also purchased from Sigma-Aldrich Corporation. Neomycin sulfate,
streptomycin, and 2, 3, 5-triphenyltetrazolium hydrochloride (TTC)
were also purchased from Sigma-Aldrich Corporation. Lactobacillus helveticus (CICC 20275) was purchased from China Center of Indu-
ctrial Culture Collection (Beijing, China). A Bacterial Genomic
DNA Extraction Kit was purchased from Takara Bio., Inc. (Noji-
gashi, Kusatsu, Shiga, Japan). All the ExCell enzyme-linked immu-
nosorbent assay (ELISA) kits were purchased from ExCell
Biotechnology Corp., Ltd. (Shanghai, China). Anti-GABA receptor
antibodies were purchased from Abcam (Ann Arbor, MI, USA).

2.2. Animals and treatments

Animals: All the animal experiments were approved by the Ethical Committee of Animal Experiments of China Pharmaceutical
University. Healthy Sprague–Dawley rats (220 ± 10 g) were provided by
Shanghai Super-B&K Laboratory Animal Corp., Ltd. (Shanghai,
China). Ethical procedures were conducted by following the princi-
plies of Reduction, Replacement, and Refinement (the 3 Rs rule).
All animals were kept in an environmentally controlled breeding room
(temperature: 20–24°C, humidity: 40–70%, and a 12-h dark/light
cycle) and fed with standard laboratory food and water for about
5 days before starting the experiments. Before each experiment, all
the rats were fasted for 12 h with free access to water. In addition, the I/R
rat model was prepared by a middle cerebral artery occlusion
method as per previous reports [26]. The pseudo germ-free (PGF)
rat model was established by intragastrical administration of neomycin
cysteine combined with streptomycin [26].

Drug administration: Rb1 was dissolved in saline and intra-
gastrically administered to rats at a dose of 50 mg/kg once a day.
Pre-treatment lasted for 6 d, and the rats were given a single
administration (50 mg/kg) after reperfusion on the 7th day. Rats in
the vehicle group were administered with saline using the same
protocol mentioned previously.

Infarct volume analysis: Twenty-six hours after cerebral
infarction (2-h ischemia and 24-h reperfusion), the rats
were anesthetized and killed by rapid decapitation. Brains were removed
and sectioned into standard coronal slices (2-mm thick). The
sections were immediately immersed in TTC medium, which was
prepared by dissolving TTC (0.125% w:v) in a buffer solution con-
aining 62.5 mM tri-S-HCl, 13 mM MgCl2, and 1.5% dimethylforma-
ide (temperature: 24°C, humidity: 70%, and a 12-h dark/light
cycle) and fed with standard laboratory food and water for about
5 days before starting the experiments. Before each experiment, all
the rats were fasted for 12 h with free access to water. In addition, the I/R
rat model was prepared by a middle cerebral artery occlusion
method as per previous reports [26]. The pseudo germ-free (PGF)
rat model was established by intragastrical administration of neomycin
cysteine combined with streptomycin [26].

In the present study, we aimed to elucidate the apparent
contradiction between the pharmacokinetics and pharmacody-
namics of Rb1 by studying neuroprotective mechanisms of Rb1 in
rats subjected to I/R-induced focal cerebral injury.

2. Materials and methods

2.1. Chemicals and standards

Rb1 was purchased from Nanjing Sart Science & Technology
Development Co., Ltd (Nanjing, Jiangsu, China). Saclofen (Sac; a
GABAA receptor antagonist) and streptomycin (a GABAA receptor
antagonist) were purchased from Sigma-Aldrich Corporation (St.
Louis, MO, USA). Rabbit polyclonal anti-GABAA (α2, β2, and γ2
subunits) and anti-GABAB (1b and 2 subunits) antibodies were also purchased from Sigma-Aldrich Corporation. Neomycin sulfate,
streptomycin, and 2, 3, 5-triphenyltetrazolium hydrochloride (TTC)
were also purchased from Sigma-Aldrich Corporation. Lactobacillus helveticus (CICC 20275) was purchased from China Center of Indu-
ctrial Culture Collection (Beijing, China). A Bacterial Genomic
DNA Extraction Kit was purchased from Takara Bio., Inc. (Noji-
gashi, Kusatsu, Shiga, Japan). All the ExCell enzyme-linked immu-
nosorbent assay (ELISA) kits were purchased from ExCell
Biotechnology Corp., Ltd. (Shanghai, China). Anti-GABA receptor
antibodies were purchased from Abcam (Ann Arbor, MI, USA).

