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Research Article

Ginsenoside Rh2 reduces depression in offspring of mice with maternal toxoplasma infection during pregnancy by inhibiting microglial activation via the HMGB1/TLR4/NF-κB signaling pathway

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ABSTRACT

Background: Maternal *Toxoplasma gondii* (*T. gondii*) infection during pregnancy has been associated with various mental illnesses in the offspring. Ginsenoside Rh2 (GRh2) is a major bioactive compound obtained from ginseng that has an anti-*T. gondii* effect and attenuates microglial activation through toll-like receptor 4 (TLR4)/nuclear factor-kappa B (NF-κB) signaling pathway. GRh2 also alleviated tumor-associated or lipopolysaccharide-induced depression. However, the effects and potential mechanisms of GRh2 on depression-like behavior in mouse offspring caused by maternal *T. gondii* infection during pregnancy have not been investigated.

Methods: We examined GRh2 effects on the depression-like behavior in mouse offspring, caused by maternal *T. gondii* infection during pregnancy, by measuring depression-like behaviors and assaying parameters at the neuronal and molecular level.

Results: We showed that GRh2 significantly improved behavioral measures: sucrose consumption, forced swim time and tail suspended immobility time of their offspring. These corresponded with increased tissue concentrations of 5-hydroxytryptamine and dopamine, and attenuated indoleamine 2,3-dioxygenase or enhanced tyrosine hydroxylase expression in the prefrontal cortex. GRh2 ameliorated neuronal damage in the prefrontal cortex. Molecular docking results revealed that GRh2 binds strongly to both TLR4 and high mobility group box 1 (HMGB1).

Conclusion: This study demonstrated that GRh2 ameliorated the depression-like behavior in mouse offspring of maternal *T. gondii* infection during pregnancy by attenuating the excessive activation of microglia and neuroinflammation through the HMGB1/TLR4/NF-κB signaling pathway. It suggests that GRh2 could be considered a potential therapy in preventing and treating psychiatric disorders in the offspring mice of mothers with prenatal exposure to *T. gondii* infection.

1. Introduction

Toxoplasma gondii (*T. gondii*) infection can be congenitally or postnatally acquired, and affects the health of humans and animals [1]. Numerous clinical studies have been reported that maternal *T. gondii* infection during pregnancy can cause schizophrenia [2–6] or autism [7–9] in offspring. Until now, there has been little research on the association between maternal *T. gondii* infection during pregnancy and depression in offspring.

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Microglia are the main resident macrophages in the brain and the first line of defense of the central nervous system (CNS) [10]. They have been shown to be involved in various neuropsychiatric diseases [11–15]. High mobility group box 1 (HMGB1) is an evolutionarily conserved chromosome protein that promotes innate and adaptive immune responses [16]. Toll-like receptor 4 (TLR4) is a HMGB1 receptor and is critical for the inflammatory processes mediated by HMGB1 [17,18]. Our previous studies have shown that chronic unpredictable mild stress (CUMS)-induced microglia activation resulted in over-production of neuroinflammation to cause host depressive-like behaviors through the HMGB1/TLR4/nuclear factor-kappa B (NF-κB) signaling pathway [14,15]. *T. gondii* infection-induced depressive-like behaviors also involved the TLR4/NF-κB signaling pathway [13]. Recent animal studies have shown that immunological responses to the *T. gondii* antigen in pregnant females can cause depressive- and anxiety-like behavior in their offspring [19]. Studies have shown that maternal *T. gondii* infection during pregnancy is an important factor in the initiation of innate immune activation in neonates and is associated with the prevalence of schizophrenia in offspring [6]. However, it is not known whether the HMGB1/TLR4/NF-κB pathway is involved in the depressive behavior in the offspring following maternal *T. gondii* infection during pregnancy. Therefore, it is suggested that blocking the HMGB1/TLR4/NF-κB signaling pathway, to inhibit microglial activation and neuroinflammation, may be a potential pharmacological target to reduce depression-like behavior in the offspring of mothers infected with *T. gondii* during pregnancy.

Pyrimethamine, sulfadoxine and leucovorin are used to treat infected fetuses and spiramycin (SPI) is used to prevent the transmission of maternal *T. gondii* infection during pregnancy to offspring [20]. However, these drugs are often caused adverse effects [21,22]. Therefore, it is necessary to explore anti-*T. gondii* drugs that have high efficiency and low toxicity. Ginseng (*Panax ginseng* Meyer) has been used in producing tonics and drugs in Asia for thousands of years [23]. The main component of red ginseng, ginsenoside Rh2 (GRh2), is characterized by anti-inflammatory, antioxidant and neuroprotective effects [24]. We have previously reported that the effects of toxoplasmic encephalitis were countered by GRh2 by its action in suppressing the activation of microglia and neuroinflammation through the TLR4/NF-κB signaling pathways [12]. Animal studies have demonstrated that GRh2 alleviated lipopolysaccharide-induced depressive [25] or tumor-related depression [26]. However, it is not clear whether GRh2 has an inhibitory effect on the depression-like behavior in the offspring of mothers infected with *T. gondii* during pregnancy. In this study, we used SPI as a positive control drug to compare with the effects of GRh2 on prenatal exposure to *T. gondii* infection-induced offspring depression-like behavior and the underlying mechanisms.

2. Materials and methods

2.1. Reagents and materials

Shanghai YuanYe Biotechnology Co., Ltd. (Shanghai, China) supplied 20(S)-ginsenoside Rh2 (purity ≥ 98%). Dalian Meilun Biological Technology Co., Ltd. (Dalian, China) provided SPI which served as a control drug. The sodium carboxymethyl cellulose (0.5% w/v) (Sangon Biotech Co., Ltd., Shanghai, China) was used to suspend GRh2 or SPI. Nissl staining solution was supplied by Xi'an Hat Biotechnology Co., Ltd (Xi'an, China). Primary antibodies used in immunohistochemical staining against tyrosine hydroxylase (TH), HMGB1, NeuN, Annexin V, ionized calcium-binding adapter molecule-1 (Iba-1), TLR4 and phospho-NF-κB p65 (p-NF-κB p65) were purchased from Abcam Inc. (Cambridge, MA, USA). The antibodies used for Western blot against TLR4, indoleamine 2,3-dioxygenase (IDO), NF-κB p65, p-NF-κB p65, myeloid differentiation primary response gene 88 (MyD88), tumor necrosis factor-alpha (TNF-α), α-tubulin and β-actin were obtained from Cell Signaling Technology Inc. (Beverly, MA, USA), antibodies against Toll/IL-1R domain-containing adaptor-inducing IFN-β (TRIF), TH, HMGB1, interferon-gamma (IFN-γ), Iba-1, inducible nitric oxide synthase (iNOS), NF-κB inhibitor-alpha (IκB-α) and p-IκB-α were procured from Abcam (Cambridge, MA, USA).

