Ginsenoside Rg1 ameliorates depressive-like behavior by inhibiting NLRP3 inflammasome activation in mice exposed to chronic stress

Hui He
Sichuan Provincial Center for Mental Health, University of Electronic Science and Technology of China

Xiaofang Xie
Chengdu University of Traditional Chinese Medicine

Xixi Kang
University of Electronic Science and Technology of China

Jinqiang Zhang
Guizhou University of Traditional Chinese Medicine

Lu Wang
The Fourth People's Hospital of Chengdu, Mental Health Center of Chengdu

Nan Hu
University of Electronic Science and Technology of China

Lei Xie
Sichuan Provincial Center for Mental Health, University of Electronic Science and Technology of China

Cheng Peng
Chengdu University of Traditional Chinese Medicine

Zili You
Sichuan Provincial Center for Mental Health, University of Electronic Science and Technology of China

Research Article

Keywords: Ginsenoside Rg1, Antidepressant, Microglia, NLRP3 inflammasome, Neurogenesis

Posted Date: March 9th, 2023

DOI: https://doi.org/10.21203/rs.3.rs-2643409/v1

License: This work is licensed under a Creative Commons Attribution 4.0 International License. Read Full License
Abstract

Objectives: Microglia-mediated inflammatory process is recognized as a target in the treatment of depression. Ginsenoside Rg1 (GRg1), the active ingredient of traditional ginseng, regulates microglial phenotypes to resist stress-induced inflammatory responses. Here we used a mouse model of stress-induced depression to investigate the involvement of microglial Nod-like receptor protein 3 (NLRP3) in the antidepressant effects of GRg1.

Methods: Male C57BL/6J mice were exposed to chronic mild stress (CMS) for three weeks, followed by intraperitoneal injection of GRg1 (20 mg/kg) or the antidepressant imipramine (20 mg/kg) for another three weeks. Depressive-like behaviors were assessed by sucrose preference test, forced swimming test, and tail suspension test. Microglial phenotypes were assessed in terms of morphological features and cytokine profiles; inflammasome activity, in terms of levels of complexes containing NLRP3, apoptosis-associated speck-like protein containing CARD (ASC) and caspase-1; and neurogenesis, in terms of numbers of proliferating, differentiating, and mature neurons identified by immunostaining.

Results: GRg1 reduced abnormal animal behaviors caused by CMS, such as anhedonia and desperate behaviors, without affecting locomotor behaviors. GRg1 also reduced the number of ASC-specks, implying inhibition of inflammasome activation, which was associated with weaker activation of pro-inflammatory microglia. At the same time, GRg1 rescued impairment of hippocampal neurogenesis in vivo and in vitro, which correlated with modulation of microglial phenotypes.

Conclusion: GRg1 appears to exert antidepressant effects by preventing stress from activating the NLRP3 inflammasome in microglia, promoting a proneurogenic phenotype and allowing adult hippocampal neurogenesis.

Highlights

1. GRg1 inhibits microglial activation to alleviate depressive-like behaviors in mice exposed to chronic mild stress.
2. NLRP3 inflammasome is involved in GRg1-induced modulation of microglial phenotypes.
3. The pro-neurogenesis effect of GRg1 is associated with the inhibition of microglial proinflammatory profiles.

1 Introduction

Major depressive disorder (MDD) is the most common psychiatric disorder and a major cause of disability worldwide [1]. In 30–50% of patients, clinically available therapeutics against MDD fail to relieve depressive symptoms or they induce intolerable side effects [2, 3], which reflects in part the biological heterogeneity of depression [4]. Therefore, more effective, individualized treatments for MDD are urgently needed.
Accumulating evidence indicates that immune mechanisms contribute to pathology of MDD, which may help explain the heterogeneity in clinical manifestations and the differences in therapeutic responses of patients [5]. Some antidepressant drugs, such as minocycline or antibodies against cytokine receptors, improve depression symptoms by inhibiting immune responses [6]. Targeting the microglia, the resident immune cells in the brain, to provide precise treatment strategies for MDD may avoid systemic immune imbalance [7], since microglia mediate neuroinflammatory responses to stress in the central nervous system (CNS) [8]. Nod-like receptor protein 3 (NLRP3) inflammasome is a crucial molecular platform in microglia to detect stress-induced signaling involving damage-associated molecular patterns (DAMPs) [9]. In the presence of stress-induced DAMPs, NLRP3 inflammasome complex with “apoptosis-associated speck-like protein (ASC) and caspase-1 to form an inflammasome in microglia [9], promotes the secretion of IL-1β and IL-18 [10]. The secretion of these proinflammatory cytokines ultimately leads to neuronal damage [10, 11]. NLRP3, the sensors of microglial cells, response to stress by the increasing of expression and assembly, which contribute to stress-induced neuroinflammatory and behavioral outcomes [12]. These considerations imply that blocking activation of the NLRP3 inflammasome in microglia may in turn reduce neuroinflammation and risk of MDD.

