Oridonin relieves depressive-like behaviors by inhibiting neuroinflammation and autophagy impairment in rats subjected to chronic unpredictable mild stress

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Abstract
Major depressive disorder (MDD) is a severe life-threatening disorder with increasing prevalence. However, the mechanistic interplay between depression, neuroinflammation, and autophagy is yet to be demonstrated. This study investigated the effect of Oridonin on CUMS-induced depression, neuroinflammation, and autophagy impairment. Male 4-week-old Sprague–Dawley rats were subjected to chronic unpredictable mild stress (CUMS), some of which were injected with Oridonin, fluoxetine (FLX), or their combination at different durations of CUMS. CUMS significantly increased the levels of cytokines (IL-1β, IL-18, and caspase-1), reduced autophagy-related protein levels (Beclin-1, p62, Atg5, and LC3B), and caused microglia cells activation. Oridonin prevented and reversed the depressive-like behavior. Furthermore, it has a stronger and longer-lasting antidepressant effect than FLX. And the antidepressant effect of Oridonin in combination with fluoxetine was greater than that of high-dose fluoxetine alone. In addition, Oridonin significantly normalized autophagy-related protein levels, and reduced levels of cytokines by blocking the interaction between NLRP3 and NEK7. Similarly, Oridonin abolished levels of cytokines and reversed autophagy impairment in LPS-activated BV2 cells. All these results supported our hypothesis that Oridonin possesses potent antidepressant action, which might be mediated via inhibition of neuroinflammation and autophagy impairment by blocking the interaction between NLRP3 and NEK7.

KEYWORDS
autophagy, depressive-like behavior, NEK7, neuroinflammation, NLRP3, Oridonin

1 INTRODUCTION
Major depressive disorder (MDD) is a life-threatening and debilitating mental disorder characterized by depressed mood, ruminative thoughts, anhedonia, cognitive dysfunction, vegetative symptoms, and even a high suicidal tendency (Alexopoulos, 2005; Kessler & Bromet, 2013). Accumulating evidence has shown that neuroinflammation is associated with the etiology of depression (Franklin, Xu, & Duman, 2018; Slavich & Irwin, 2014). Experimental and clinical studies have indicated that proinflammatory cytokines, including IL-1β,...
(interleukin 1β) and IL-18 (interleukin 18), were positively associated with depressive symptoms (Rosa et al., 2004; Wu et al., 2020). And, several studies showed that elevation in brain IL-1β level is both necessary and sufficient for producing a high incidence of depression, and reducing brain IL-1 levels may have potent anti-depressive actions (Ellul, Boyer, Groc, Leboyer, & Fond, 2016; Goshen et al., 2008). The NLRP3 inflammasome is one multiprotein complex that serves as platform for the activation of caspase-1, leading to the processing and secretion of IL-1β and IL-18 (Gross, Thomas, Guarda, & Tschopp, 2011). Therefore, depressive-like behaviors required a functional NLRP3 inflammasome (Alcocer-Gomez et al., 2016; Zhang et al., 2015). Microglia in the central nerve system (CNS) act as the first line of broader immune response to pathogen-associated molecular patterns. When stimulated by external factors, microglia can be activated via TLR-like or NOD-like receptors, and then released immune molecules, including proinflammatory cytokines, chemokines, and reactive oxygen species (Walsh, Muruve, & Power, 2014), which leads to the development of depressive symptoms (Wong et al., 2016).

Autophagy is a catabolic process that degrades the cytosolic constituents through autophagosome formation. It regulates cytokine production and secretion, inflammasome activation, and clearance of accumulating as well as invading pathogens (Deretic & Levine, 2018). In the last decade, numerous studies have further indicated that autophagy can regulate NLRP3 inflammasome activation through various mechanisms (Saitoh & Akira, 2016). On the contrary, a few studies have also shown that NLRP3-deficient mice have increased autophagy levels at baseline and under stress conditions (Kim et al., 2018; Zhang et al., 2014). In addition, caspase-1 was also reported to regulate the autophagic process through cleavage of other substrates (Jabir et al., 2014; Yu et al., 2014). Studies have evidenced the activation/inhibition of autophagy and excessive activation of microglia to have a close relationship with depression, but the role of autophagy in depression pathogenesis is ambiguous (Shih et al., 2019; Tan et al., 2018). Therefore, scholars across the world postulated that future procedures targeting the NLRP3 inflammasome may have promising effects in the prevention and treatment of depression (Kaupmann et al., 2017; Xu et al., 2016).

Oridonin (Ori), a diterpenoid isolated from Rabdosia rubescens, has multiple biological properties, especially anti-inflammatory and neuro-regulatory activities (Lin et al., 2019; Xu, Li, Zhang, Schluesener, & Zhang, 2019). Although it has been suggested that Ori may be involved in antidepressant effects through PPAR-γ/AMPA receptor signaling pathway, its specific targeting mechanism has not been clarified (Liu & Du, 2020). Recently, one finding suggested that Ori can be used to treat a variety of inflammatory diseases by blocking the interaction between NLRP3 and NEK7 (He et al., 2018). NIMA-related kinase 7 (NEK7), which plays a crucial role in mitosis entry, cell cycle progression, cell division, and mitotic processes, has been proven to be a vital mediator during inflammasome activation in macrophages (Shi et al., 2016). In our previous study, Ori effectively reduced insulin resistance in dysglycemia comorbid depression model by inhibiting peripheral inflammation (Liang, Zheng, Xie, Xiao, & Wang, 2021). However, the molecular mechanism of Ori in the treatment of neuroinflammation-induced autophagy impairment and depressive-like behaviors is yet to be elucidated. Therefore, we investigated whether Ori can attenuate depressive-like behaviors by inhibiting neuroinflammation and autophagy impairment. For better appraisal, we also compared the anti-depressive effects of Ori with those of fluoxetine (FLX, as classic antidepressants). In addition, in order to verify whether be used as a complementary treatment for antidepressants, we tested the total effects of the combined application of these two drugs.