2.2. Mice and T. gondii maintenance

Twenty male and 30 female 9-week-old BALB/c mice (20–24 g weight) were provided by Changchun Yisi Experimental Animal Co. Ltd., China [SPF, SCXK (JI) Jilin, China, 2011-0004]. *T. gondii* cysts of the avirulent Fukaya strain (Graduate School of Medicine, Chiba University, Japan) used in our present study were maintained in C57BL/6 mice (Changchun Yisi Experimental Animal Co. Ltd., China) by serial oral inoculation passage. All care and experimental procedures of animals were conducted in accordance with the Guide for the Care and Use of Laboratory Animals (resolution number 201501022). Approval of animal studies was obtained from Animal Research Committee of Yanbian University.

2.3. Experimental designs

2.3.1. Prenatal maternal T. gondii infected and drug administration

The BALB/c mice were divided into groups of three female with two male per cage overnight. The next day, each female mouse vaginal suppository was checked. The vaginal suppository day was designated gestational day (GD)0, and each mouse was housed separately. Twenty five pregnant mice were randomly divided into 5 groups (n = 5 each): the normal control group (N, uninfected), the negative control group (NC, infection), the GRh2 groups (50 or 100 mg/kg body weight of GRh2) and the SPI group (100 mg/kg body weight of SPI). *T. gondii* Fukaya strain was administered as 8 cysts/mouse by oral gavage on GD5, except to mice in the N group, and the drugs were administered intragastrically, perorally, once daily from GD9 to GD18. Pregnant mice delivered their pups naturally on GD21, and the day of birth recorded as postnatal day (PD)0. The entire experimental schedule is shown in Fig. 1A.

2.3.2. Behavioral evaluation and sample preparation in offspring

After weaning, the sucrose preference test (SPT), tail suspension test (TST), and forced swimming test (FST) were conducted to evaluate the 10 male offspring from each group on PD36 to PD42. All mice were sacrificed after the behavioral evaluations. The entire experimental schedule is shown in Fig. 1A.

2.4. Sucrose preference test

This experiment was conducted as previous described [14,15,27]. Briefly, the mice in each group were acclimatized to drink from 2 bottles (1% sucrose or tap water) in individual cages for 2 days. Two bottles containing tap water or 1% sucrose solution were given to the mice for 1 h after being deprived of water and food for 24 h. The calculation of sucrose preference was carried out as: sucrose preference = sucrose intake (g)/[sucrose intake (g) + water intake (g)] × 100%.

2.5. Tail suspension test

The TST was conducted as described in previous studies (https://pubmed.ncbi.nlm.nih.gov/32964428/) [15]. Quantification of the cumulative immobility time in the last 4 min of a 6-min tail

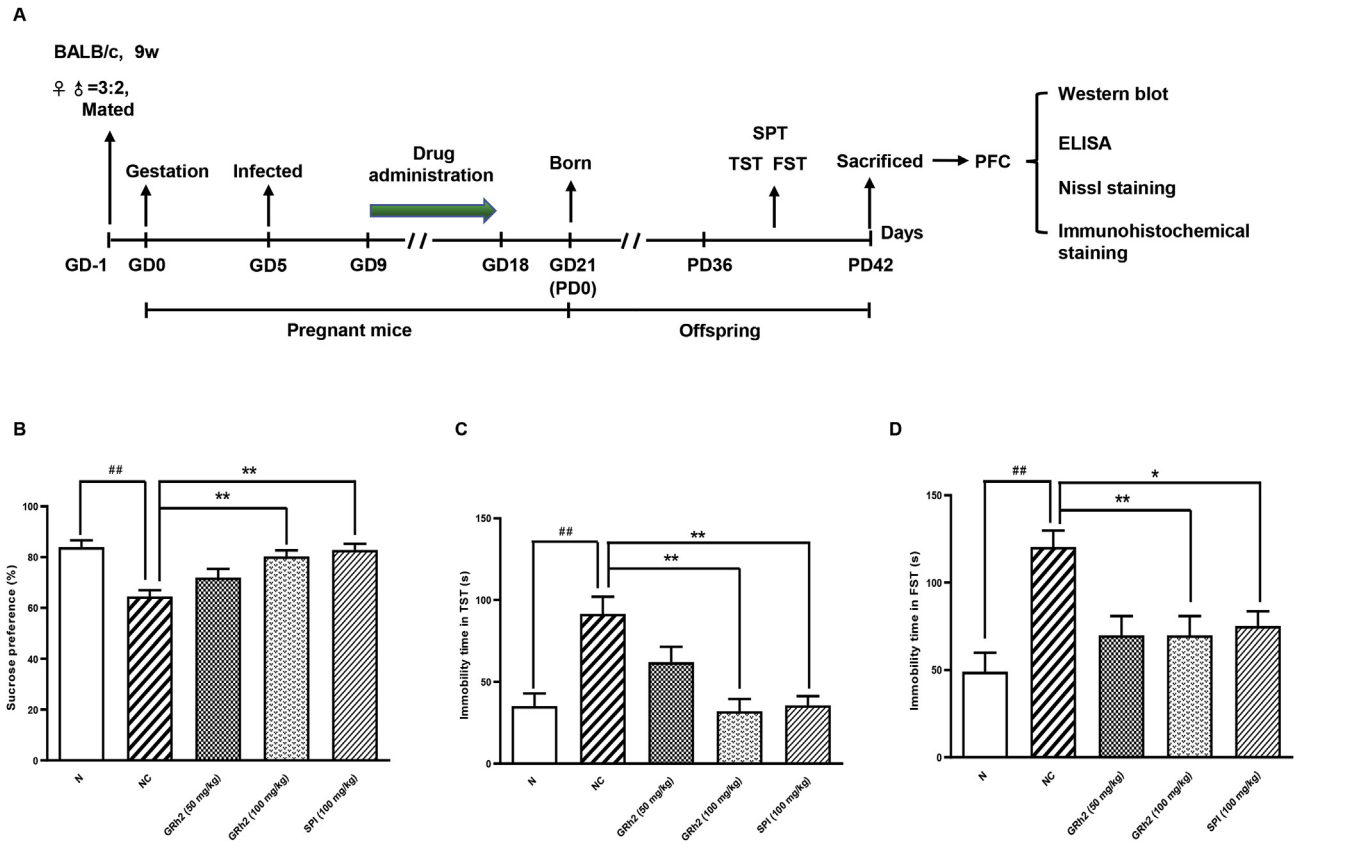

Fig. 1. The ameliorating effects of GRh2 on depression-like behaviors in the offspring mice. (A) A schematic diagram of our experimental procedures. (B) Sucrose preference in the SPT. Immobility time in the TST (C) and FST (D). All values were presented as the mean ± SEM. n = 10/group. ##$p < 0.01$ *vs*. N group; *$p < 0.05$, **$p < 0.01$ *vs*. NC group.

suspension of mice (held by adhesive tape 1 cm from the tail tip at 50 cm above the floor) was performed.