Microglia exert either detrimental or beneficial effects on neurogenesis, during which newborn neurons contribute to memory, cognition, and mood regulation [13]. The impairment of adult hippocampal neurogenesis is associated with the pathogenesis of MDD [14, 15]. Indeed, antidepressants inhibit inflammatory mediators released by microglia, increase the survival of neural precursor cells in the dentate gyrus (DG), reverse hippocampal dysfunction, promote neurogenesis, and adjust stress response, all of which can improve depressive-like behaviors [6, 13, 16]. Enhancing a pro-neurogenic phenotype of microglia contributes to stress resilience in CMS-exposed mice [17, 18]. Inhibiting pro-inflammatory activation of microglia has been linked to greater neurogenesis [5, 16]. Therefore, therapeutic strategies against MDD might target on switching of microglial phenotypes.

Traditional Chinese medicines offer promising alternatives for treatment of heterogeneous mood disorders [19]. In traditional Chinese medicine, ginseng has long been used to treat mood disorders [19–21], and its major active ingredients, ginsenosides, appear to be effective against depression [20, 22, 23]. In particular, the ginsenoside Rg1 (GRg1), a protopanaxatriol-type saponin, exerts its effects by influencing multiple processes in the brain, with few side effects [24]. GRg1 exerts neuroprotective effects by modulating the monoamine neurotransmitter system and the hypothalamus-pituitary-adrenal axis [25], by upregulating neurotrophic factors [26], and exerting anti-inflammatory and anti-apoptotic effects [22, 27–29]. Recent studies have indicated that GRg1 attenuated stress-induced microglial overactivation [28, 30, 31]. This led us to explore the molecular mechanisms underlying the modulation of microglial phenotypes of GRg1 in an animal model of stress-induced depression, which may be influenced by the activity of the NLRP3 inflammasome.

Here we test the hypothesis that GRg1 modulates microglia-mediated neurogenesis to promote stress resilience through actions on microglial NLRP3 signaling. In this study, we examined the effects of GRg1 on depressive-like behaviors in chronic mild stress (CMS)-exposed mice. Then, we investigated whether
inhibition of the NLRP3 pathway is required for the alteration of phenotypes in GRg1-treated microglia in vivo and in vitro. Finally, we observed the contribution of hippocampal neurogenesis to stress vulnerability in GRg1-administered mice. The results indicate the therapeutic potential of GRg1 in the treatment of stress-related mental disorders. This investigation provides new insights of microglial inflammasome pathway underlying the antidepressant effects of GRg1.

2 Material And Methods

2.1 Animals

Eight-week-old (weighing 18–22 g) male C57BL/6J mice supplied by Chengdu Dossy Experimental Animal Co. Ltd. (Chengdu, China) were acclimatized for 14 days prior to the experiments in a standard laboratory animal facility (25°C, 12-h light/dark cycle) with food and water ad libitum. The reason for not choosing female animals is that female mouse sex hormones can confound experimental results. All experimental procedures were approved by the Ethics Committee of the University of Electronic Science and Technology of China and performed in strict accordance with the Guidelines for the Care and Use of Laboratory Animals from the US National Institutes of Health (8th edition, revised 2010).

2.2 CMS treatment

At nine weeks of age, mice were transferred to individual cages and exposed to CMS for three weeks following a previously described protocol [5]. Each mouse was subjected to two or more of the following stressors per day: food and water deprivation (12 h), reversal of day-night cycle (12 h), tail pinch (5 min), strobe lighting (12 h), wet cage (24 h), empty cage (24 h), tilted cage (45°, 24 h), soiled bedding (24 h), and/or restraint (2 h).

2.3 Pharmacological treatments

GRg1 (99.92% pure; Chengdu Alfa Biotechnology, Chengdu, China) and imipramine (IMI, Sigma-Aldrich, Germany) were dissolved separately in 0.9% saline to concentrations of 20 mg/mL. Forty male mice were randomly assigned to five treatment conditions (8 mice per condition): two control groups not exposed to CMS that received either saline solution (control group) or GRg1 (GRg1 group), and three CMS-exposed groups that received saline (CMS), GRg1 (CMS-GRg1), or imipramine (CMS-IMI). Both drugs were administered intraperitoneally (i.p.) at 20 mg/kg daily for 3 weeks. Control groups received the same volume of saline. After three weeks of treatment, mice underwent behavioral tests and were sacrificed for collection of brain tissues as described below.

2.4 Behavioral measurements

2.4.1 Sucrose preference test (SPT)

The SPT was performed as described previously [32]. Single-caged mice were deprived of food and water for 24 h, then given ad libitum access to 1% sucrose solution (A) or drinking water (B) for 2 h, before and
after which the volume of the solutions was measured. The sucrose preference (%) of each mouse was calculated for each week according to the formula: \[\frac{\text{Vol. A}}{\text{Vol. A} + \text{Vol. B}} \times 100\%\]. The experimenter was unaware of the grouping of the mice.