2.3. Measurement of proinflammatory cytokine levels using ELISA

Tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), and
interleukin-1β (IL-1β) levels on the experimental stroke side were
quantified using ExCell ELISA kits (ExCell Biology) as per the
manufacturer's instructions. In brief, the brain tissue was homog-
einized in phosphate-buffered solution (PBS, pH 7.4). The protein
centrations of each sample was determined using a BCA Protein
Assay Kit (Beyotime, Shanghai, China).
2.4. Lac.H cultivation and colonization

Lac.H (CICC 20275, Lac) was inoculated into de Man, Rogosa, and Sharpe medium containing 8 g/L of beef extract, 4 g/L of yeast extract, 10 g/L of proteose peptone, 20 g/L of glucose, 5 g/L of sodium acetate, 2 g/L of triammonium citrate, 0.2 g/L of magnesium sulfate, 0.05 g/L of manganese sulfate, 2 g/L of dipotassium hydrogen phosphate, and 1 g/L of polysorbate 80. After incubating at 37°C for 12 h, Lac.H was collected by centrifuging at 5000 g (4°C) for 15 min and resuspending at a concentration of 1 x 10^9 colony-forming unit (CFU)/mL in de Man, Rogosa, and Sharpe medium. Lac.H was then stored as frozen aliquots at -70°C until use. Lac.H-colonized rats were prepared by intragastrically administering 1 x 10^9 CFU of Lac.H once per day for 15 consecutive days before I/R surgery, followed by one dose after reperfusion.

2.5. The influence of GABA receptor antagonists on the neuroprotective effects of Rb1 and Lac.H

To investigate the effects of GABA receptors on the neuroprotective effect of Rb1 and Lac.H, 0.2 mg kg^-1 of bicuculline (Bic; a GABAA receptor antagonist) was intraperitoneally administrated to rats, whereas 0.1 mg kg^-1 of Sac (a GABAB receptor antagonist) was intravenously administrated to rats 30 min before I/R surgery. The rats in the vehicle group were treated with an isotonic medium.

2.6. Quantitative analysis of Rb1 based on LC–MS/MS

Sample collection and preparation: Both I/R model and normal rats were administrated with Rb1 intragastrically at a dose of 50 mg/kg. The rat plasma, striatum, and hippocampus were collected at 1, 6, and 24 h after intragastrical administration of Rb1 at a dose of 50 mg/kg. All the tissues (~0.1 g) were homogenized in 1 mL of water in an ice bath. The tissue homogenates were extracted using n-butanol as previously described [26].

Instruments, parameters, and conditions: The LC–MS/MS 8050 system (Shimadzu; Tokyo, Japan) was used to analyze Rb1 in a biological matrix. Chromatographic separation was performed on a C18 reversed-phase LC column (Thermo Hypersil GOLD ODS 5 μm; 50 mm x 2.1 mm I.D., Thermo Scientific, USA). The optimized MS-operating parameters were as follows: 3 L/min of nebulizing gas, 10 L/min of heating gas, and 250°C desolvation temperature. Quantification was performed using multiple reaction monitoring acquisition mode by monitoring the precursor ion to product ion transitions of m/z 1143.7/945.6 for Rb1 and m/z 815.5/779.4 for digoxin (IS). The collision energy values for Rb1 and IS were 53 and 30, respectively. The present assay was fully validated in our previous study with respect to linearity, sensitivity, intraassay and interassay precision and accuracy, recovery, and matrix effect [35].

2.7. Western blot analysis of GABA receptors

The hippocampus and striatum were homogenized in radioimmunoprecipitation assay lysis and extraction buffers containing 1 mmol/L of the protease inhibitor phenylmethylsulfonyl fluoride(Beyotime). Protein concentration was determined using a BCA Protein Assay Kit (Beyotime). The proteins were separated on a 4–14% gel using tris-glycine sodium dodecyl sulfate polyacrylamide gelelectrophoresis (SDS-PAGE) and then transferred onto a nitrocellulose membrane. The blots were washed with tris-buffered saline with Tween 20 (TBST) buffer and then blocked in TBST buffer supplemented with 5% nonfat milk powder for 1 h at room temperature. The blots were then incubated with either rabbit polyclonal anti-GABA_A α2 subunit antibody (1:1000, ab72445), rabbit anti-GABA_A β2 subunit antibody (1:30000, ab16213), rabbit anti-GABA_A γ2 subunit antibody (1:1000, ab16213), rabbit anti-GABA_B 1b subunit antibody (1:1000, ab166604), or rabbit anti-GABA_B 2 subunit antibody (1:1000, ab52248) overnight at 4°C. After washing the blots thrice with TBST, the membranes were incubated with horseradish peroxidase–conjugated goat anti-rabbit IgG antibody (1:10000) for 60 min and then washed again. Membrane-bound secondary antibodies were detected using chemiluminescence.
2.8. Real-time Polymerase Chain Reaction (PCR) analysis of GABA receptors