2.6. Forced swimming test

The FST was conducted based on earlier methodology (https://pubmed.ncbi.nlm.nih.gov/32964428/) [15]. A clear cylinder (diameter: 20 cm, height: 45 cm) full of water (depth: 15 cm, 24–26°C) was used for mice FST. The water was replaced after each trial. Mice were individually placed in the transparent cylinder for 6 min, and cumulative immobility time was quantified during the last 4 min.

2.7. Nissl staining and immunohistochemical staining

The prefrontal cortex (PFC) sections (8 μm) of mounted brain tissues were cut using a freezing microtome (CM1900; Leica Microsystems, Wetzlar Germany). The immunohistochemical and Nissl staining were conducted on the bases of the methods reported previously [12,14,15].

2.8. Molecular docking

The binding pattern between the GRh2 and the human HMGB1 or TLR4 was declared by the molecular docking study using Autodock Vina 1.1.2 [28]. The TLR4 and HMGB1 3D structures were deposited in the RCSB protein databank (http://www.rcsb.org/pdb/). ChemBioDraw Ultra 14.0 and ChemBio3D Ultra 14.0 software were used to draw the GRh2 3D structure. The docking input files were generated by AutoDockTools 1.5.6 package [29,30]. The Vina docking score was employed to estimate the best-scoring position with visual analysis conducted by PyMOL 1.7.6 software (http://www.pymol.org/).

2.9. ELISA

The levels of 5-hydroxytryptamine (5-HT) and dopamine (DA) in PFC were measured using kits for ELISA assay (MLBIO, China) based on protocols of the manufacturer.

2.10. Western blotting

The total lysates prepared from PFC (20 mg) samples were subjected to Western blot analysis for using the indicated antibodies The immunoreactive bands were visualized as described previously [12,14,15].

2.11. Statistical analysis

Data were analyzed by GraphPad Prism 8 software (GraphPad Software, San Diego, CA, USA), and one-way ANOVA test, followed by Tukey *post hoc* test, was used to compare the differences among multiple groups. Data are expressed as mean ± SEM. $p < 0.05$ was considered of significance.

3. Results

3.1. GRh2 ameliorates depression-like behaviors in the offspring mice

The behavioral tests including SPT, TST and FST of offspring mice were conducted in the 6th week after birth to explore the GRh2 effects on their depression-like behavior. As shown in Fig. 1B, the sucrose preference was significantly lower in the NC group compared with N mice, whereas treatment with GRh2 (100 mg/kg) or SPI (100 mg/kg) markedly reversed the reduction in the percentage of the sucrose preference (Fig. 1B). Fig. 1C and D showed that the time of immobility in the TST and FST significantly increased in the NC mice in comparison with that in the N mice. The analytical data revealed that, as with SPI administration, GRh2 ameliorates depression-like behaviors, assessed by SPT, TST and FST, in the offspring mice compared with the NC group. The effect of GRh2 treatment was dose-dependent in each test but the effects of the low dose (50 mg/kg) of GRh2 were not significantly different. Thus, 100 mg/kg dose of GRh2 was used for follow-up experiments.

3.2. GRh2 reverses monoamine neurotransmitter changes and alters the levels of IDO and TH protein expression of the PFC in the offspring mice

The protein levels of PFC 5-HT and DA expression were notably reduced in the infected offspring (5-HT: 233.73 ± 7.46 ng/mL; DA:135.86 ± 4.37 pg/mL) in comparison of those in the N mice (5-HT: 291.38 ± 10.58 ng/mL; DA:109.02 ± 3.76 pg/mL). However, the monoamine levels in the group of GRh2 (5-HT: 271.38 ± 7.01 ng/mL; DA:127.13 ± 3.52 pg/mL) or SPI (5-HT: 288.70 ± 8.99 ng/mL; DA:128.72 ± 5.64 pg/mL) treatment were comparable to those in the N group (Fig. 2A and B). Western blot was performed to detect the PFC protein levels of IDO and TH expressions (Fig. 2C). The expression of IDO was markedly enhanced, whereas the expression of TH was significantly lower in NC group in comparison of that in N group. GRh2 or SPI could significantly restore the immunoreactivity of these proteins towards normal levels. Similar results were observed in immunohistochemical staining. The protein levels of TH immunoreactivity in the NC group were decreased in comparison of those in the N group, while GRh2 or SPI opposed these trends (Fig. 2D).

3.3. GRh2 inhibits neuronal injury in the PFC of the offspring mice

The effects of GRh2 on PFC neuron loss in the offspring mice were explored in this study. Nissl stained neurons in the PFC from the N group were large, had clear outlines and abundant cytoplasmic Nissl bodies. In contrast, a clear percentage of neurons in the NC group were damaged with swollen or shrunken, even pyknotic cell bodies, unclear boundaries and fewer cytoplasmic Nissl bodies. After either GRh2 or SPI administration the neuronal damage was less than in the NC group (Fig. 3A). The numbers of Nissl bodies, swollen, shrunken and pyknotic cell bodies in the PFC were markedly reduced in the NC mice in comparison with that in the N mice. However, the decrease of Nissl body swollen, shrunken and pyknotic cell bodies numbers were markedly inhibited by GRh2 or SPI (Fig. 3B). Next, we used double immunohistochemistry staining to detect Annexin V expression in PFC neurons (Fig. 3C and D). The Annexin V-positive neurons of PFC were significantly increased in the NC group in comparison of those in the N group. However, only a marginal increase in Annexin V-positive neurons was seen in the offspring mice treated with GRh2 or SPI.