2.4.2 Tail suspension test (TST)

The tip of the mouse tail was fixed with medical tape, and mice were hung on a shelf 30 cm above the ground. A high-definition camera was used to record the movements of the animal during 6 min. The latency to abandon the struggle to escape for the first time and the total time spent immobile during the 6 min were recorded. The experimenter was unaware of the grouping of the mice.

2.4.3 Forced swimming test (FST)

The FST was performed as previously described [32]. Mice were placed into a beaker 25 cm high and 12 cm in diameter that contained 18 cm of water at 25°C. The latency to abandon the struggle to escape for the first time during 6 min was recorded, as was total immobility time during the last 4 min. The water was changed between animals to avoid odor interference. The experimenter was unaware of the grouping of the mice.

2.4.4 Open field test (OFT)

Mice were placed in the center of a rectangular chamber (50 × 50 × 50 cm) and allowed to explore the area for 5 min. The time spent in the center (25 × 25 cm) and the total distance traveled by the mice were recorded using OFT100 software (Taimeng Tech, Chengdu, China). The experimenter was unaware of the grouping of the mice.

2.4.5 Locomotor activity test (LAT)

The LAT was performed to evaluate spontaneous activity in mice. Animals were placed in six different areas and allowed to acclimatize for 1 min, after which they were left for 10 min to behave freely, during which they were recorded on video. The time spent standing and moving was determined (Techman Software ZZ6, Chengdu, China). Between animals, the experimental area was cleaned with 75% alcohol to avoid odor interference. The experimenter was unaware of the grouping of the mice.

2.5 Cell culture

2.5.1 Primary microglial cultures

Primary microglia were isolated from the brains of neonatal C57BL/6J mice (P0–P3) [33]. Briefly, mice were rapidly decapitated using surgical scissors to minimize pain and stress, and the heads were sterilized with 75% ethanol to avoid contamination. After brain was quickly transferred into cold phosphate-buffered saline (PBS; catalog no. G4202, Servicebio, Chengdu, China). The meninges were peeled away, and the brain was placed in as little pre-cooled PBS as possible, to which was added digestion solution (8 U/mL papain and 125 U/mL DNase). This mixture was gently shaken continuously in a water bath at 37 °C for 20 min, with strong shaking once at 10 min and again at 20 min. Tissue was
filtered through a 70-µm cell strainer and collected into a 50 mL centrifuge tube. Cells were centrifuged at 200 g for 10 min, resuspended in 10 mL DMEM/F-12 (1:1) basic medium (Gibco, Chengdu, China), and 5×10^6 cells were plated in square flasks. The medium was changed after 48 h.

On day 10 of primary culture, microglia were pre-treated with 20 µM GRg1 for 30 min, then treated for 3 h with PBS alone or containing 50 ng/mL lipopolysaccharide (LPS; Sigma-Aldrich, Shanghai, China) and 0.25 mM ATP (ATP; InvivoGen, San Diego, CA, USA). The cultures were then processed for immunocytochemistry, reverse transcription-quantitative PCR (RT-PCR) or western blot analyses as described below.

### 2.5.2 Neural stem cell (NPC) cultures

Hippocampi were isolated from three neonatal C57BL/6J mice 0–3 days old. Treatment of infant mice was following the procedure described above in method 2.5.1, and subsequently quickly placed in pre-cooled PBS, digested using enzyme A at 37°C for 10 min, and centrifuged at 4 °C for 10 min. The supernatant was resuspended in DMEM/F-12 (1:1) basic medium. The cells were plated in dishes and incubated for three days in high-glucose DMEM/F12 containing 40 ng/ml N2 (Gibco, Chengdu, China, Cat# 17502-048), and 80 ng/ml B27 supplement (Gibco, Chengdu, China, Cat# 17504-04), 20 ng/mL epidermal growth factor (FGF2), and 20 ng/mL fibroblast growth factor (EGF). To stimulate differentiation, the medium was changed to differentiation medium: high-glucose DMEM/F12, 40 ng/ml N2, 80 ng/mL B27 supplement, and 10% fetal bovine serum (catalog no. 10099141, Gibco, Australia).