### 2 | MATERIALS AND METHODS

#### 2.1 | Animals

Male 4-week-old Sprague–Dawley rats (80–100 g) were purchased from the Center for Experimental Animal at Wuhan University, Wuhan, China. All rats were housed individually under a standard 12 h light/dark schedule (22–24°C with 50–60% humidity) with lights on (150 lx intensity) at 08:00 a.m. For acclimation, the rats were treated with 1% sucrose solution (weight/volume) for 7 days. All experiment protocols were performed in accordance with the experimental protocols approved by the Laboratory Animal Welfare & Ethics Committee of Renmin Hospital of Wuhan University (Certificate no. 20190430).

#### 2.2 | Chronic unpredictable mild stress (CUMS) protocols

The procedure used for the chronic unpredictable mild stress (CUMS) was adapted from our past research (H. Wang, Xiao, Wang, & Wang, 2020). Rats in the CUMS group were subjected to various mild stressors for 4 and 6 weeks, respectively. The stressors applied included the following: cage tilting (45°) for 24 h, damp sawdust (200 mL of water in a cage) for 24 h, swimming in water at 45°C for 5 min, swimming in water at 5°C for 5 min, tails clamped for 1 min, 24 h of water deprivation, 12 h of overnight illumination, cage shaking for 10 min, making noise for 10 min and 24 h of food deprivation. During the stress procedure, two different stressors were applied each day, and the sequence of stressors changed every 3 days. Control rats were housed under the condition of the same breed but with no exposure to the above stressors.

#### 2.3 | Drug administration and experiment design

Ori (purity ≥98%, CAS: 28957-04-2) purchased from Albabiotech company (Chengdu, China) was dissolved in dimethylsulfoxide (DMSO, Sigma, St. Louis, MO) and then diluted in saline to a concentration of 20 mg/mL. The structure of the Ori is shown in Figure 1a. FLX (Sigma-Aldrich, St. Louis, MO, USA) was dissolved in phosphate-
buffered saline to obtain a stock solution of 5 mg/mL (PBS, pH 7.4). The doses of Ori administered intraperitoneally were adopted from previous studies of Alzheimer’s disease (Wang et al., 2014; Wang et al., 2016). All rats were randomly selected and given intraperitoneal injection of Ori and FLX.

Two patterns of drug application were employed in this study: prophylactic and therapeutic treatment. According to our preliminary experimental results (Figure 1b–e), the rats subjected to mild stressors developed depressive symptoms from the third week. Thus, in the prophylactic treatment, drugs were given after rats were subjected to mild stressors at first day until third weekend. In this paradigm, the rats were treated with Ori at 5, 10, or 20 mg/kg and FLX at 10 or 18 mg/kg once per day for 3 weeks. In addition, FLX at 10 mg/kg together with varied doses of Ori in the drug combination study. The FST was performed at the fourth weekend, SPT at third and fourth weekend and body weight measurement once per week. For the therapeutic treatment, the rats received daily treatment with Ori at 5, 10, or 20 mg/kg once per day from the fourth weekend to the sixth weekend during CUMS procedure. The FST, SPT, and body weight measurements were performed at the sixth weekend (Figure 1f).

2.4 Behavioral tests

All behavioral tests, including sucrose preference test and forced swim test, were all carried out in the dark phase (19:00–22:00 p.m.).

2.4.1 Sucrose preference test

The sucrose preference test (SPT) is a widely used method of evaluating depressive-like behavior in animals (Willner, 2017). According to this literature, food and water were deprived 20 h before SPT. During the test, the rats were allowed to drink 1% sucrose solution (250 ml) and the same volume of water for 2 h. The one bottle of sucrose solution was changed into water on the second day. Fluid consumptions were checked after the 2 h test. Prior to the CUMS, baseline preference for sucrose was recorded, and only rats that showed a sucrose preference >65% were included in this study. Sucrose preference proportion = sucrose solution consumption/(sucrose solution consumption + tap water consumption) * 100%.

2.4.2 Forced swim test

The Forced swim test (FST) is one of the most commonly used animal models for assessing antidepressant-like behavior (Slattery & Cryan, 2012). According to literature, the FST setup consisted of a cylinder (40 cm height × 28 cm internal diameter). In the pretest, the rats were individually placed in the cylinder with water at 25 ºC filled to 30 cm. The rats were removed after 15 min, dried, and returned to their home cages. Then on the test day, they were immersed in a swimming tank for 6 min one by one. Immediately after the testing, rats were removed from the water, gently dried with paper towels,
and placed inside a cage warmed by a heating pad. The duration of climbing and immobility was videotaped and analyzed. The immobility time was defined as floating time in the water without struggling and only performing the movements to keep the head above water during the final 4 min. And Climbing time was defined as upward-directed movement time of the forepaws.