3.4. GRh2 improves PFC microglia activation and inflammation in the offspring mice

To investigate the binding mode between GRh2 and HMGB1 at the molecular level, molecular docking study was performed (Fig. 4A–i). The GRh2 molecule was located at the hydrophobic pocket of HMGB1, forming strong hydrophobic bonds to Pro-105, Pro-106, Ala-108, Phe-109, Phe-110 and Trp-140. Notably, one hydrogen bond interaction was observed between GRh2 and residue Pro-105, with a bond length of 2.5 Å, forming the main interaction between GRh2 and HMGB1 (Fig. 4A–ii). Compared with the N group, the protein levels of Iba-1 (a microglia-specific marker), HMGB1, IFN-γ, TNF-α and iNOS expression were markedly increased in the NC mice, whereas these effects were significantly less in the GRh2 and SPI treated groups (Fig. 4B). As shown in Fig. 4C, immunohistochemistry staining showed that the relative numbers of Iba-1-positive cells and relative fluorescence intensity of Iba-1-stained microglia in the PFC of NC group were significantly lower compared with the N group. In contrast, these changes were significantly less in groups administered GRh2 or SPI administration. Double immunohistochemistry staining was conducted to examine the expressions of HMGB1 in the Iba-1-positive cells of PFC (Fig. 4D and E). The HMGB1 expressions in the Iba-1-positive cells of the NC mice were clearly higher compared with those in the uninfected N mice. The administration of either GRh2 or SPI markedly inhibited the protein levels of HMGB1 in the Iba-1-positive cells of the PFC.

3.5. GRh2 inhibits the TLR4/NF-κB signaling pathway in the PFC of the offspring mice

Molecular docking studies were performed to investigate the binding mode between GRh2 and TLR4 at the molecular level (Fig. 5A–i). The GRh2 glucose group was located at the hydrophobic pocket, forming stable hydrophobic bonds with Leu-293, Tyr-296, Tyr-292, and Val-318 in the pocket (Fig. 5A–ii). Importantly, the main interactions between the GRh2 and the TLR4 were three hydrogen bonds between the GRh2 and residues Leu-293 (bond length 2.3 Å) and Lys-362 (2.9 and 3.0 Å).

The GRh2 effects on the levels of TLR4, MyD88 and TRIF protein expression were assessed using Western blotting. The protein expressions of TLR4 and MyD88 in the NC mice were markedly increased than those in the N mice (Fig. 5B). Nevertheless, the administration of either GRh2 or SPI greatly reduced the protein expressions of TLR4 and MyD88. However, the expression of TRIF was unaffected by any groups (Fig. 5B). These results suggested that GRh2 could inhibit the TLR4/MyD88 signal cascade in the PFC of offspring mice.

Furthermore, the immunohistochemical double staining analyses showed that higher TLR4 expression in Iba-1-positive cells were found in the NC mice in comparison with those in the N mice, whereas treatment with GRh2 or SPI notably inhibited the TLR4 expression in Iba-1-positive cells of the PFC (Fig. 5C).

Western blot and double immunohistochemistry staining of the mice PFCs were carried out to further determine whether GRh2 promotes the activation of NF-κB p65 via suppressing IκB-α phosphorylation. Fig. 5D showed that the protein levels of p-IκB-α and p-NF-κB p65 expression were markedly increased in the NC mice in comparison of those in the N mice, whereas their protein levels were markedly decreased by GRh2 and SPI treatment compared with those in the NC group. PFC double immunohistochemical staining analyses of the protein levels of p-NF-κB p65 expression in Iba-1-positive cells showed they were extremely elevated in the NC mice, and this trend was significantly lower after GRh2 or SPI administration (Fig. 5E).