### 2.6 Western blotting

The experimental mice were deeply anesthetized using pentobarbital sodium (50 mg/kg, i.p.; R&D Systems, Minneapolis, USA, Cat# 4579/50), perfused transcardially with 0.9% normal saline, and brain tissue was quickly removed. Total protein was extracted from mouse hippocampi and primary microglial cultures. The total concentration of soluble protein was estimated using the bicinchoninic acid protein assay (Pierce Thermo Scientific, Waltham, MA, USA), and samples were diluted as needed with 1 × RIPA buffer (Beyotime, Chengdu, China) in order to ensure equal loading onto 10% sodium dodecyl sulphate-polyacrylamide gels. The fractionated proteins were transferred to a polyvinylidene difluoride membrane (Merck Millipore, Darmstadt, Germany), which was blocked with 5% non-fat dry milk, then incubated overnight at 4°C with primary antibodies against the following proteins: IL-1β (1:500; bs-20449R, Bioss, Beijing, China), NLRP3 (1:1000; AG-20B-0014, AdipoGen, San Diego, CA, USA), caspase-1 (1:1000; AG-20B-0042-C100, AdipoGen), ASC (1:1000; sc-514414, Santa Cruz Biotechnology, Santa Cruz, CA, USA), glyceraldehyde 3-phosphate dehydrogenase (GAPDH; 1:1000; GB11002, Servicebio), or β-actin (1:2000; GB11001, Servicebio). After washing Tris-buffered saline containing Tween-20 (TBST), membranes were incubated with the corresponding secondary antibodies, either rabbit immunoglobulin (Ig) G (1:10000; catalog no. ab6721, Abcam, Cambridge, UK) or mouse IgG (1:10000; ab6789, Abcam). Antibody binding was determined by fluorescence detection using the BeyoECL Plus kit (catalog no. P0018S, Beyotime) and quantitated using Image J software (version 1.45, US National Institutes of Health, Bethesda, MD, USA).
2.7 RNA extraction and RT-PCR

Mice were anesthetized with 50 mg/kg pentobarbital (R&D Systems, MI, USA), hippocampi were quickly collected on ice, and total RNA was extracted using Trizol (Invitrogen Life Technologies, CA, USA). The RNA was reverse-transcribed with the First Strand cDNA Synthesis Kit (TaKaRa, Beijing, China), and cDNA was stored at -80°C until analysis using RT-qPCR and commercial reagents (catalog no. 2206210A, TaKaRa) in a Bio-Rad CFX 96 thermocycler (Hercules, California, USA). The cycle conditions were 95°C for 10 min, followed by 38 cycles at 95°C for 3 s, annealing at the appropriate temperature for 30 s, and extension at 72°C for 5 min. Gene expression was analyzed using the $2^{-\Delta\Delta Ct}$ method relative to the expression of β-actin. Primer pairs were used for amplification of genes in Table S1.

2.8 Immunofluorescence

Immunofluorescence was used to evaluate the expression of ASC and the microglial marker Iba1 in sections of mouse hippocampus or in primary cultures. Briefly, the mice were deeply anaesthetized with pentobarbital sodium and tissue slices or cultured cells were fixed with 4% paraformaldehyde, washed three times in PBS (5 min per wash), incubated with 0.3% Triton X-100 for 15 min, and blocked for 1 h with QuickBlock™ Blocking Buffer for Immunol Staining (Beyotime). Samples were incubated overnight at 4°C with primary antibodies against ASC (1:100; sc-514414, Santa Cruz Biotechnology) and Iba1 (1:400; ab5076, Abcam), washed with PBS, incubated for 1 h at 37°C with secondary antibodies, washed three times with PBS (5 min/time), then incubated with 4’,6-diamidino-2-phenylindole (DAPI) for 5 min. Cells were observed and captured with an inverted fluorescent microscope.

The samples were mounted on slides and imaged under a fluorescence microscope (Carl Zeiss, Jena, Germany).

2.9 Statistical analysis

Data were expressed as mean ± standard error of the mean (SEM) of at least five replicates. Data were plotted and statistically analyzed using GraphPad Prism version 7.0 (GraphPad Software, San Diego, CA, USA). Normal distribution of data was checked using the Shapiro-Wilk test, and differences between two groups were assessed for significance using the unpaired $t$ test with Welch’s correction or a non-parametric Mann-Whitney test. Differences among three or more groups were assessed using one- or two-way analysis of variance and Tukey’s post hoc tests.

3 Results

3.1 GRg1 ameliorates depressive-like behaviors induced by CMS

The structure of GRg1 is shown (Fig. 1A). Mice were subjected to CMS for three weeks, then mice were treated with GRg1 (20 mg/kg) or IMI (20 mg/kg) as positive control for another three weeks (Fig. 1B).
CMS reduced sucrose preference by 38 ± 4.5%, confirming the validity of our depression model, which was reversed by GRg1 treatment, similar to the effects of IMI treatment (Fig. 1C). After CMS, mouse weight decreased significantly, and GRg1 restored body weight to values similar to controls within 21 days of treatment (Fig. 1D) FST and TST are the most effective behavioral measures tests to detect antidepressant activity. GRg1 treatment significantly reversed the increased immobility time due to CMS in the TST (Fig. 1E) and FST (Fig. 1F), similar to IMI. To rule out the possibility that the behavioral capacity impairments exhibited by the stressed animals were due to spontaneous mobility, we performed OFT and LAT in experimental animals. At the same time, GRg1 increased the percentage of time spent in the center in the OFT (Fig. 1G). Neither CMS nor the treatments affected spontaneous mobility, since all animal groups showed similar results in the OFT and LAT (Fig. 1H). The effects of GRg1 were not observed in control mice, suggesting that GRg1 acts only on stress-induced pathways.