The rat hippocampus and striatum were dissected and isolated under cold conditions. RNA was extracted and isolated as per standard procedures using TRIzol reagent (Takara, Kyoto, Japan). Purity and concentration of RNA were determined using a dual-beam UV-Vis spectrophotometer (BioTek, Winooski, VT, USA). Total RNA (1 μg) was reverse transcribed to cDNA using the PrimeScript RT Reagent Kit (Takara). Real-time PCR was performed using a Real-Time PCR detection system (Bio-Rad) with SYBR Green Real-Time PCR Master Mix (Bio-Rad) as per the manufacturer’s instructions. Specific primers for rat GABA<sub>A</sub> receptor subunits (α2, β2, and γ2), GABA<sub>B</sub> receptor subunits (1b and 2), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) are listed in Supplemental Table 1.

2.9. Real-time PCR analysis of gut microbiota

Total genomic DNA of intestinal microbiota was extracted using the Bacterial Genomic DNA Extraction Kit, Ver. 3.0 (Takara). Real-time PCR for microbiota genomic DNA was performed on a thermal cycler with the following parameters: initial denaturation at 94°C for 5 min; 40 cycles of denaturation at 94°C for 15 s, annealing at 59°C for 30 s, and elongation at 72°C for 30 s. The primers are shown in Supplemental Table 2.

3. Results

3.1. Pharmacokinetics of Rb1 in control and I/R rats

As illustrated in Fig. 1, Rb1 concentrations in the rat hippocampus and striatum collected at 1 h, there was no significant difference in concentrations of Rb1 between the control and I/R groups (p > 0.05). At 6 h, Rb1 concentrations in the hippocampus and striatum of I/R model rats tended to be higher than those of the control rats, but the differences were not significant (p > 0.05). At 24 h, the difference of the Rb1 concentrations in the hippocampus and striatum between the control and I/R groups was also not significant (p > 0.05).

In rat plasma, concentrations of Rb1 in the I/R model rats were significantly higher than those in control rats at 6 h and 24 h after drug administration. Ratios of brain to plasma concentrations were calculated to compare exposure of Rb1 in plasma and the brain. As shown in Supplemental Table 2, intracephalic exposure to Rb1 was very low because all ratios were much lower than 0.1.

3.2. Neuroprotective effects of Rb1 mediated by intestinal microbiota

As shown in Figs. 2A, 2B, cerebral infarcts were pronounced after I/R surgery, and infarct volumes were significantly reduced by pretreatment with 50 mg/kg of Rb1 for 7 consecutive days. Compared with the I/R group, infarct size was significantly decreased from 22.83 ± 7.33% to 5.95 ± 2.47% by Rb1. Neurological deficits were evaluated by scoring specific behaviors; as shown in Fig. 2C, the
mean neurological deficit score of the control group was significantly lower than that of the I/R group. Furthermore, the increase in the neurological deficit score caused by I/R surgery could be greatly reduced by pretreatment with Rb1 \((p < 0.001)\). In addition, levels of IL-1, IL-6, and TNF-\(\alpha\) in the stroke region were much higher in the I/R group than in the control group \((p < 0.05)\). However, after pretreatment with Rb1, levels of IL-1, IL-6, and TNF-\(\alpha\) were significantly decreased when compared with untreated I/R model rats \((p < 0.05)\) (Figs. 2D–2F).