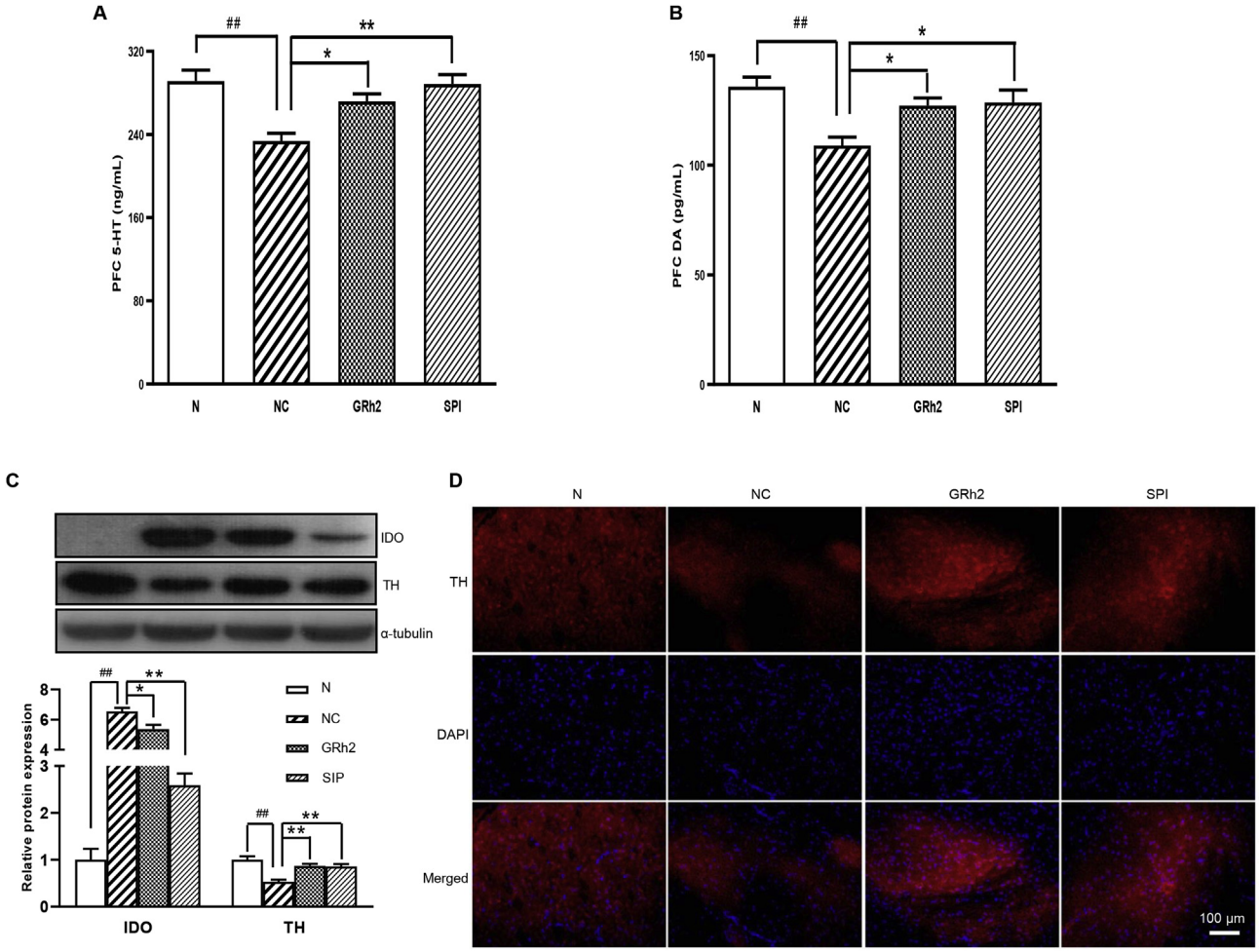

Fig. 2. Effects of GRh2 on PFC IDO and monoamines in the offspring mice. (A, B) ELISA measurements of the levels of 5-HT (A) and DA (B) expression in the PFC. (C) Western blot to detect the expression of IDO and TH in the PFC. (D) Representative immunohistochemical staining images of TH in the PFC. Results (n = 5/group) were expressed as mean ± SEM. ##$p < 0.01$ *vs.* N group; *$p < 0.05$, **$p < 0.01$ *vs.* NC group.

4. Discussion

In this study, we found that GRh2 treatment of adult female mice exposed to *T. gondii* infection during pregnancy reversed depressive behavior in their offspring. It also attenuated microglial activation and neuroinflammation through HMGB1/TLR4/NF-κB signaling pathway in the offspring.

Parasitic infections have had adverse effects on the health of tens of millions of pregnant women and fetuses [31]. A recent review of the literature indicated that *T. gondii* infection may be associated with neuropsychiatric disorders in both mothers and newborns (including anxiety, schizophrenia spectrum disorders and depression) [32]. Wang et al. [33] found that mice with congenital toxoplasma infection showed more severe depression and stereotyping compared with those with postnatal acquired infection. To date, few studies have been conducted on the mental behavior effects and their underlying mechanisms in offspring without vertical transmission of prenatal exposure to *T. gondii* infection. Al Malki et al. [9] reported that prenatal exposure to *T. gondii* infection could cause childhood autism, whether or not they are infected by the vertical transmission of *T. gondii*. The latest animal experiments have proved that the immune response to toxoplasma antigens during pregnancy may lead to anxiety-like and depression-like behaviors in their offspring, but the specific mechanism has not been clarified yet [19]. A new type of congenital toxoplasmosis called “uninfected congenital toxoplasmosis” has been proposed by Yano et al. [34], in which intrauterine growth retardation of fetuses occurs without fetal *T. gondii* infection *in utero*. We also found that *T. gondii* were not detected in the brains of mouse offspring of mothers with *T. gondii* infection during pregnancy (data not shown), however, the offspring showed depression-like behavior. A previous study showed that some maternal chronic infections can influence the neonatal immune response [6]. However, the effects and underlying pathogenic mechanism of depression-like behavior in mouse offspring of maternal *T. gondii* infection during pregnancy remains unclear.

Our previous study found that microglia activation in the PFC was critical for the occurrence and progression of depression [14,15]. Animal studies found that the pro-inflammatory *in utero* milieu triggered by maternal infection not only had a negative effect on neurodevelopment but could also influence the onset of mood disorders in offspring. These negative effects may result from programming individuals for a pro-inflammatory immune phenotype via activation of the neonatal immune system [35]. Our previous studies showed that GRh2 can inhibit the neuroinflammation induced by *T. gondii* infection, reducing the damage to neurons in the hippocampus and cortex [12]. We also found that in the CUMS mouse model, sertraline inhibited inflammation by inhibiting the