3.2 GRg1 attenuates microglial activation in hippocampus of CMS-exposed mice

In the CNS, microglia-mediated neuroinflammation is a major contributor to MDD. The microglia-specific marker Iba1 was used to examine microglial morphology (Fig. 2A). In the hippocampus of CMS-exposed mice, the total number and area of microglia was significantly increased and the numbers of microglial branches was decreased, and these effects were significantly reversed by GRg1 treatment (Fig. 2B-2D). Using Sholl analysis [34], we found that CMS induced in hippocampal microglia a pro-inflammatory phenotype characterized by amoeboid morphology, smaller cell perimeter and increased cell solidity (Fig. 2E), as well as smaller critical radius and shorter maximal length of dendrites per microglia (Fig. 2F). In contrast, GRg1 restored microglial morphology to that observed in the control group. These findings suggest that GRg1 significantly relieves stress-induced neuroinflammation by inhibiting microglial activation in hippocampus. Therefore, it may be a therapeutic target through which GRg1 alleviates depressive-like behaviors.

3.3 GRg1 inhibits NLRP3 inflammasome induced-neuroinflammation in CMS-exposed mice.

We examined the expression of inflammatory factors in the mouse hippocampus of CMS-exposed mice. CMS-exposed mice showed increased expression of pro-inflammatory cytokines IL-1β and IL-18 in the hippocampus, which GRg1 treatment suppressed (Fig. 3A-B). In addition, we found that GRg1 treatment significantly attenuated NLRP3 inflammasome activation, reflected in the reduced levels of NLRP3, cleaved caspase-1 and cleaved IL-1β. These changes were validated at the protein level using western blotting (Fig. 3F-3H). These experiments indicate that GRg1 reversed the increase in proinflammatory factors, revealing its role in inhibiting neuroinflammation in CNS.

Next, we examined changes in the ASC adaptor protein, a core element of NLRP3 inflammasome activation. We found that CMS induced ASC aggregation in microglia, whereas the percentage of
ASC+/Iba1+ cells was significantly decreased after GRg1 treatment (Fig. 4A). By western blotting, we found that GRg1 treatment significantly reduced ASC expression in CMS mice (Fig. 4B). Next, we examined the co-localization of ASC and the microglial marker Iba1, which was defined as the overlap between the two fluorescent signals at the pixel level (Fig. 4C-4E). For visualization of the results, we plotted the fluorescence intensity of ASC and Iba1 immunostaining (Fig. 4F). Our results clearly reveal that CMS-exposed mice increased co-localization between ASC and Iba1, which was reversed by GRg1 treatment.

3.4 GRg1 blocks CMS-induced ASC speck formation and NLRP3 inflammasome activation in primary cultures of microglia

All these in vivo results were explored in greater detail using primary microglial cultures, which we stimulated with LPS and ATP to simulate stress. Based on the CCK8 assay, GRg1 did not affect viability of primary microglia (Fig. 5A). We used multiple GRg1 concentrations (10, 20, 40, 80 µM) to treat primary microglia from the LPS-ATP group. GRg1 treatment, especially at 20 µM, significantly downregulated levels of mRNAs encoding the inflammasome-related inflammatory factors IL-1β, IL-18, and NLRP3 (Fig. 5B-5D).

Consistent with our results in mice, stressing primary microglia in culture upregulated fluorescence intensity and protein levels of NLRP3, which GRg1 reversed (Fig. 5E-5F). Compared with the PBS group, levels of mature IL-1β and mature caspase-1 were increased in the culture medium of the LPS-ATP group, which GRg1 treatment significantly reversed. LPS-ATP treatment also increased cytoplasmic levels of IL-1β precursor, which GRg1 did not reduce. Neither LPS-ATP nor GRg1 altered cytoplasmic levels of caspase-1 precursor (Fig. 5G). LPS-ATP treatment significantly upregulated levels of ASC and induced its aggregation in microglia, which GRg1 reversed (Fig. 5H-5I). These results suggest that GRg1 prevents formation of the NLRP3 inflammasome in response to stress, and that it does so by blocking recruitment of the adaptor protein ASC.

3.5 GRg1 enhances hippocampal neurogenesis in stressed mice and primary cultures of microglia

The above experiments indicated that GRg1 inhibited a pro-inflammatory microglial phenotype under stress conditions. Given that such inhibition has been linked to enhanced hippocampal neurogenesis, we wonder whether GRg1 stimulates neurogenesis. CMS reduced numbers of BrdU+ cells (proliferating cells), BrdU+-DCX+ cells (proliferating nascent neurons) and NeuN+ cells (mature neurons), while GRg1 restored these numbers to values similar to those in control mice. In fact, GRg1 increased the ratio of the number of BrdU+-DCX+ cells to the number of BrdU+ cells (Fig. 6A), indicating greater differentiation of newborn neurons in the dentate gyrus. CMS significantly reduced the number of nascent neurons and their branch
lengths, which GRg1 reversed (Fig. 6B). CMS also reduced the number of mature neurons, which GRg1 restored to control values (Fig. 6C).