2.5 | Sacrifice and sample preparation

After completion of behavioral tests, all rats were sacrificed by decapitation. Hippocampus were dissected and flash-frozen in liquid nitrogen immediately after decapitation. The remaining rats were transcardially perfused with 0.9% saline, then with 4% paraformaldehyde in phosphate buffer. And then, the brains were rapidly collected and fixed in 4% paraformaldehyde for 1 week. Finally, the brains were embedded in paraffin. Serial 5 μm sections were cut in the coronal plane.

2.6 | Enzyme-linked immunosorbent assays

Serum IL-1β, IL-18, and corticosterone levels were measured, respectively, by Rat Interleukin 1β, IL-1β ELISA Kit (CSB-E08055r, CUSABIO, Wuhan, China), Rat Interleukin 18, IL-18 ELISA Kit (CSB-E04610r, CUSABIO, Wuhan, China) and Rat Corticosterone, CORT ELISA Kit (CSB-E07014r, CUSABIO, Wuhan, China). All procedures are performed according to the manufacturer’s instructions (Lai, Feldman, & Clark, 2005).

2.7 | Immunostaining and morphometric analysis

According to the previous study (Rho & Swanson, 1989), the Paraffin-embedded brain sections were rinsed in 0.01 M PBS (pH 7.3) 3 times (10 min for each) and blocked in 0.01 M PBS containing 10% normal donkey serum and 0.3% Triton X-100 for 1 h at RT. And then, the sections were incubated for 1 h at RT and for 48 h at 4°C with primary antibodies: goat anti-Iba1 antibody (1:500; ab5076, Abcam, USA), mouse anti-GFAP (1:1000; MAB3420, Merck, Germany), mouse anti-NeuN (1:1000; MAB377, Merck, Germany) and rabbit anti-NEK7 antibody (1:800; NBP1-31110, NOVUS, USA) in PBS containing 0.3% (v/v) Triton X-100, 0.25% (w/v) λ-carrageenan, and 5% (v/v) donkey serum (PBS-XCD). All sections were washed three times in 0.01 M PBS (10 min each), and then were incubated for 1.5 h at RT with donkey anti-goat IgG/FITC antibody (1:1000; bs-0294D-FITC, BIOSS, China), donkey anti-rabbit IgG/Cy3 antibody (1:1000; bs-02950-Cy3, BIOSS, China) and goat anti-mouse IgG/FITC antibody (1:1000; bs-02966G-FITC, BIOSS, China), respectively. Finally, all sections were air-dried and coverslipped with a mixture of 0.05 M PBS containing 50% (v/v) glycerin and 2.5% (w/v) triethylenediamine. The confocal images were obtained, and digital images were captured using a Fluoview laser scanning confocal microscopes (Olympus) equipped with the FV1000 (Ver.1.7a) software.

2.8 | Western blot analysis

The hippocampus and prefrontal cortex were homogenized in the ice-cold RIPA lysis buffer (P0013B, Beyotime Biotechnology, China) with protease inhibitor cocktail (P1005, Beyotime Biotechnology, China) and phosphatase inhibitor (P1050, Beyotime Biotechnology, China). The protein concentrations were estimated using the bicinchoninic acid (BCA) method (P0010S, Beyotime Biotechnology, China). The centrifuged lysates were added with 5X loading buffer (P0015, Beyotime Biotechnology, China) at 4:1 volume ratio, and then heated in boiling water for 10 min. Equal amounts of protein samples were loaded and separated on 10% or 12% SDS-PAGE gels (10% or 12% TGX FastCast Kit, BIORAD, USA). The proteins were electroblotted onto a polyvinylidene difluoride membrane (0.22 μm; PVDF, Millipore, USA). Afterwards, the blots were blocked with 0.1% Tween-20 solution (TBS-T) containing 3% bovine serum albumin (BSA) and incubated with appropriate primary antibodies: NEK7 antibody (ab133514, Abcam, USA), anti-IL-1β beta antibody (ab9722, Abcam, USA), IL18BP antibody (NB200-201, NOVUS, USA), NLRP3 antibody (PA5-79740, THERMO, USA), anti-caspase-1 + p10 + p12 antibody (ab179515, Abcam, USA), anti-β-actin (14600-1-AP, Proteintech Group, China), anti-ATG5 (10181-2-AP, Proteintech Group, China), anti-Beclin-1 (11306-1-AP, Proteintech Group, China), anti-β-actin (18420-1-AP, Proteintech Group, China), and GAPDH antibody (AF1186, Beyotime Biotechnology, China). The immunoblots were then incubated with the appropriate horseradish peroxidase (HRP)-conjugated secondary antibodies at RT for 1.5 h. The immunoblotting bands were visualized by incubation with ECL reagent (P0018FS, BIORAD, USA) and exposed to X-ray film (BIORAD). For analysis, the levels of target proteins were normalized against those of GAPDH (Martin et al., 2004).

2.9 | Immunoprecipitation

For detecting the interaction of NEK7 and NLRP3 by co-immunoprecipitation (Co-IP)(Ponzielli et al., 2008). According to the manufacturer’s instructions (P2012, Beyotime Biotechnology, China), the centrifuged lysates were incubated with a 1:200 dilution of anti-NEK7 or anti-NLRP3 antibody overnight, at 4°C. Then, 40 μl Protein A/G Agarose (Beyotime, China) was added and incubated for an additional 4 h in a shaker. The immune complexes were boiled in the sample buffer after washing with PBS five times. The samples were then immunoblotted with anti-NLRP3 or anti-NEK7, respectively.