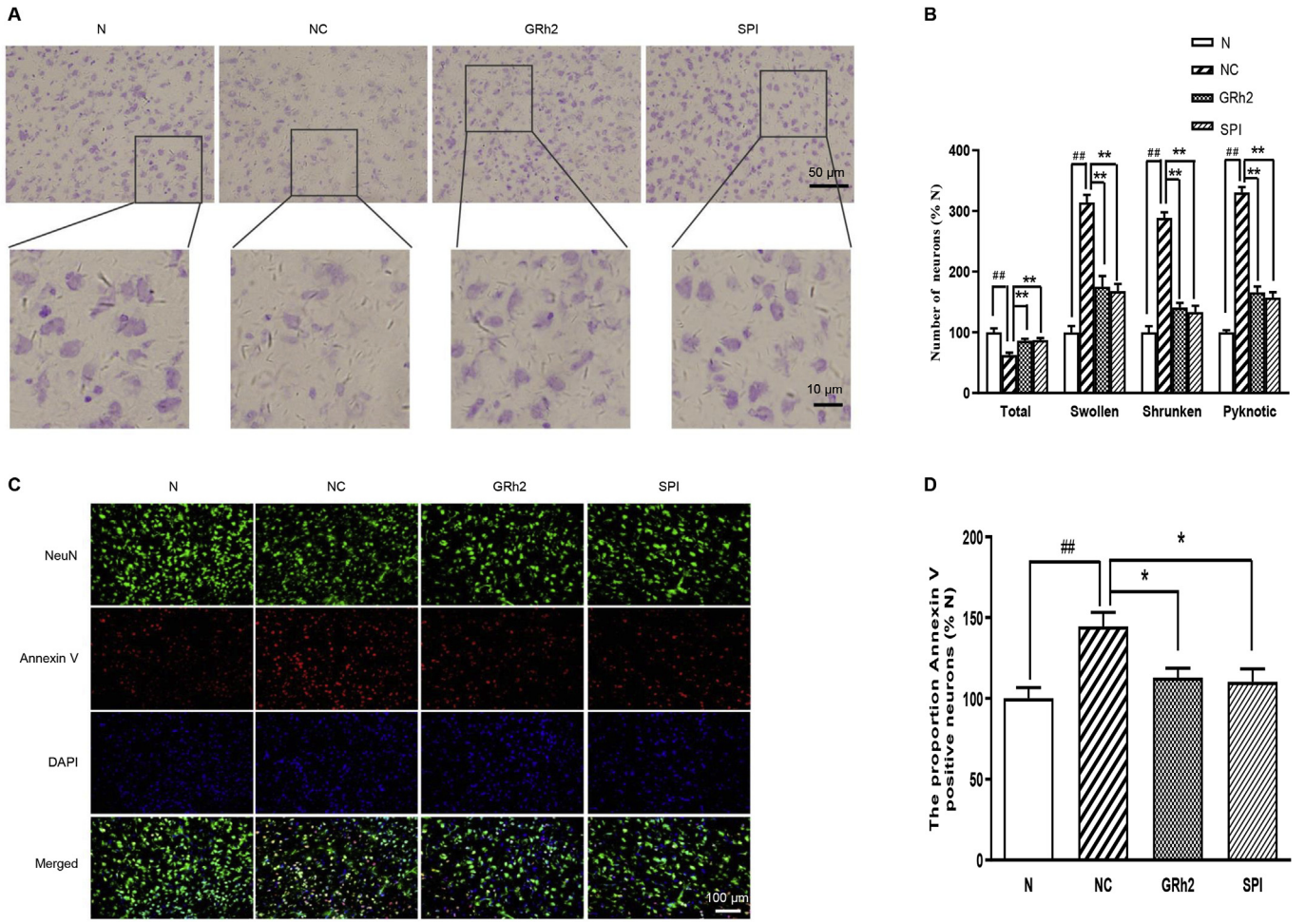

Fig. 3. Effects of GRh2 on PFC neuron loss and neuronal Annexin V expression in the offspring mice. (A) Nissl staining in the PFC. (B) The quantitative results of relative neuron density, swollen, shrunken and pyknotic cell bodies in the PFC. (C) Immunohistochemistry detection of Annexin V (red) within NeuN (green) in the PFC. (D) The proportion of Annexin V positive neurons. Values (n = 5/group) were expressed as mean ± SEM. ##$p < 0.01$ *vs.* N group; *$p < 0.05$, **$p < 0.01$ *vs.* NC group.

activation of microglia cells and the expression of inflammatory factor TNF-α, further preventing the development of depression [11]. In this study, we found the expression levels of Iba-1 and inflammatory cytokines (HMGB1, TNF-α, iNOS, and IFN-γ) were increased in the PFC of offspring whose mothers were infected with *T. gondii* during pregnancy. Whereas GRh2 inhibited both the activation of microglia and the inflammation response. This suggests that GRh2 interferes with the onset of depression in offspring, possibly by inhibiting the maternal inflammation of *T. gondii* infection during pregnancy. We also found that apoptosis and neuronal damage in the PFC of mouse offspring of maternal *T. gondii* infection during pregnancy was inhibited by GRh2.

Previous results showed that the depression-like behaviors can be caused by *T. gondii* infection and that the protein levels of both 5-HT and DA expression in the brains were reduced in the acute infectious stage [13,36]. Over activation of inflammation in the brain and neurotransmitter disorders may be underlying causes of neurobehavioral disorders in infected hosts [37]. Previous studies have found that IFN-γ, TNF-α and HMGB1 stimulate the production of IDO, resulting in reduced 5-HT production in the brain and thereby inducing depression-like behavior [15,36]. Another report demonstrated that immune enhancement in response to the *T. gondii* infection caused the activation of IDO and inflammation linked to anhedonic and depression-like behaviors [13,36]. However, DA synthesis relies on the conversion of tyrosine to L-dihydroxyphenylalanine by the rate-limiting enzyme TH [38]. *T. gondii* can also reduce the expression of TH in the neurons of the superior cervical ganglion [39]. A recent study found that pregnant women with depression had low levels of dopamine and serotonin, and subsequently gave birth to babies with depression-like symptoms, also with lower levels of DA and 5-HT [40,41]. Our previous study found that compared with healthy mice, the levels of TH and DA expression of the PFC were reduced in the chronic unpredictable mice exposed to mild stress [14]. The levels of 5-HT and DA can be suppressed by iNOS increase leading to the depression-like behavior [14,15]. In our present study, the IDO expression levels of the PFC can be increased and down-regulation of the TH levels decreased in the mouse offspring affected by prenatal exposure to *T. gondii* infection. Consequently, the depression-like behavior of offspring was induced due to the decreased 5-HT and DA expression in the PFC. However, GRh2 treatment suppressed both the increased protein levels of IDO expression and the reduced TH, 5-HT, and DA expression in the PFC of the offspring of mothers infected with toxoplasma during pregnancy. However, Goodwin et al. [42] reportedly found no differences of the protein levels of cerebral 5-HT and DA expression between the congenital toxoplasma infection offspring mice and the uninfected mice. The difference in findings for 5-HT and DA may be attributable to their use of 8-week-old male and female CD-1 offspring mice and the use of the VEG strain (type III genotype) of *T. gondii* on GD11 to infect