To test whether GRg1 induces the production of extracellular factors that induce neurogenesis, we exposed primary cultures of NPCs to conditioned medium from primary microglial cultures that had been pre-treated (or not) with GRg1, then stressed with LPS and ATP (Fig. 6D). As expected, the conditioned medium from stressed microglia reduced the numbers of proliferative cells (BrdU\(^+\)), glial cells (GFAP\(^+\)) and mature neurons (MAP2\(^+\)). Conditioned medium from microglia that were treated with GRg1 led to levels of all three cell types that were similar to the levels in untreated NPCs cultures (Fig. 6E-6F).

4 Discussion

Microglia are implicated in stress-induced mental and behavioral disorders, so targeting microglia-mediated neuroinflammation is a focus of current pharmacology effects to treat MDD. In this study, we confirm that GRg1 has potent antidepressant effects in CMS-exposed animals, and we show in vivo and in vitro that these therapeutic effects involve regulation of microglial phenotype by attenuating NLRP3 signaling, which contributes to promotion of hippocampal neurogenesis.

Ginseng is widely used as a tonic herbal in Asian countries, and its various components show distinct pharmacological properties. In clinical practice, ginseng appears to provide benefits to MDD patients while triggering milder adverse effects on cognition and sexual dysfunction than popular antidepressants [22]. In the present study, the sucrose preference experiment was used to verify the successful establishment of the CMS model, which is widely used as an animal model of depression [32]. To examine the antidepressant activity of GRg1, we used FST and TST, the classical behavioral experiments for assessing antidepressant effects in animal models of depression [33, 34]. These tests showed that GRg1 significantly reduced anhedonia and desperation behaviors in CMS-exposed mice. We also found that GRg1 did not alter the behavior of unstressed control animals, suggesting its safety. This reinforces the potential of GRg1 as a clinical treatment for depression.

Microglia-mediated neuroinflammation is a biomarker and of inflammation-related depression and target for treatment treat [5, 35, 36]. The hippocampus is a target structure of stress-induced depression [28, 31], and we found that CMS triggered pro-inflammatory changes in microglia in that brain region, leading to high production of IL-1\(\beta\) and IL-18, which GRg1 reversed. These results confirm previously reported anti-inflammatory effects of GRg1 in animal models of depression [28]. The NLRP3 inflammasome appears to be a central mediator of the neuroinflammatory processes during the pathogenesis of depression. Microglia are dynamically influenced by stress-induced CNS environmental signals, during which NLRP3 inflammasome play a critical role. As the description of two-step model of NLRP3 priming and activation, the priming step is manifested in NF-\(\kappa\)B-mediated overexpression of NLRP3, and the activation step is required of NLRP3 oligomerization and inflammasome assembly to allow the processing of pro-IL-1\(\beta\) and pro-IL-18 to their mature and secreted forms [37]. Zhang et al reported that GRg1 inhibited NF-\(\kappa\)B which triggered the production of NLRP3 in CMS-treated rats [31]. In the present research, we showed that GRg1
exerts its antidepressant effects by inhibiting the aggregation of ASC and assembly of NLRP3 inflammasome. In animal models of depression, activity of the NLRP3 inflammasome in hippocampus correlates positively with depressive-like behaviors [38, 39]. We further found that GRg1 treatment significantly reduced the cleavage of caspase-1, and release of IL1β and IL-18. Our study provides further suggest that GRg1 could prevent neuroinflammatory processes by inhibiting ASC recruitment which is necessary for inflammasome assembly.

Promotion of neurogenesis is likely to be important to antidepressant benefits [16, 40]. Normally, microglia facilitate the proliferation of NPCs to support the integration of neuronal circuits, whereas stress-induced microglia inhibit hippocampal neurogenesis [20, 41]. Microglia-mediated neuroinflammation is an important mechanism leading to abnormal neurogenesis in depression. Inhibiting the activation of microglia may be a common way for antidepressants to alleviate neurogenesis and exert therapeutic effects [14, 18]. Here we show that GRg1 increased progenitor cell proliferation in stressed mice, leading to neurogenesis. Our experiments in vivo and in vitro suggest that GRg1 polarizes microglia towards a pro-neurogenic phenotype, which we demonstrated directly by culturing NPCs in conditioned medium from GRg1-treated activated microglia. Therefore, our study suggested that GRg1 conferred with resilience stress in CMS-exposed mice through neurogenesis improvement, which was at least in part dependent on microglial functions.