2.10 | Cell culture and treatment

Mouse microglial (BV2 cells) purchased from China Center for Type Culture Collection (Wuhan, China), were maintained in Dulbecco’s Modified Eagle Medium (DMEM) containing 10% fetal bovine serum (FBS) and incubated in a humidified atmosphere of 5% CO2 and 95% air at 37°C. The cells (passages from 10 to 15) were pretreated with
Ori (respectively, 5, 10, 20, or 40 μmol/L) for 12 h followed by incubation with LPS (1 μg/mL) for 24 h.

2.11 Cell viability analysis

Cell viability was measured using MTT (C0009, Beyotime Biotechnology, China) assay (Cai et al., 2021). The microglia cells were plated in cell culture plates at a density of 1.5 × 10^5 cells/well and cultured overnight. Various concentrations of Ori (respectively, 5, 10, 20, or 40 μmol/L) were added to each well and incubated for 12 h. According to the manufacturer’s instructions, the MTT solution (5 mg/mL) was added to each well and incubated in a humidified atmosphere of 5% CO2 and 95% air at 37°C for 4 h. Thereafter, the formazan solution was added and incubated at 37°C for 3 h. After the formazan crystals had dissolved, absorbance was read at 570 nm.

2.12 Limulus amebocyte lysate (LAL) test

The effect of Ori on LPS activity was measured by using the LAL test (Rat Lipopolysaccharides ELISA Kit, CSB-E14247r, CUSABIO, Wuhan, China) (Lamprou et al., 2022). Briefly, a series of concentrations of the Ori (5, 10, 20, and 40 μmol/L) were incubated with LPS (1 μg/mL) for 30 min at 37°C. The absorbance was measured at 450 nm after the addition of 100 μl of the chromogenic substrate.

2.13 Statistical analysis

All data were analyzed with GraphPad Prism 6 software (San Diego, CA, USA) and are presented as the mean ± SD. The normality of the data was tested using the D’Agostino and Pearson omnibus normality test. In addition, the data from behavioral tests at one point in time and Western blot analysis were analyzed by Student’s t-test for two-group comparisons or one-way analysis of variance (ANOVA) followed by Dunnett’s multiple comparison test for the various CUMS groups. p-Values less than 0.05 were considered statistically significant.

3 RESULTS

3.1 The effects of prophylactic and therapeutic Ori treatment on depressive-like behavior induced by CUMS

In prophylactic treatment, the experimental rats were subjected to CUMS and drug administration simultaneously for 3 weeks. To determine the potential anti-depressive effect of Ori, behavioral analyses were performed. Firstly, no significant differences were detected between CUMS and Veh in all tests. As compared to Control, CUMS rats showed significantly increased immobility time (t = 11.71, p < .001. Figure 2a) and decreased climbing time in the FST (t = 9.216, p < .001. Figure 2b). In addition, in comparison with control group, CUMS also showed fewer percentage of sucrose preference (t = 13.71, p < 0.001. Figure 2c) and lighter body weight (t = 8.56, p < .001. Figure 2d) at fourth weekend. However, both 5 mg/kg and 10 mg/kg of Ori significantly decreased immobility time in CUMS rats compared with CUMS + Veh (respectively, t = 3.918, p < .05; t = 9.114, p < .001) (Figure 2a). And only 10 mg/kg of Ori significantly increased climbing time (t = 3.749, p < .05. Figure 2b). Similarly, sucrose preference levels were higher in both 5 mg/kg and 10 mg/kg of Ori groups when compared to CUMS + Veh group at fourth weekend (respectively, t = 2.949, p < .05; t = 4.051, p < .001. Figure 2c). Meanwhile, no significant differences were detected between 5 or 10 mg/kg and CUMS + Veh in body weight gain measurement (Figure 2d). Interestingly, the depression-like behaviors in the CUMS rats were not further improved by 20 mg/kg of Ori in the FST and SPT, as well as in body weight gain.

In therapeutic treatment, no significant differences were detected between CUMS and CUMS + Veh in all tests. Meanwhile, a visual but insignificant improvement in depression-like behaviors in response to 5 mg/kg of Ori was observed in immobility time and climbing time at sixth weekend (Figure 2e,f), at which time a significant increase in sucrose preference level compared with CUMS + Veh (t = 2.945, p < .05. Figure 2g). Therapeutic treatment with 10 mg/kg of Ori caused substantial inhibition of the depression-like behaviors compared with CUMS + Veh in all test (immobility time, t = 8.384, p < .001; climbing time, t = 4.578, p < .05; sucrose preference level, t = 5.626, p < .001. Figure 2e-g). Same as prophylactic administration, the depression-like behaviors in the CUMS rats were also not improved by 20 mg/kg of Ori therapeutic treatment. In addition, no significant differences were detected among all doses of Ori compared with CUMS + Veh in body weight gain measurement (Figure 2h). Taken together, these results suggest that both prophylactic and therapeutic application of Ori are able to relieve the depression-like behaviors induced by CUMS, and between the two protocols, the prophylactic treatment was more effective considering the initial effective dose.

