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

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Research

Keywords: depressive-like behavior, autophagy, neuroinflammation, Oridonin, NEK7, NLRP3

Posted Date: May 5th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-442417/v1

Abstract

Background: Major depressive disorder (MDD) is a common, chronic, and severe life-threatening disorder with increasing prevalence**.** Although neuroinflammation is associated with the etiology of depression, the mechanistic interplay between depression, neuroinflammation, and autophagy is yet to be demonstrated. This study investigated the effect of Oridonin on CUMS-induced neuroinflammation, depression, and autophagy impairment.

Methods: Male 4-week-old Sprague-Dawley rats were subjected to chronic unpredictable mild stress (CUMS) for 4 and 6 weeks respectively, some of which were injected with Oridonin, fluoxetine (FLX) or their combination at different durations of CUMS. After CUMS procedure, sucrose preference test and Forced swim test were used to evaluate depressive-like behaviors. Concomitant neuroinflammation changes and autophagy impairments were examined in the hippocampus.

Reults: In this study, we found that chronic stress resulted in depressive-like behaviors and caused neuroinflammation and autophagy impairment. In particular, CUMS treatment significantly increased the levels of cytokines (IL-1β, IL-18 and Caspase-1), reduced autophagy-related protein levels (Beclin-1, p62, Atg5 and LC3B), caused microglia cells activation. Oridonin treatment can prevent and reverse the depressive-like behavior induced by CUMS in prophylactic and therapeutic administration, but there is ceiling effect in the treatment process. Furthermore, 10mg/kg dose of Ori has a stronger and longer lasting antidepressant effect than the 10mg/kg dose of fluoxetine. And the antidepressant effect of Ori in combination with fluoxetine was greater than that of high-dose fluoxetine alone. In addition, Ori treatment significantly normalized autophagy-related protein levels, and reduced levels of cytokines via blocking the interaction between NLRP3 and NEK7. Further, we investigated the role of autophagy in LPS-activated BV2 cells and levels of cytokines. Similarly, Oridonin treatment abolished and reversed levels of cytokines and autophagy-related protein levels**.**

Conclusions: All these results supported our hypothesis that Ori possesses potent anti-depressive action, which might be mediated via inhibition of neuroinflammation and autophagy impairment through blocking the interaction between NLRP3 and NEK7.

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[1, 2]. Accumulating evidence has shown that neuroinflammation is associated with the etiology of depression[3, 4]. 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[5, 6]. And, several studies showed that elevation in brain IL-1β level is both necessary and sufficient for producing the high incidence of depression and reducing brain IL-1 levels may have potent antidepressive actions[7, 8]. Microglia in the central nerve system (CNS) act as the

first-line of broader immune response to pathogen-associated molecular patterns. In response to stressful stimuli, a complex network of activation pathways induced the microglia to release immune molecules, including cytokines, chemokines and reactive oxygen species[9], which leading to the development of depressive symptoms[10].

Macroautophagy 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[11]. In the last decade numerous studies have further indicated that autophagy can regulate NLRP3 inflammasome activation through various mechanisms[12]. Furthermore, NLRP3 was also shown to modulate autophagy. In addition, updated studies proved that depressive-like behaviors required a functional NLRP3 inflammasome[13, 14]. The NLRP3 inflammasome is one multiprotein complex that serve as platforms for the activation of caspase-1, leading to the processing and secretion of IL-1β and IL-18[15]. A few studies have also shown that NLRP3-deficient mice have increased autophagy levels at baseline and under stress conditions[16, 17]. Besides NLRP3, caspase-1 was also reported to regulate the autophagic process through cleavage of other substrates[18, 19]. 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[20, 21]. Therefore, scholars across the world postulated that future procedures targeting the NLRP3 inflammasome may have promising effects in the prevention and treatment of depression[22, 23].

Oridonin (Ori), a diterpenoid isolated from Rabdosia rubescens, has multiple biological properties, especially anti-inflammatory and neuroregulatory activities[24, 25]. It can attenuate behavioural deficits in Alzheimer's disease (AD)[26–28], which means that Ori has the potential application against neurodegenerative disorders. However, the targeting mechanism of Ori is still unknown. Recently, one finding suggested that Ori can be used to treat a variety of inflammatory diseases through blocking the interaction between NLRP3 and NEK7[29]. Previous studies demonstrated that NEK7, one of eleven NEK kinases found in vertebrates, was implicated in mitotic spindle formation and separation of centrosomes (with NEK6 and NEK9)[30–34]. Furthermore, a new observation revealed that NEK7, by direct interaction with NLRP3, is also an important component of the NLRP3 inflammasome, contributing to its assembly in macrophages[35]. However, the molecular mechanism of Ori in the treatment of neuroinflammation-induced autophagy impairment and depressive-like behaviors is yet to be elucidated. Therefore, this study aimed to evaluate the antidepressant-like and neuroprotective effects of Ori. Furthermore, we investigated whether Ori can attenuate neuroinflammation and autophagy impairment. For better appraisal, we also compared the anti-depressive effects of Ori with those of fluoxetine (FLX) and tested the total effects of the combined application of these two drugs.