Taken together, these results demonstrate that Rb1 exerts prominent after establishing the I/R model in PGF rats. Cerebral infarct volume was 20.86 \(\pm\) 4.48% by pretreatment with Rb1 \((p < 0.001)\). Thus, the relative populations of Lac.B, Lac.H, and Lac.R in the I/R rats to those in the control group, respectively, were downregulated by I/R surgery, and Rb1 treatment could significantly upregulate the populations of Lac.H \((p < 0.05)\). After pretreatment with Rb1 for 7 consecutive days, the abundances of Lac.B, Lac.H, and Lac.R increased by 2.13, 5.18, 15.26, and 3.30 folds relative to the control group, respectively. Hence, Rb1 had the most significant effect on Lac.H in I/R rats. Then, populations of Lac.H in the control, I/R, I/R + Rb1, PGF + I/R, and PGF + I/R + Rb1 groups were compared to further investigate the role of Lac.H in Rb1 neuroprotection. The relative populations of Lac.H were obtained by calculating the ratios of the probiotics abundances in I/R, I/R + Rb1, PGF + I/R, or PGF + I/R + Rb1 rats to those of the corresponding control group rats. Clearly, the relative populations of Lac.H in the I/R + Rb1 rat groups were significantly higher than those in the control group rats \((p < 0.05)\) (Fig. 3B). Thus, Rb1 treatment could significantly reverse the reduction of Lac.H caused by I/R surgery \((p < 0.05)\). The lack of increase of Lac.H in PGF mice treated with Rb1 could be related to the action of antibiotics neomycin sulfate and streptomycin.

### 3.4. Neuroprotective effects of Lac.H in I/R model rats

Lac.H was cultured and colonized in rats by intragastrically administrating \(1 \times 10^{8}\) CFU of Lac.H once per day for 15 consecutive days, and then neuroprotective effects of Lac.H were evaluated. As shown in Figs. 4A–4C, Lac.H colonization greatly reduced the volume of infarcts caused by I/R surgery, and the increase in neurological deficit score caused by I/R surgery was significantly attenuated by colonization with Lac.H \((p < 0.001)\). The levels of IL-1, IL-6, and TNF-\(\alpha\) in the I/R group were significantly down-regulated by Lac.H \((p < 0.05)\) (Figs. 4D–4F).

### 3.5. Effects of Lac.H on GABA receptor expression in the hippocampus

Previous studies have revealed that lactic acid bacteria, such as Lac.R, could directly affect neurotransmitter receptors in normal, healthy animals [19]. However, changes in GABA\(_B\) receptor subunit expression induced by I/R surgery and Lac.H colonization were not...
3.6 Roles of GABA<sub>A</sub> and GABA<sub>B</sub> receptors in neuroprotective effects of Lac<H>

A GABA<sub>A</sub> receptor antagonist (bicuculline, Bic) and GABA<sub>B</sub> receptor antagonist (Sac) were used to inhibit GABA receptors by intraperitoneal injection before I/R surgery. As shown in Figs. 6A, 6B, Lac<H> colonization greatly reduced infarct volumes caused by I/R surgery, and both Bic and Sac reduced the effect of Lac<H> on the infarct volume. The mean cerebral infarct volumes in the I/R, I/R+Lac<H>, I/R+Lac<H>+Bic, and I/R+Lac<H>+Sac groups were 7.38 ± 2.47%, 16.0 ± 2.30%, and 10.94 ± 3.63%, respectively. The mean neurological deficit scores of the I/R, I/R+Lac<H>, I/R+Lac<H>+Bic, and I/R+Lac<H>+Sac groups were 1.00 ± 0.35, 2.38 ± 0.45, and 1.50 ± 0.42, respectively (Fig. 6C). Measurement of inflammatory factor levels demonstrated that IL-1β, IL-6, and TNF-α levels in the I/R+Lac<H>+Bic group were significantly higher than those in the I/R+Lac<H> group, whereas no significant difference was found between the I/R+Lac<H> and I/R+Lac<H>+Sac groups.

3.7 Effects of Rb1 on GABA receptor expression

The effects of Rb1 on expression of GABA receptors were investigated by measuring the protein and RNA expression levels of GABA<sub>A</sub> receptors (α2, β2, and γ2) and GABA<sub>B</sub> receptors (R1b and R2) in the rat hippocampus using Western blot real-time PCR analysis. As shown in Figs. 7A, 7B, pretreatment with Rb1 significantly attenuated decreases in protein expression levels of GABA<sub>A</sub> subunits (α2, β2, and γ2) in two brain regions of the I/R group (p < 0.05). Although protein expression of the GABA<sub>B</sub> subunits was also regulated by Rb1, differences between the I/R and I/R+Rb1 groups were not significant (p > 0.05). Besides, the results of the determination of relative RNA expression indicated that the variation trend of RNA expression of GABA<sub>A</sub> receptors was in accordance with that of proteins (Fig. 7C).