3.2 Comparison of the anti-depressive effects of Ori, FLX, and their combination

Next, we compared the anti-depressive effect of Ori with that of FLX and examined the total effects of these two drugs delivered in the prophylactic treatment. In addition to the usual dose of 10 mg/kg, a dose of 18 mg/kg was adopted because this is the maximum effective value for the treatment of depression in animal experiments (Almeida, Duarte, Oliveira, & Crestani, 2015; David et al., 2009; Wang et al., 2020). There was a significant group effect in the immobility time (F[6,49] = 36.89, p < .001), climbing time (F[6,49] = 26.63, p < .001) and SPT (F[6,49] = 11.03, p < .001). Compared with CUMS + Veh rats, both prophylactic application of FLX at 10 mg/kg and 18 mg/kg significantly decreased the immobility time (respectively, p < .001; p < .001. Figure 3a) and increased climbing time (respectively, p < .05; p < .001. Figure 3b) in the FST, meanwhile they
Prophylactic treatment

(A) Forced swim test

(B) Forced swim test

(C) Consumptions of sucrose water in the SPT.

(D) Body weight gain.

Therapeutic treatment

(E) Forced swim test

(F) Forced swim test

(G) Consumptions of sucrose water in the SPT.

(H) Body weight gain.

The following groups were used: Control, CUMS, CUMS + Vehicle, CUMS + Ori (5 mg/kg), CUMS + Ori (10 mg/kg), and CUMS + Ori (20 mg/kg). The data (mean ± SEM) were analyzed by one-way analysis of variance (ANOVA), n = 8. *p < .05, **p < .001 versus Control group; #p < .05, ##p < .001 versus CUMS + Veh group.
increased sucrose preference in the SPT at fourth weekend (respectively, \( p < .05 \); \( p < .001 \). Figure 3c). The extents of improvement in anti-depressive by FLX at 10 mg/kg were significantly weaker compared with those caused by Ori at 10 mg/kg in all tests (immobility time, \( p < .05 \); climbing time, \( p < .05 \); sucrose preference level, \( p < .05 \) Figure 3a–c). Ori and FLX were administered in combination to examine the total effects of these two drugs. Our results showed that supplementation with Ori at 5 or 10 mg/kg dose-dependently enhanced the anti-depressive effect of FLX (10 mg/kg) alone in all tests, especially 10 mg/kg dose of Ori (immobility time, \( p < .001 \); climbing time, \( p < .001 \); sucrose preference level, \( p < .001 \) Figure 3a–c). Interestingly, further observation revealed the anti-depressive effect of Ori (10 mg/kg) plus FLX (10 mg/kg) was even apparently better than that of FLX at maximum effective dose (18 mg/kg) (immobility time, \( p < .05 \); climbing time, \( p < .05 \); sucrose preference level, \( p < .05 \) Figure 3a–c). However, no significant differences were detected among all groups in body weight gain measurement (Figure 3d). Altogether, these results suggest that the anti-depressive effects of Ori at 10 mg/kg are stronger than those of FLX at 10 mg/kg. In addition, Ori is a potent adjuvant to increase the effects of FLX.

### 3.3 Ori impeded microglial activation in the prefrontal cortex and hippocampus

Microglial activation in the prefrontal cortex and hippocampus is associated with the development of depression (Colasanti et al., 2016; Pan, Chen, Zhang, & Kong, 2014). Therefore, we investigated the change of microglia induced by CUMS and examined the effects of Ori treatment on the prefrontal cortex and hippocampus. According to immunostaining and morphometric analysis, there was no significant difference in Iba-1 (a marker of microglia) expression levels.
between CUMS and CUMS + Veh in prefrontal cortex. In addition, CUMS and CUMS + Veh exhibited significantly increased expression levels of Iba-1 in the prefrontal cortex compared with Control group (respectively, $t = 5.994, p < .001; t = 6.114, p < .001$), suggesting the activation of microglia in the prefrontal cortex of CUMS rats. Ori at 10 mg/kg significantly decreased expression levels of Iba-1 ($t = 3.334, p < .05$). However, FLX at 10 mg/kg could not significantly decrease expression levels of Iba-1 (Figure 4a,b). Similarly, there was no significant change for Iba-1 expression levels between CUMS and CUMS + Veh in hippocampus. Both CUMS and CUMS + Veh exhibited significantly increased expression levels of Iba-1 in the hippocampus compared with Control group (respectively, $t = 15.41, p < .001; t = 17.36, p < .001$), while prophylactic application of FLX at 10 mg/kg and Ori at 10 mg/kg remarkably inhibited the increase of Iba-1 compared with CUMS + Veh group (respectively, $t = 4.901, p < .05; t = 6.206, p < .001$) (Figure 4c,d). The above results show that Ori may impede microglial activation in the prefrontal cortex and hippocampus, and FLX also possesses similar function in the latter.

### 3.4 Ori alleviated NLRP3 inflammasome activation in hippocampus and peripheral tissues

To determine the activation of the NLRP3 inflammasome in the hippocampus, we detected its subsequent productions including IL-1β, IL-18, and caspase-1 (Figure 5a). As was displayed, relative protein levels of IL-1β, IL-18, and caspase-1 p10 were significantly upregulated in both CUMS (respectively, $t = 5.59, p < .001; t = 6.414, p < .001; t = 4.914, p < .001$) and CUMS + Veh (respectively, $t = 7.216, p < .001; t = 6.414, p < .001; t = 6.037, p < .001$) compared with Control group. And no significant differences were detected between CUMS and CUMS + Veh. In addition, both Ori (IL-1β, $t = 5.345$, **p < .001** versus Control group; **p < .001** versus CUMS + Veh group; IL-18, **p < .001** versus Control group; **p < .001** versus CUMS + Veh group; caspase-1 p10, **p < .001** versus Control group; **p < .001** versus CUMS + Veh group).
p < .05; IL-1β, t = 3.889, p < .05; caspase-1 p10, t = 3.53, p < .05) and FLX (IL-1β, t = 4.025, p < .05; caspase-1 p10, t = 3.354, p < .05) hindered those upregulations (Figure 5b–d). Altogether, Ori impeded NLPR3 inflammasome activation, and FLX also has the ability to inhibit the levels of IL-1β and caspase-1 p10, except for IL-1β.