In conclusion, GRg1 enhances stress resistance by promoting hippocampal neurogenesis, which is partially dependent on the microglial proneurogenic phenotype and involving inhibition of NLRP3 inflammasome activation. Our results confirm and extend the idea that antidepressant efficacy involves alteration of microglial phenotypes and promotion of neurogenesis, and provided a therapeutic approach for GRg1 treatment of depression in clinical practice.

Abbreviations

ASC, Apoptosis-Associated Speck-Like Protein; ATP, adenosine triphosphate; BrdU, Bromodeoxyuridine; CM, Conditioned medium; CMS, Chronic mild stress; CNS, Central nervous system; DAPI, 4′,6-Diamidin-2-phenylindol; DG, Dentate gyrus; FST, Force swimming test; GFAP, glial fibrillary acidic protein; GRg1, Ginsenoside Rg1; IL, Interleukin; IMI, Imipramine; LAT, Locomotor activity test; LPS, Lipopolysaccharide; MAP2, microtubule-associated proteins; MDD, Major depressive disorder; NLRP3, nucleotide-binding oligomerization domain-like receptor protein 3; OFT, Open field test; PBS, Phosphate-buffered saline; RT-qPCR, Reverse transcription-quantitative PCR; SPT, Sucrose preference test; TST, Tail suspension test.

Declarations

Ethics approval and consent to participate

All animals care and experimental procedures were approved by the Ethics Committee of the University of Electronic Science and Technology of China and all procedures were performed in accordance with
National Institutes of Health guidelines for the care and use of laboratory animals and approved by the Institutional Animal Care and Use Committee of the University of Electronic Science and Technology of China.

Consent for publication

Not applicable

Data Availability Statement

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

Competing interests

The authors declare no conflicts of interest.

Acknowledgments

We are grateful to A. Chapin Rodríguez for help in revising the manuscript. Thanks to all the contributors.

Funding

This work was supported by the Sichuan Science and Technology Program (grant nos. 2022ZYD0070 and 2022NSFSC0590), the Open Research Fund of State Key Laboratory of Southwestern Chinese Medicine Resources (grant no. SKLTCM2022016), and the Medico-Engineering Cooperation Funds from the University of Electronic Science and Technology of China (grant no. ZYGX2021YGLH217).

Authors' contributions

Zili You and Hui He designed the research. Hui He carried out the experiments and performed data analysis. Xixi Kang, Nan Hu, Lu Wang and Lei Xie participated part of the experiments. Hui He and Xixi Kang wrote the manuscript. Zili You, Jinqiang Zhang, Cheng Peng and Xiaofang Xie revised the manuscript. All the authors have read and approved the final manuscript.

References


**Figures**
GRg1 ameliorates depressive-like behaviors in mice exposed to chronic mild stress (CMS). (A) Chemical structure of GRg1. (B) Experimental timeline to assess the effects of GRg1 on depressive-like behaviors induced by CMS. (C-H) Analyses of animals treated with saline solution (CON), 20 mg/kg GRg1 (GRg1), and three CMS-exposed groups that received saline (CMS), GRg1 (CMS-GRg1), or imipramine (CMS-IMI). Comparison of sucrose preference ratios in the different treatment groups every week. (D) Comparison of mouse body weights. (E) Immobility time and latency of mice in the tail suspension test at week 6. (F) Immobility time and latency of mice in the forced swimming test at week 6. (G) Time spent in the center and distance traveled by mice in the open field test at week 6. (H) Spontaneous activity and standing time
of mice in the locomotor activity test at week 6. \( n = 8 \) per group, \(* P < 0.05, ** P < 0.01, *** P < 0.001 \) vs. the control group; \# \( P < 0.05, ## P < 0.01, ### P < 0.001 \) vs. the CMS group. \( P \) values were obtained by one-way analysis of variance with Tukey's multiple-comparisons test.

Figure 2

**GRg1 attenuates microglial activation in the hippocampus of mice exposed to chronic mild stress (CMS).**

(A) Immunofluorescence staining of microglia using Iba1 in the dentate gyrus (DG), CA1, and CA3 regions of the hippocampus. (B-D) Numbers of (B) Iba1\(^+\) cells and (C) branches as well as (D) area of Iba1\(^+\) cells in the DG, CA1, and CA3 regions. (E) Cell perimeter and cell solidity (convex area/microglia area), based on two-dimensional analysis of individual cells in the hippocampus. (F) Critical radius (top) and the
dendritic maximum (bottom) of microglia evaluated by Sholl analysis. n = 6 per group, * P < 0.05, ** P < 0.01, *** P < 0.001 vs. the control group; # P < 0.05, ## P < 0.01, ### P < 0.001 vs. the CMS group. P values were obtained by one-way analysis of variance with Tukey’s multiple-comparisons test.