The effects of Rb1 and Lac<H> on expression of GABA receptors were also investigated by measuring the protein and RNA expression levels of GABA<sub>A</sub> receptors (α2, β2, and γ2) and GABA<sub>B</sub> receptors (R1b and R2) in the rat striatum. As shown in Fig. S1, both Rb1 and Lac<H> could upregulate expression of the GABA<sub>A</sub> subunits (α2, β2, and γ2), and the regulation extent of Lac<H> was significantly greater than that of Rb1. Although protein and RNA expression of the GABA<sub>B</sub> receptor subunits could also be upregulated by Rb1 or Lac<H>, the regulation extent was much less than that on the GABA<sub>A</sub> receptors.

3.8 Roles of GABA<sub>A</sub> and GABA<sub>B</sub> receptors in neuroprotective effects of Rb1

The effects of GABA receptor antagonists on neuroprotective effects of Rb1 were investigated. As shown in Figs. 8A, 8B, mean cerebral infarct volumes in the I/R, I/R+Rb1, I/R+Rb1+Bic, and I/R+Rb1+Sac groups were 19.29 ± 2.31%, 5.95 ± 2.47%, 16.64 ± 2.30%, and 8.85 ± 3.62%, respectively. The mean neurological deficit score was also significantly affected by Bic. The neurological deficit scores in the I/R, I/R+Rb1, I/R+Rb1+Bic, and I/R+Rb1+Sac groups were 2.70 ± 0.57, 1.00 ± 0.35, 2.30 ± 0.45, and 1.40 ± 0.42 (Fig. 8C), respectively. Moreover, IL-1β, IL-6, and TNF-α levels in the I/R+Rb1+Bic group were significantly higher than those in the I/R+Rb1 group, whereas no significant difference was found between the I/R+Rb1 and I/R+Rb1+Sac groups.
Fig. 5. The expressions of GABA receptors in the rat hippocampus. (A) Protein band. (B) Gray-scale analysis. (C) RNA expression. **, p < 0.01 vs. control; ***, p < 0.001 vs. control; *, p < 0.05 vs. I/R; **, p < 0.01 vs. I/R; ***, p < 0.01 vs. I/R. GABA, gamma-aminobutyric acid; I/R, ischemia/reperfusion; Lac.H, Lactobacillus helveticus.

Fig. 6. Effects of GABA receptor antagonists on efficacy of Lac.H (n = 5 per group). (A) Coronal sections of TTC-stained brains. (B) Infarct volume. (C) Neurology deficit score. (D) IL-6 levels. (E) IL-1β levels. (F) TNF-α levels. *, p < 0.05; **, p < 0.01; ***, p < 0.001. Bic, bicuculline; GABA, gamma-aminobutyric acid; I/R, ischemia/reperfusion; Lac.H, Lactobacillus helveticus; Sac, saclofen; TTC, 2, 3, 5-triphenyltetrazolium hydrochloride.
Fig. 7. The protein and RNA expression of GABA receptors in the rat hippocampus. (A) Protein band. (B) Gray-scale analysis. (C) Relative RNA expression. **, \( p < 0.01 \) vs. control; ***, \( p < 0.001 \) vs. control; 
#, \( p < 0.05 \) vs. I/R; ***, \( p < 0.001 \) vs. I/R; ###, \( p < 0.01 \) vs. I/R. GABA, gamma-aminobutyric acid; I/R, ischemia/reperfusion.

Fig. 8. Effects of GABA receptor antagonist. (A) Coronal sections of TTC-stained brains. (B) Infarct volume. (C) Neurology deficit score on efficacy of Rb1 \((n = 5)\). (D) IL-1\( \beta \) levels. (E) IL-6 levels. (F) TNF-\( \alpha \) levels. *, \( p < 0.05 \); **, \( p < 0.01 \); ***, \( p < 0.001 \). Bic, bicuculline; GABA, gamma-aminobutyric acid; I/R, ischemia/reperfusion; Sac, saclofen; TTC, 2, 3, 5-triphenyltetrazolium hydrochloride.
4. Discussion