The ELISA results showed that chronic stress enhanced levels of serum IL-1β, IL-18, and corticosterone, which indicated in the CUMS (respectively, t = 8.736, p < .001; t = 6.208, p < .001; t = 12, p < .001) and CUMS + Veh (respectively, t = 12.94, p < .001; t = 5.72, p < .001; t = 10.05, p < .001) compared with Control. However, injection of Ori normalized serum IL-1β (t = 6.462, p < .001), IL-18 (t = 3.646, p < .05) and corticosterone (t = 12.48, p < .001) when comparing with CUMS + Veh. The function of FLX were similar with Ori in regulating serum IL-1β (t = 3.52, p < .05) and corticosterone (t = 9.701, p < .001) (Figure 5e–g). There were no significant differences between CUMS and CUMS + Veh in ELISA results.
A strong association among autophagy, neuroinflammation, and depression has been reported (Oakes, Davies, & Collins, 2017; Su et al., 2016; D. Wang et al., 2017). Thus, we investigated autophagy during CUMS-induced neuroinflammation and depressive-like conditions. Western blot results showed that CUMS treatment altered the expression of autophagy-related signaling molecules including Beclin-1, p62, Atg5, and LC3B, while Ori treatment reversed these changes (Figure 6a). As shown in Figure 6b, relative protein level of Beclin-1 was significantly downregulated in both CUMS \((t = 6.472, p < .05)\) and CUMS + Veh \((t = 6.932, p < .05)\) groups compared with Control group, while Ori treatment \((t = 3.608, p < .05)\) increased expression level of Beclin-1 compared with CUMS + Veh group. Further, both Ori \((t = 6.045, p < .05)\) and FLX \((t = 4.207, p < .05)\) treatments upregulated expression level of P62 compared with CUMS + Veh group, respectively (Figure 6c). As shown in Figure 6d, relative protein level of Atg5 were significantly downregulated in both CUMS \((t = 4.664, p < .05)\) and CUMS + Veh \((t = 5.104, p < .05)\) groups compared with Control group, while both Ori \((t = 6.108, p < .05)\) and FLX treatments \((t = 4.175, p < .05)\) increased expression levels of Atg5 compared with CUMS + Veh group, respectively. In addition, the upregulated expression levels of LC3BII observed in the Ori \((t = 5.127, p < .05)\) and FLX \((t = 4.707, p < .05)\) treatments, respectively (Figure 6e). Finally, no significant differences were detected between CUMS and CUMS + Veh in all autophagy-related signaling molecules.

To explore the molecular mechanisms underlying the effects of Ori, we investigated the interaction between NLRP3 and NEK7 in hippocampus. Firstly, double immunofluorescent staining showed that NEK7 immunoreactivity was mainly double-labeled with Iba-1 (microglia) but not with GFAP (astrocytes) and NeuN (neurons) (Figure 7a). According to immunoprecipitation and subsequent immunoblotting analyses, Ori treatment dose-dependently blocked the interaction between NLRP3 and NEK7 (Figure 7b). Co-immunoprecipitation of NEK7 and NLRP3 was then detected (Figure 7c, 5 mg/kg group, \(t = 21.86, p < .001\); 10 mg/kg group, \(t = 32.2, p < .001\). Figure 7e, 5 mg/kg group, \(t = 22.39, p < .001\); 10 mg/kg group, \(t = 55.6, p < .001\). As shown in Figure 7d and f, furthermore, western blotting analysis showed no alteration in expressions of NEK7 or NLRP3 with Ori treatment, suggesting that Ori could not decrease endogenous levels of NEK7 or NLRP3.
3.7 Ori dose-dependently inhibited inflammatory cytokines in lipopolysaccharide-activated BV2 microglia

The BV2 cells were pretreated with Ori for 12 h followed by incubation with LPS for 24 h (Figure 8a). Before investigating the effects of Ori on inflammatory response of BV2 cells induced by LPS, we first assayed LPS activity and cytotoxicity by treating BV2 cells with Ori at various concentrations, respectively (5, 10, 20, and 40 μmol/L). As shown in Figure 8b, LPS activity decreased in a dose-dependent manner in response to Ori, and the biological activity of LPS was inhibited by about 26% at 40 μmol/L ($t = 8.362, p < .001$). Ori was not cytotoxic at concentrations below 20 μmol/L, but it decreased cell viability to 93.62% and 92.27% at 40 μmol/L in DMSO group and LPS group, respectively ($t = 3.889, p < .05$; $t = 3.015, p < .05$ vs. DMSO group) (Figure 8c). Due to Ori at 40 μmol/L has certain toxicity to cells, the final concentration of ≤ 20 μmol/L was selected for subsequent experiments.