2. Materials And Methods

2.1Animals

Male 4-week-old Sprague-Dawley rats 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.

2.2 Chronic unpredictable mild stress (CUMS) protocols

The procedure used for the chronic unpredicted mild stress (CUMS) was adapted from our past research[36]. 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 24h, 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 with the same breed but with no exposure to the above stressors.

2.3Drug administration and Experiment design

Ori purchased from alfabiotech company (Chengdu, China) was dissolved in dimethylsulfoxide (DMSO, Sigma, St. Louis, MO) and then diluted in saline to a concentration of 20 mg/ml. 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[26, 27].

Two patterns of drug application were employed in this study: prophylactic and therapeutic treatment. According to our preliminary experimental results (Fig. 1A, 1B, 1C and 1D), the rats subjected to mild stressors developed depressive symptoms from the third week. Thus, in the prophylactic treatment, drugs were given after rats 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 three weeks. In addition, FLX at 10mg/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 fourth weekend to sixth weekend during CUMS procedure. The FST, SPT and body weight measurement were performed at the sixth weekend (Fig. 1E).

2.4Behavioral tests

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

Sucrose preference test

The sucrose preference test (SPT) is a widely used method of evaluating depressive-like behavior in animals[37]. 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 2h. 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%.

Forced swim test

The Forced swim test (FST) is one of the most commonly used animal models for assessing antidepressant-like behavior[38]. According to literature, the FST setup consisted of a cylinder (40 cm height ×28cm internal diameter). In the pretest, the rats were individually placed in the cylinder with water at 25°C 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.

2.5Sacrifice 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 one week. Finally, the brains were embedded in paraffin. Serial 5 μm sections were cut in the coronal plane.

2.6Enzyme-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 according to the manufacturer's instructions.

2.7Immunostaining and morphometric analysis

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; MAB3402, 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 were then 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-0295D-Cy3, BIOSS, China) and Goat Anti-Mouse IgG/FITC antibody (1:1000; bs-0296G-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.

2.8Western 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 (0.22μm; 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-1 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-LC3 (14600-1-AP, Proteintech Group, China), Anti-ATG5 (10181-2-AP, Proteintech Group, China), Anti-Beclin-1 (11306-1-AP, Proteintech Group, China), Anti-P62/SQSTM1 (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, Beyotime Biotechnology, China) and exposed to X-ray film (BIORAD). For analysis, the levels of target proteins were normalized against those of GAPDH.

2.9Immunoprecipitation

For detecting the interaction of NEK7 and NLRP3 by co-immunoprecipitation (Co-IP). 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.

2.10Cell culture and treatment

The BV2 microglia 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 were pretreated with Ori (respectively, 5, 10, 20 or 40 umol/L) for 12h followed by incubation with LPS (1ug/ml) for 24h.

2.11Cell Viability Analysis

Cell viability was measured using MTT (C0009, Beyotime Biotechnology, China) assay. The microglia cells were plated in cell culture plates at a density of 1.5 × 103 cells/well and cultured overnight. Various concentrations of Ori (respectively, 5, 10, 20 or 40 umol/L) were added to each well and incubated for 24 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 3h. After the formazan crystals had dissolved, absorbance was read at 570 nm.

2.12Limulus 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). Briefly, a series of concentrations of the Ori (5, 10, 20 and 40 umol/L) were incubated with LPS (1ug/ml) for 30 min at 37°C. The absorbance was measured at 450 nm after the addition of 100 ul of the chromogenic substrate.

2.13Statistical Analysis

All data were analyzed with GraphPad Prism 6 software (San Diego, CA, USA) and are presented as the mean ± SD. Data were mainly analyzed using Student's t test for two-group comparisons. 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

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 three weeks. To determine the potential antidepressive effect of Ori, behavioral analyses were performed. As compared to Control, both CUMS and CUMS + Veh rats showed significantly increased immobility time (respectively, t = 11.71, $P < 0.001$; t = 18.33, $P < 0.001$. Figure 2A) and decreased climbing time in the FST (respectively, t = 9.216, $P < 0.001$; t = 10.44, $P < 0.001$. Figure 2B). In addition, in comparison with control group, both CUMS and CUMS + Veh also showed fewer percentages of sucrose preference (respectively, t = 13.71, $P < 0.001$; t = 10.39, $P < 0.001$. Figure 2C) and lighter body weight (respectively, t = 8.56, $P < 0.001$; t = 7.058, $P < 0.001$. Figure 2D). 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, $P < 0.05$; t = 9.114, $P < 0.001$) (Fig. 2A). And only 10 mg/kg of Ori significantly increased climbing time (t = 3.749, $P < 0.05$. Figure 2B). Similarly, sucrose preference levels were higher in both 5 mg/kg and 10 mg/kg of Ori groups when comparing to CUMS + Veh group at fourth weekend (respectively, t = 2.949, $P < 0.05$; t = 4.051, $P < 0.001$. Figure 2C). Meanwhile, no significant differences were detected between 5 or 10 mg/kg and CUMS + Veh in body weight gain measurement (Fig. 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, 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 (Fig. 2E and 2F)., at which time a significant increase in sucrose preference level compared with CUMS + Veh (t = 2.945, $P < 0.05$. 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, $P < 0.001$; climbing time, t = 4.578, $P < 0.05$; sucrose preference level, t = 5.626, $P < 0.001$. Figure 2E, 2F and 2G). 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 (Fig. 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.