Figure 3

GRg1 inhibits neuroinflammation mediated by the NLRP3 inflammasome in mice exposed to chronic mild stress (CMS). (A-D) Expression of IL-1β, IL-18, NLRP3, and Caspase-1 in the hippocampus, quantified by real-time quantitative PCR and normalized to the expression of GAPDH, n = 6 per group. (E-H) Levels of IL-1β, IL-18, NLRP3, and Caspase-1 in the hippocampus, based on western blotting. Levels were normalized to those of β-actin, n = 5 per group. * P < 0.05, ** P < 0.01, *** P < 0.001 vs. the control group; # P < 0.05, ## P < 0.01, ### P < 0.001 vs. the CMS group. P values were obtained by one-way analysis of variance with Tukey’s multiple-comparisons test.
Figure 4

**GRg1 blocks ASC speck formation and oligomerization in microglia during chronic mild stress (CMS).** (A) Immunofluorescence staining of hippocampal sections against Iba1 (red), ASC (green), and DAPI (blue). The bar chart shows numbers of ASC⁺ microglia, n = 5 per group. (B) Levels of ASC in the hippocampus by western blotting. Levels were normalized to those of β-actin, n = 5 per group. (C) Zoomed-in views of the yellow dashed regions in panel C. (D) Schematic for co-immunostaining against Iba1 and ASC in order to identify the proportion of microglia containing ASC specks. (E) Plots of pixel intensity for the “merge” images in panel C. * P < 0.05, ** P < 0.01, *** P < 0.001 vs. the control group; # P < 0.05, ## P < 0.01, ### P < 0.001 vs. the CMS group. P values were obtained by one-way analysis of variance with Tukey’s multiple-comparisons test.
**Figure 5**

**GRg1 inhibits the activation of the NLRP3 inflammasome in primary microglial cultures.** (A) The viability of primary microglia after treatment with different concentrations of GRg1 for 24 h, as measured using the CCK8 kit. (B-D) Expression of IL-1β, IL-18, NLRP3 in the primary microglia, by real-time quantitative PCR. Data were standardized to control cultures treated with phosphate-buffered saline (PBS). (E) Immunofluorescence staining of primary microglia for NLRP3 (green). Nuclei were stained with DAPI (blue). The bar chart shows the fluorescence intensity of NLRP3 in the primary microglia, n = 3 per group. (F) Levels of NLRP3 in primary microglia relative to levels of GADPH, as measured by western blotting, n = 5 per group. (G) Levels of pro-IL-1β and pro-caspase-1 in cytoplasm and levels of cleaved IL-1β and
caspase-1 in supernatant of primary microglia, as examined using western blotting. Levels were normalized to those of GADPH, n = 5 per group. (H) Immunofluorescence of primary microglia against ASC (green) and Iba1 (red). Nuclei were stained using DAPI (blue). Plots of pixel intensity are shown underneath the corresponding image. (I) Levels of ASC in primary microglia, by western blotting. Levels were normalized to those of β-actin, n = 5 per group. * P < 0.05, ** P < 0.01, *** P < 0.001 vs. control (PBS) group; # P < 0.05, ## P < 0.01, ### P < 0.001 vs. the LPS-ATP group. P values were obtained by one-way analysis of variance with Tukey's multiple-comparisons test (A-D) and two-way analysis of variance with Tukey's multiple-comparisons test (F-I).

**Figure 6**
GRg1 partially relaxes the inhibition of hippocampal neurogenesis during chronic mild stress (CMS). (A) Immunofluorescence staining of brain sections against bromodeoxyuridine (BrdU, green) and doublecortin (DCX, red) to reveal different cell populations in dentate gyrus of the hippocampus. Nuclei were stained with DAPI (blue), n = 4 per group. (B) Immunofluorescence staining of sections against DCX (red), with nuclear staining by DAPI (blue). Numbers of DCX\(^+\) cells and length of neuronal branches are shown, n = 4 per group. (C) Immunofluorescence staining against NeuN (red), with nuclear staining by DAPI (blue), n = 4 per group. (D-F) Experimental procedure in which primary cultures of neural stem cells (NPCs) were incubated with conditioned medium from microglia (M-CM) that had been pretreated (or not) with GRg1, then stressed with LPS and ATP. The cells were cultured for 12 h in the conditioned medium, n = 6 per group. (E) During the last 2 h of incubation, BrdU (green) was added to label proliferating cells. Nuclei were stained with DAPI (blue), n = 6 per group. (F) Immunofluorescence staining against MAP2 (red) and GFAP (green), n = 6 per group. Nuclei were stained with DAPI (blue). * P < 0.05, ** P < 0.01, *** P < 0.001 vs. control (PBS) group; # P < 0.05, ## P < 0.01, ### P < 0.001 vs. the LPS-ATP group. P values were obtained by two-way analysis of variance with Tukey's multiple-comparisons test.

**Supplementary Files**

This is a list of supplementary files associated with this preprint. Click to download.

- DataFiles.docx
- Figure4forchecking.pdf
- Figure6forchecking.pdf
- WesternBlotPDFforChecking.pdf
- figure2forchecking.pdf
- TableS1.docx