To further investigate whether Ori has any inhibitory effects on the NLRP3 activation in BV2 cells induced by LPS, we assessed the expression levels of IL-1β and IL-18 using ELISA. Our results showed that expression levels of IL-1β and IL-18 were significantly increased following treatment with LPS compared with control (respectively, $t = 11.07, p < .001$; $t = 8.586, p < .001$). In addition, treatment with Ori 10 μmol/L decreased the expression levels of IL-1β and IL-18 compared with DMSO group (respectively, $t = 7.4, p < .05$; $t = 3.932,$
Furthermore, treatment with Ori 20 μmol/L has stronger inhibition of IL-1β and IL-18 expressions compared with DMSO group (respectively, \( t = 11.77, p < .001; t = 5.782, p < .001 \) (Figure 8d,e)). Above results showed that Ori dose-dependently inhibited inflammatory cytokines in BV2 cells induced by LPS.

### 3.8 Ori suppressed LPS-induced autophagy impairment in lipopolysaccharide-activated BV2 microglia

Meanwhile, we also investigated autophagy-related proteins in LPS-activated BV2 microglia. As shown in Figure 9a, LPS treatment altered the expression of autophagy-related proteins including Beclin-1, p62, Atg5, and LC3B, while Ori treatment reversed these changes. According to immunoblotting analyses, expression level of Beclin-1 compared with DMSO group at 5 μmol/L, 10 μmol/L, and 20 μmol/L (respectively, \( t = 5.093, p < .05; t = 3.865, p < .05; t = 7.903, p < .05 \) (Figure 9b)). Further, 5 μmol/L, 10 μmol/L, and 20 μmol/L Ori treatments increased expression levels of P62 compared with DMSO group, respectively \( (t = 5.313, p < .05; t = 4.802, p < .05) \) (Figure 9c). And both 10 μmol/L and 20 μmol/L Ori treatments increased expression levels of Atg5 compared with DMSO group, respectively \( (t = 4.615, p < .05; t = 5.444, p < .05) \) (Figure 9d). As shown in Figure 9e, 5 μmol/L, 10 μmol/L, and 20 μmol/L Ori treatments increased expression levels of LC3BII compared with DMSO group, respectively \( (t = 3.818, p < .05; t = 4.243, p < .05; t = 4.069, p < .05) \).

### 4 DISCUSSION

The link between excessive inflammatory response and autophagy impairment is considered a hallmark of various neurodegenerative...
diseases including Parkinson’s disease (PD) and Alzheimer’s disease (AD) (El Sayed & Ghoneum, 2020; Yao et al., 2019). However, the mechanistic interaction between neuroinflammation, autophagy, and depression is still largely unknown. Moreover, several studies showed that Ori has not only anti-inflammatory effect, but it has the function of regulating autophagy (Xu et al., 2019; Yao et al., 2017). Thus, this study aimed to determine whether autophagy and neuroinflammation mediate the antidepressant effects of Ori.

Although Ori has recently been recommended as a potential drug for the treatment of depression (Liu & Du, 2020), its mechanism of action and dosage have not been thoroughly studied. In the present study, we found that Ori treatment is able to inhibit chronic cytokine-mediated inflammatory responses characterized by activation of NLRP3 inflammasome and consequent productions (IL-1β, IL-18, and caspase-1) in the brain. In addition, it ameliorates microglia activity and most importantly autophagy impairment induced by CUMS, which may underlie its anti-depressive effects. We tend to attribute this effect partially to the function of blocking the interaction between NLRP3 and NEK7 via Ori directly binding with NLRP3. Furthermore, it is also confirmed that Ori is a potent adjuvant to increase the anti-depressive effects of FLX. In this study, both prophylactic and therapeutic treatments with Ori effectively improved depressive-like behaviors induced by CUMS, with the effects of the former being more pronounced than the latter. However, our results showed that Ori could not improve depressive-like behaviors at 20 mg/kg. A previous study demonstrated that Ori did not attenuate the memory impairment in AD mice at 20 mg/kg (Wang et al., 2016), which also confirmed our finding through other models. Therefore, it seems that Ori has the dose ceiling effect in anti-depressive treatment. In the prophylactic paradigm, interestingly, the effects of Ori still on depressive-like behaviors of rats after withdrawal 1 week, although we applied Ori for the first 3 weeks throughout CUMS treatment. This demonstrated that Ori affected depression with a slow onset and offset property. Therefore, Ori may be an ideal candidate for preventing the occurrence of depression when used at the early stages of chronic stress. FLX, as the first specific serotonin reuptake inhibitor, has been an essential medicine for major depression in clinics (Zhou et al., 2020). Furthermore, it is also frequently employed in animal experiments to explore the efficacy of other new drugs as a reference (Ma, Wang, Xu, Wang, & Wang, 2018; Szewczyk et al., 2019). In this study, our results showed that the anti-depressive effect of Ori at 10 mg/kg was slightly stronger than that of FLX at 10 mg/kg in all behavioral tests. In addition, the combined application of Ori with FLX dose-dependently enhanced the anti-depressive of FLX alone, with the most prominent effect of Ori observed at 10 mg/kg, which is even better than maximum effective dose of FLX alone.