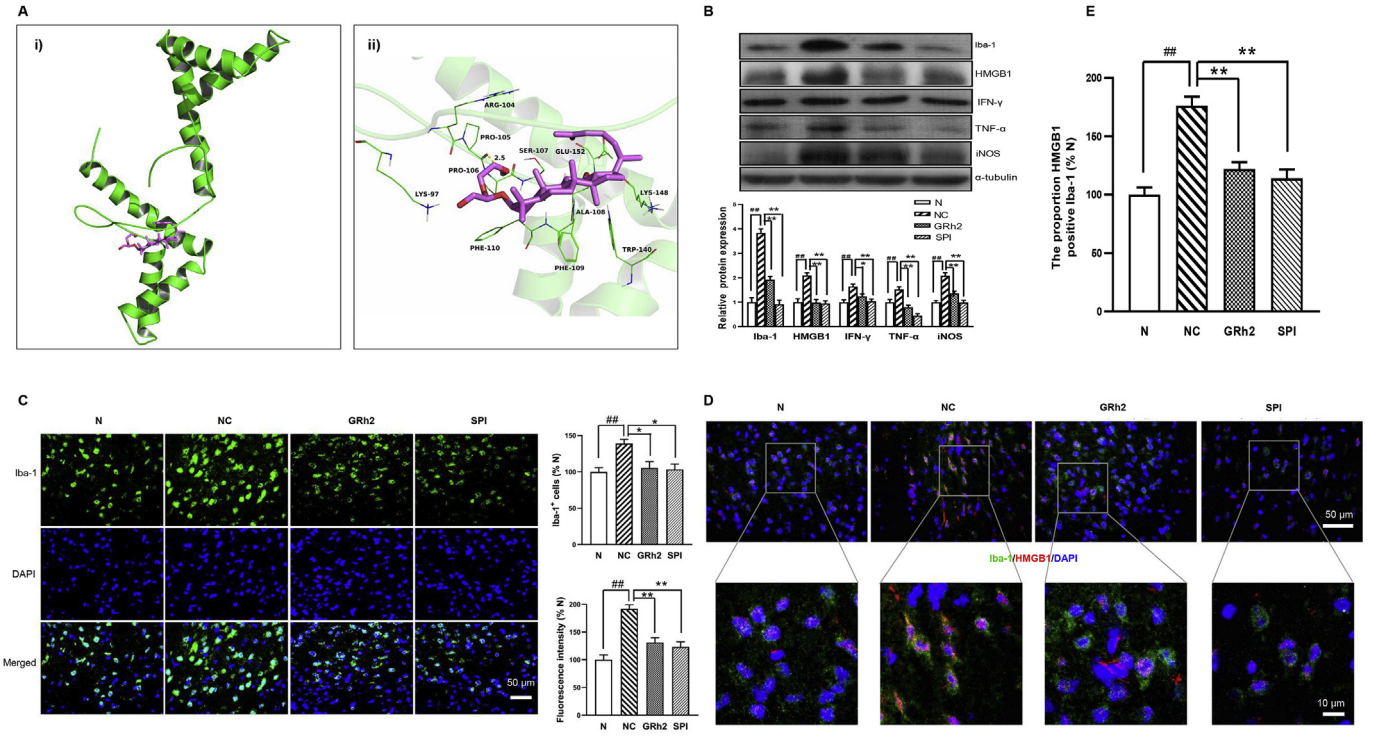

Fig. 4. Effects of GRh2 on microglial activation and inflammatory response in the PFC of offspring mice. (A) Molecular docking analysis between GRh2 and HMGB1, (i) the overall view and (ii) magnified view. (B) Protein expressions of PFC Iba-1, HMGB1, IFN-γ, TNF-α and iNOS assessed by Western blot n = 5/group. (C) Immunohistochemistry of Iba-1 (green) expression in the PFC. Relative numbers and fluorescence intensities of Iba1-positive cells were quantified by ImageJ program.(D) Immunohistochemistry indicated HMGB1 (red) and Iba-1 (green) expression in the PFC. (E) The proportion of HMGB1 in Iba1-positive cells were quantified by ImageJ software. Results (n = 5/group) were presented as mean ± SEM. ##$p < 0.01$ *vs.* N group; *$p < 0.05$, **$p < 0.01$ *vs.* NC group.

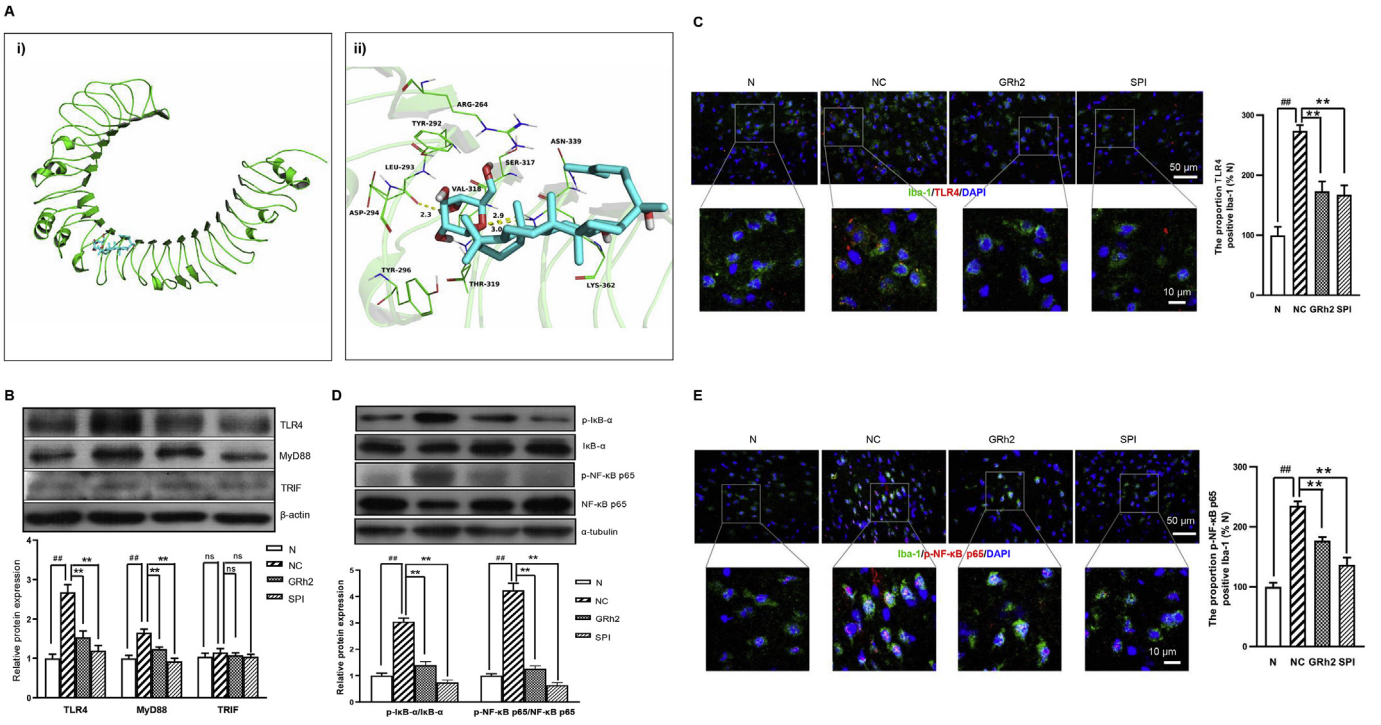

Fig. 5. GRh2 suppresses TLR4/NF-κB signaling pathway in the PFC of offspring mice (A) Molecular docking analysis between GRh2 and TLR4 in the overall view (i) and magnified view (ii). (B) Western blots of TLR4 and MyD88 protein expressions. n = 5/group. (C) Immunohistochemical staining was performed to measure the levels of PFC TLR4 (red) and Iba-1 (green) expression. The proportion of TLR4 in Iba1-positive cells were quantified by ImageJ software. n = 5/group. (D)The protein levels of PFC p-IκB-α, IκB-α, p-NF-κB p65 and NF-κB p65 were evaluated by Western blot. n = 5/group. (E) Representative images of PFC p-NF-κB p65 (red) and Iba-1 (green) were displayed, the proportion of p-NF-κB p65 in Iba1-positive cells were quantified by ImageJ software. Values (n = 5/group) were expressed as mean ± SEM. ##$p < 0.01$ *vs.* N group; **$p < 0.01$ *vs.* NC group.