Ginsenosides are the major bioactive component in Panax and other types of ginseng, including American ginseng Panax quinquefolius, Korean ginseng Panax ginseng, and Chinese ginseng Panax notoginseng. [28,36] Rb1, one of the most abundant PPD ginsenosides, exhibits various pharmacological activities, including neuroprotective, antitumor, cardiovascular-protective, acute renal injury-protective, lung injury-protective, and antiaging effects, in many in vitro and in vivo models. [29–33] In the present study, all concentrations at 1, 6, and 24 h were found to be much lower than 100 ng/g, and the Rb1 concentration ratios of brain to plasma were far lower than 0.1. To date, the apparent contradiction between the pharmacokinetics and pharmacodynamics of Rb1 has not been elucidated fully. In our previous study, we found that ginsenosides could promote various metabolic processes, such as oxidation, dehydrogenation, demethylation, and deglycosylation, in an intestinal microbiota incubation system, and metabolism of ginsenosides was greatly affected by the intestinal microflora. [36]

Given that intestinal microflora can significantly influence the pharmacokinetics of Rb1 in vitro and in vivo, we hypothesized that intestinal microflora can also affect the pharmacology of Rb1. As shown in Fig. 5, neuroprotective effects of Rb1 differed greatly between conventional and PCF rats. Effects of Rb1 on cerebral infarct volume; neurological deficit score; and levels of IL-1, IL-6, and TNF-α in I/R rats were attenuated by coadministering an anti-inflammatory cocktail. Thus, the gut microbiota plays a key role in mediating neuroprotective effects of Rb1.

Research using probiotics to improve CNS function has increased significantly over the last 15 years. Both the vagus and enteric nerves are thought to be involved in gut–brain interactions and can be affected by certain probiotics including Bif. L, Bif. D, Lac. B, Lac. H, and Lac. R. [41.] In the present study, the relative abundances of Bif. L, Bif. D, Lac. B, Lac. H, and Lac. R were quantified to determine which probiotics dominate neuroprotective effects of Rb1. As shown in Fig. 3, the effect on Lac. H was the most prominent one, with Rb1 increasing abundance of Lac. H by 15.26 folds. Therefore, in the present study, Lac. H was chosen as the most promising therapeutically probiotic for cerebral I/R. In the past few years, Lac. R (B-1) was found to exert significant effects on associated behavioral and physiological responses by altering the expression of specific GABA receptors in certain areas of the brain. In addition, Lac. R was shown to change levels of neurometabolites including glutamate and glutamine, total N-acetyl aspartate and N-acetyl aspartyl glutamic acid, and GABA. [19] However, neuroprotective effects of Lac. H remained unexplored. To investigate these effects, we colonized Lac. H in rats by intragastrically administrating 1 × 10^9 CFU of Lac. H once per day for 15 consecutive days. As shown in Fig. 4, cerebral infarct volume; neurological deficit score; and levels of IL-1, IL-6, and TNF-α in I/R rats were greatly improved by Lac. H.

GABA is the primary inhibitory neurotransmitter of the CNS. We previously demonstrated that PNE could enhance Bif. L levels in I/R rats. Herein, expression levels of GABA_A receptor subunits (α2, β2, and γ2) and GABA_B receptor subunits (1b and 2) in the hippocampus and striatum of control, I/R, I/R+Lac. H, and I/R+Rb1 rats were assessed to investigate neuroprotective mechanisms of Lac. H and Rb1. As shown in Figs. 5 and 7 and S1, pretreatment with Rb1 and Lac. H could attenuate decreases in protein and mRNA levels of GABA_A (α2, β2, and γ2) and GABA_B (1b and 2) receptor subunits caused by I/R surgery. However, Rb1 effects on the GABA_B receptor subunits were not as significant as Rb1 effects on the GABA_A receptor subunits, especially α2 and γ2.

Overall, the findings of the present study suggest that I/R surgery downregulates the population of certain probiotics (Bif. L, Bif. D, Lac. B, Lac. H, and Lac. R). After pretreatment with Rb1, the relative abundance of specific probiotics can be significantly enhanced, and Lac. H is upregulated far more that the other studied probiotics. Enhanced Lac. H levels can then upregulate the expression of GABA_A (α2, β2, and γ2) and GABA_B (1b and 2) receptor subunits in the rat hippocampus and striatum. Upregulation of GABA_B receptors may play a crucial role in mediating neuroprotective effects of Rb1 and Lac. H.

Acknowledgments

This study was supported by the National Nature Science Foundation of China (81374054, 81573559, 81530098), the Nature Science Foundation of Jiangsu Province (BK20171395), and the National Key Special Project of Science and Technology for Innovation Drugs of China (2017ZX09301013).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jgr.2018.09.002.