The NLRP3 is an intracellular signaling molecule that binds to ASC upon activation, and then interacts with pro-caspase-1 to create a complex referred to as the inflammasome, which leads to the

![Detection of related protein levels of autophagy-related proteins in LPS-activated BV2 cells. (a) Densitometry analyses of the bands. (B-E) Western blot analysis of Beclin-1 (b), p62 (c), Atg5 (d), and LC3B (e) protein expression. The following groups were used: Control, DMSO, CUMS+Ori (5 mg/kg), CUMS+Ori (10 mg/kg), and CUMS+Ori (20 mg/kg). The data (mean ± SEM) were analyzed by Student’s t-test for two-group comparisons, n = 3–4. The experiment was repeated three times. *p < .05, **p < .001 versus Control group; #p < .05 versus DMSO group](image)
activation of IL-1β, IL-18, and caspase-1. In recent years, more and more evidence indicated that activation of NLRP3 inflammasomes is involved in altered prefrontal cortex and hippocampal function and consequent mood disorders of neuropsychiatric states, which can be ameliorated by their pharmacological antagonisms (Liu, Li, Su, Wang, & Jiang, 2019; Pan et al., 2014). NEK7 is an important component of the NLRP3 inflammasome in macrophages. However, it is not known that whether NEK7 is also expressed in brain cells. In this study, our findings showed that it was highly expressed in microglia but not in astrocytes and neuron-like cells. Furthermore, Wu et al. (Wu et al., 2020) indicated that IL-18 expression was mainly found in microglia at a later phase of post-stroke depression. Therefore, we believe that NEK7 binding NLRP3 activated IL-1β, IL-18, and caspase-1 maturation in microglia at a later phase of depression. In this study, prophylactic application of Ori significantly reduced neuroinflammation in the hippocampus, as manifested by suppression of microglial activation, reversal of cytokine levels, and blocking the interaction between NEK7 and NLRP3.

Previous studies on the role of autophagy in depression yielded inconclusive results (Jia & Le, 2015; Song et al., 2017; Tan et al., 2018). However, some recent animal experiments have shown that CUMS reduced the expression of autophagy-related proteins (Shu et al., 2019; Zhao et al., 2017). Therefore, one of our aims was to investigate the mechanistic relationship among neuroinflammation, depression, and autophagy function after Ori treatment. In this study, our results indicated that CUMS decreased the expression of autophagy-related proteins. And CUMS-induced depressive-like behavior and neuroinflammation are associated with autophagy impairment via NLRP3 inflammasome activation. Furthermore, Ori, as well as fluoxetine treatment, significantly reversed the expression of these proteins, suggesting that Ori plays a key role in improving autophagy impairment in microglia cells under stress conditions. The mechanism regulating autophagy might be involved in tissue or cell types.

Currently, there are many inhibitors of NLRP3 inflammasome, but the mechanism of action is different. BAY11–7082, as NF-kB inhibitor, indirectly inhibited NLRP3 inflammasome activation to alleviate neuroinflammation (Jiang, Li, He, Zhou, & Zhu, 2017). In addition, MCC950, another small-molecule inhibitor, has been used to treat NLRP3-associated autoinflammatory and autoimmune diseases (Coll et al., 2015). And several observations demonstrated that MCC950 completely abrogated neuroinflammation (Chivero et al., 2021; Fu et al., 2020; Huang et al., 2021). It directly interacts with the Walker B motif within the NLRP3 NACHT domain, thereby inhibiting NLRP3 activation and inflammasome formation (Coll et al., 2019). According to the latest research, we found that Oridonin forms a covalent bond with the cysteine279 of NLRP3 in NACHT domain to block the interaction between NLRP3 and NEK7, thereby inhibiting NLRP3 inflammasome assembly and activation (He et al., 2018). Thus, MCC950 and Ori may have a similar pharmacological mechanism. Ori is a commonly used traditional Chinese medicine for the treatment of inflammatory diseases and a high-lipophilic small molecule diterpene compound that passes through the blood–brain barrier through passive diffusion. Therefore, we believed that blocking the interaction between NLRP3 and NEK7 by Ori-mediated is common cause for the anti-depressive effect.

In conclusion, our study demonstrated that chronic stress stimuli strongly induce pro-inflammatory cytokines including IL-1β, IL-18, and caspase-1 along with depressive-like behaviors. Further NLRP3 inflammasome activation was associated with autophagy impairment under CUMS-induced stress conditions. Ori acts as an antidepressant by attenuating neuroinflammation and autophagy impairment via blocking the interaction between NLRP3 and NEK7. Our findings suggest that treatment with Ori could be a valuable therapeutic strategy to treat neuroinflammation associated with autophagy impairment and depressive-like behaviors. In addition, it is noteworthy that Ori is a potent adjuvant to increase the anti-depressive effects of FLX. However, there was a limitation in our study. We did not conduct in vivo toxicological study, because it was previously reported that oridonin has effects on liver function.

AUTHOR CONTRIBUTIONS
Liang Liang and Gaohua Wang performed design and wrote the manuscript, Liang Liang, Hui Wang, Ying Hu, Hetao Bian, and Ling Xiao performed research and analyzed data. The authors read and approved the final manuscript.

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CONFLICT OF INTEREST
The authors declare that they have no competing interests.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE
All procedures involving animals were approved and carried out according to the guidelines of the Institutional Animals Care Committee of Renmin Hospital of Wuhan University.

CONSENT FOR PUBLICATION
Not applicable.

DATA AVAILABILITY STATEMENT
The data that support the findings of this study are available from the corresponding author upon reasonable request.

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