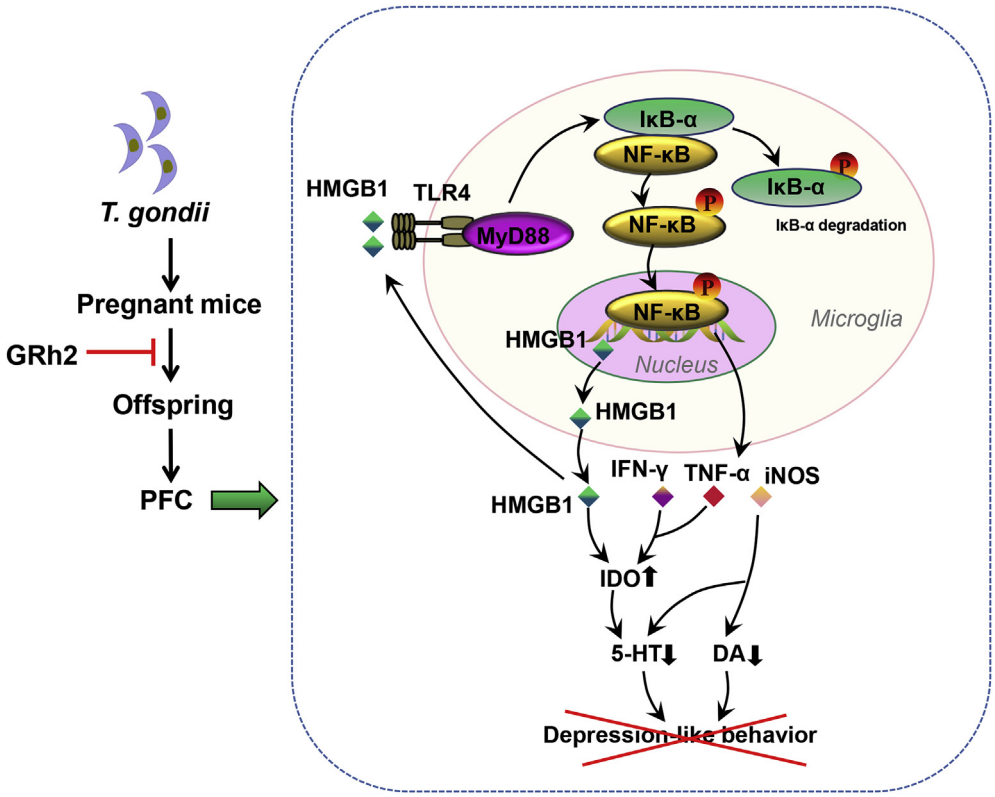

Fig. 6. Schematic diagram for the possible mechanisms by which GRh2 suppresses the maternal toxoplasma infection-induced depression-like behaviors in the offspring mice. The activation of microglia and neuroinflammation can be inhibited by GRh2 via the HMGB1/TLR4/NF-κB signaling pathway, thereby reducing the depression-like behaviors in these mice.

pregnant mice. Since it was reported that male mice may be more susceptible to subtle neurotransmitter changes than female mice [42,43], we used 6-week-old male BALB/c mice offspring and infected pregnant mice with Fukaya strain (type II genotype) of *T. gondii* on GD5 in this study. We suggest that infection at different stages of pregnancy, *T. gondii* strains, mouse species and sex might affect the monoamine transmitter levels in offspring.

T. gondii not only causes secretion of HMGB1 to induce macrophages producing TNF-α [44], but also promotes the hosts to secrete HMGB1 [18]. Our previous research found that the increased expression of TNF-α [11,13–15] and HMGB1 [14,15] in microglia could improve depression-like behavior. Mice exposed to CUMS activated their microglia to secrete HMGB1, which promoted the release of IDO and decreased the expression of 5-HT, thus inducing depression [15]. Interestingly, the HMGB1/TLR4 complex induces more HMGB1 secretion, which enhances the inflammatory process [45]. It has been reported that TLR4 may be related to the neuroinflammatory response induced by toxoplasma infection in mice [12,13] and rats [46]. NF-κB is a key transcription factor in the regulation of inflammatory responses [47]. Among of NF-κB families, NF-κB p65 is a key protein in the NF-κB signaling pathway under physiological conditions [14].

Previously, we demonstrated that the HMGB1/TLR4/NF-κB signaling pathway participated in the depression-like behaviors [14,15]. Recently, we found that depression-like behaviors in mice can result from infection by *T. gondii* through the activation of microglia and the responses to inflammation via the TLR4/NF-κB signaling pathway [13]. In our Western blot study, we detected high expression levels of TLR4, MyD88, HMGB1, NF-κB p65 and p-IκB-α in the PFCs of offspring exposed to maternal toxoplasma infection during pregnancy and that GRh2 treatment reduced this phenomenon. Double immunohistochemistry staining confirmed that the increased levels of HMGB1, TLR4 and p-NF-κB p65 expressions were found in the PFC microglia from offspring exposed to maternal toxoplasma infection during pregnancy and that GRh2 reduced the changes. In addition, we found that there was no difference in the expressions of TLR4, NF-κB, HMGB1, IFN-γ, TNF-α, iNOS, 5-HT, DA, TH and IDO in control, uninfected mice with or without GRh2 treatment (data not shown). Therefore, we hypothesize that GRh2 may treat depression-like behaviors in the mouse offspring of maternal toxoplasma infection during pregnancy through the HMGB1/TLR4/NF-κB signaling pathway. A proposed pathway for the action of GRh2 is given in Fig. 6. Further investigations might use TLR4 gene knockout mice or direct inhibition of TLR4 pathway.

In summary, our findings suggest that maternal toxoplasma infection during pregnancy causes depression-like behavior in the mouse offspring even if it is not transmitted vertically. In addition, we found that early treatment with GRh2 of *T. gondii* infection in mothers during pregnancy may reduce depression-like behavior in their offspring by alleviating the activation of microglia and inflammatory response through the HMGB1/TLR4/NF-κB signaling pathway. This provides pharmacological evidence that GRh2 could reduce psychiatric disorders in offspring exposed to the consequences of maternal *T. gondii* infection during pregnancy.

Declaration of competing interest

None.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jgr.2021.04.003.

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