Conflicts of interest

All authors have no conflicts of interest to declare.

References

[1] McFall-Ngai M, Hadfield MG, Bosch TGC, Carey HV, Domazet-Loito T, Douglas AE, Dubhliner N, Eberl G, Fukami T, Gilbert SF, et al. Animals in a bacterial world, a new imperative for the life sciences. Proc Natl Acad Sci USA 2013;110:3229–36.
[2] Eckburg PB, Bik EM, Bernstein CN, Purdom E, Deheflesen L, Sargent M, Gill SR, Nelson KE, Relman DA. Diversity of the human intestinal microbial flora. Science 2005;308:1635–8.
[3] Shin SC, Kim SH, You H, Kim B, Kim AC, Lee KA, Yoon JH, Ryu JH, Lee WJ. Drosophila microbiome modulates host developmental and metabolic homeostasis via insulin signaling. Science 2011;334:670–4.
[4] Mazzoli R, Pessione E. The neuro-endocrinological role of microbial glucose and GABA signaling. Front Microbiol 2016;7:1934.
[5] Chimeré C, Emery E, Summers DK, Keyser U, Gribble FM, Reimann F, Reimann A. Bacterial metabolite indole modulates incretin secretion from intestinal enteroeoendocrine I cells. Cell Rep 2014;9:1202–8.
[6] Cryan JF, O’Mahony SM. The microbiome-gut-brain axis: from bowel to behavior. Neurogastroenterol Motil 2011;23:187–92.
[7] Al-Asmakh M, Anuar F, Zadjali F, Rafier J, Pettersson S. Gut microbial communities modulating brain development and function. Gut Microbes 2012;3:366–73.
[8] Rhee SH, Pothoulakis C, Mayer EA. Principles and clinical implications of the gut-brain microbiota. Nat Rev Gastroenterol Hepatol 2009;6:306–14.
[9] Lyte M. Microbial endocrinology in the microbiome-gut-brain axis: how bacterial production and utilization of neurochemicals influence behavior. PLoS Pathog 2013;9:e1003726.
[10] Magwood R, Stone TW. The gut-brain axis, BNDF, NMDA and CNS disorders. NeuroChem Res 2016;41:2819–35.
[11] Tse JKY. Gut microbiota, nitric oxide and microglia as pre requisites for neurodegenerative disorders. ACS Chem Neurosci 2017;8:1438–47.
[12] Liang S, Wang T, Xu K, Luo J, Li W, Wu X, Duan Y, Jin F. Administration of Lactobacillus helveticus NS8 improves behavioral, cognitive, and biochemical aberrations caused by chronic restraint stress. Neuroscience 2015;310:561–77.
[13] Arsenneau-Bédard J, Rondeau I, Gilbert K, Girard SA, Tompkins TA, Godbout R, Nelson KE, Relman DA. Combination of Lactobacillus helveticus RO052 and Bifidobacterium longum RO175 reduces post-myocardial infarction depression symptoms and restores intestinal permeability in a rat model. Br J Nutr 2012;107:1793–9.
[14] Daveni S, Talari SA, Alaei H, Salami M. Probiotics treatment improves diabetes-induced impairment of synaptic activity and cognitive function: behavioral and electrophysiological proofs for microbiome-gut-brain axis. Neuroscience 2013;240:287–96.
[15] Lee NK, Paik HD. Bioconversion using lactic acid bacteria: ginsenosides, GABA, and phenolic compounds. J Microbiol Biotechnol 2017;27:869–77.
[16] Yunas RA, Poluektova EU, Dyachkova MS, Klimina KM, Kovtun AS, Averina OV, Orlova VS, Danilenko VN. GABA production and structure of gadv/gadc genes in Lactobacillus and Bifidobacterium strains from human microbiota. Anaerobe 2016;42:197–204.
[17] Borrelli L, Aceto S, Agnisola C, Paolo SD, Dipineto L, Stilling RM, Dinan TG, Cryan JF, Menna LF, Fiogetti A. Probiotic modulation of the microbiota-gut-brain axis and behaviour in zebrafish. Sci Rep 2016;6:30046.

[18] Wu C, Sun D. GABA receptors in brain development, function, and injury. Metabolic Brain Disease 2015;30:367–79.

[19] Bravo JA, Forsythe P, Chew MV, Escaravage E, Savignac HM, Dinan TG, Bienenstock J, Cryan JF. Ingestion of Lactobacillus strain regulates emotional behavior and central GABA receptor expression in a mouse via the vagus nerve. Proc Natl Acad Sci USA 2011;108:16050–5.

[20] Cryan JF, Kelly PH, Chaperon F, Gentsch C, Mombereau C, Lingenhoehl K, Auteri M, Zizzo MG, Serio R. GABA and GABA receptors in the gastrointestinal tract: from motility to inflammation. Pharmacol Res 2015;93:11–21.

[21] Lee MR, Yun BS, Sung CK. Comparative study of white and steamed Black-panax ginseng, P. Quinquefolium, and P. Notoginsengon cholinesterase inhibitory and antioxidative activity. J Ginseng Res 2012;36:93–101.

[22] Zheng YK, Miao CP, Chen HH, Huang FF, Xia YM, Chen YW, Zhao LX. Endophytic fungi harbored in Panax notoginseng: diversity and potential as bioinhibitory and antioxidative activity. J Ginseng Res 2012;36:93–101.

[23] Wang J, Qiao L, Li S, Yang G. Protective effect of ginsenoside Rb1 against lung injury induced by intestinal ischemia-reperfusion in rats. Molecules 2013;18:1214–26.

[24] Sun Q, Meng Q, Jiang Y, Liu H, Lei S, Su W, Duan W, Wu Y, Xie Z. Protective effect of ginsenoside Rb1 against intestinal ischemia-reperfusion induced acute renal injury in mice. Plos One 2015;8, e80859.

[25] Ahmed T, Raza SH, Maryam A, Setzer W, Braidy N, Nabavi SF, de Oliveira MR, nabavi SM. Ginsenoside Rb1 as neuroprotective agent: a review. Brain Res Bull 2016;125:30–43.

[26] Swanson RA, Morton MT, Tsao-Wu G, Savallos RA, Davidson C, Sharp FR. A semiautomated method for measuring brain infarct volume. J Cereb Blood Flow Metab 1990;10:290–3.

[27] Zhou L, Xing R, Chen L, Rao T, Wang Q, Ye W, Fu H, Wang X, Wang G. Development of a systematic approach to rapid classification and identification of notoginsenosides and metabolites in rat feces based on liquid chromatography coupled triple time-of-flight mass spectrometry. Analytica Chimica Acta 2015;867:56–66.

[28] Liu C, Hu M, Guo H, Zhang M, Zhang J, Li F, Zhong Z, Chen Y, Li Y, Xu P. Combined contribution of increased intestinal permeability and inhibited deglycosylation of ginsenoside Rb1 in the intestinal tract to the enhancement of ginsenoside Rb1 exposure in diabetic rats after oral administration. Drug Metab Dispos 2015;43:1702–10.

[29] Liu C, Hu M, Guo H, Zhang M, Zhang J, Li F, Zhong Z, Chen Y, Li Y, Xu P. Combined contribution of increased intestinal permeability and inhibited deglycosylation of ginsenoside Rb1 in the intestinal tract to the enhancement of ginsenoside Rb1 exposure in diabetic rats after oral administration. Drug Metab Dispos 2015;43:1702–10.

[30] Chen H, Xiao J, Chen H, Kang D, Shao Y, Shen B, Zhu Z, Yin X, Zhu Z, Li H, Rao T. Qualitatively and quantitatively investigating the regulation of intestinal microbiota on the metabolism of panax notoginseng saponins. J Ethnopharmacol 2016;194:324–6.

[31] Carabotti M, Scirocco A, Maselii MA, Severi C. The gut-brain axis: interactions between enteric microbiota, central and enteric nervous systems. Ann Gastroenterol 2015;28:203–9.

[32] Bienenstock J, Kunze W. Microbiota and the gut-brain axis. Nutr Rev 2015;73(Suppl 1):28–31.

[33] Barrett E, Ross RP, O’Toole PW, Fitzgerald GF, Stanton C. γ-Aminobutyric acid production by culturable bacteria from the human intestine. J Appl Microbiol 2014;113:411–7.

[34] Bharwani A, Mian MF, Surette MG, Bienenstock J, Forsythe P. Oral treatment with Lactobacillus rhamnosus attenuates behavioural deficits and immune changes in chronic social stress. BMC Med 2017;15:7.

[35] Wang H, Lee IS, Braun C, Enck P. Effect of probiotics on central nervous system functions in animals and humans: a systematic review. J Neurogastroenterol Motil 2016;22:589–605.