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 10mg/kg, a dose of 18 mg/kg was adopted because this is maximum effective value for the treatment of depression in animal experiments[36, 39, 40]. Compared with CUMS + Veh rats, both prophylactic application of FLX at 10mg/kg and 18mg/kg significantly decreased the immobility time (respectively, q = 7.509, $P < 0.001$; q = 13.7, $P < 0.001$. Figure 3A) and increased climbing time (respectively, q = 5.902, $P < 0.05$; q = 10.68, $P < 0.001$. Figure 3B) in the FST, meanwhile they increased sucrose preference in the SPT at fourth weekend (respectively, q = 4.589, $P < 0.05$; q = 9.006, $P < 0.001$. Figure 3C). The extents of improvement in anti-depressive by FLX at 10mg/kg were significantly weaker compared with those caused by Ori at 10mg/kg in all tests (immobility time, q = 4.617, $P < 0.05$; climbing time, q = 4.876, $P < 0.05$; sucrose preference level, q = 4.589, $P < 0.05$. Figure 3A, 3B and 3C). 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 (10mg/kg) alone in all tests, especially 10mg/kg dose of Ori (immobility time, q = 10.81, $P < 0.001$; climbing time, q = 9.598, $P < 0.001$; sucrose preference level, q = 8.833, $P < 0.001$. Figure 3A, 3B and 3C). Interestingly, further observation revealed the anti-depressive effect of Ori (10mg/kg) plus FLX (10mg/kg) was even apparently better than that of FLX at maximum effective dose (18mg/kg) (immobility time, q = 4.617, $P < 0.05$; climbing time, q = 4.824, $P <$

0.05; sucrose preference level, q = 4.416, $P < 0.05$. Figure 3A, 3B and 3C). However, no significant differences were detected among all groups in body weight gain measurement (Fig. 3D). Altogether, these results suggest that the anti-depressive effects of Ori at 10mg/kg are stronger than those of FLX at 10mg/kg. In addition, Ori is a potent adjuvant to increase the effects of FLX.

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[41, 42]. 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, CUMS and CUMS + Veh groups exhibited significantly increased expression levels of Iba-1(a marker of microglia) in the prefrontal cortex compared with Control group (respectively, t = 5.994, $P < 0.001$; t = 6.114, $P < 0.001$), suggesting the activation of microglia in the prefrontal cortex of CUMS rats. Ori at 10mg/kg significantly decreased expression levels of Iba-1(t = 3.334, $P < 0.05$). However, FLX at 10mg/kg could not significantly decrease expression levels of Iba-1(Fig. 4A and 4B). In addition, CUMS and CUMS + Veh groups also exhibited significantly increased expression levels of Iba-1 in the hippocampus compared with Control group (respectively, t = 15.41, $P < 0.001$; t = 17.36, $P < 0.001$), while prophylactic application of FLX at 10mg/kg and Ori at 10mg/kg remarkably inhibited the increase of Iba-1 compared with CUMS + Veh group (respectively, t = 4.901, $P < 0.05$; t = 6.206, $P < 0.001$) (Fig. 4C and 4D). Above results show that Ori may impede microglial activation in the prefrontal cortex and hippocampus, and FLX also possesses similar function in the latter.

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 (Fig. 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, $P < 0.001$; t = 6.414, $P < 0.001$; t = 4.914, $P < 0.001$) and CUMS + Veh groups (respectively, t = 7.216, $P < 0.001$; t = 6.414, $P < 0.001$; t = 6.037, $P < 0.001$) compared with Control group. In addition, both Ori (IL-1β, t = 5.345, $P < 0.05$; IL-18, t = 3.889, $P < 0.05$; Caspase-1 p10, t = 3.53, $P < 0.05$) and FLX (IL-1β, t = 4.025, $P < 0.05$; Caspase-1 p10, t = 3.354, $P < 0.05$) hindered those upregulations (Fig. 5B, 5C and 5D). Altogether, Ori impeded NLRP3 inflammasome activation, and FLX also has the ability to inhibit the levels of IL-1β and Caspase-1 p10, except for IL-18.

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, $P < 0.001$; t = 6.208, $P < 0.001$; t = 12, $P < 0.001$) and CUMS + Veh (respectively, t = 12.94, $P < 0.001$; t = 5.72, $P < 0.001$; t = 10.05, $P < 0.001$) compared with Control. However, injection of Ori normalized serum IL-1β (t = 6.462, $P < 0.001$), IL-18 (t = 3.646, $P < 0.05$) and corticosterone (t = 12.48, $P < 0.001$) when comparing with CUMS + Veh. The function of FLX were

similar with Ori in regulating serum IL-1β (t = 3.52, $P < 0.05$) and corticosterone (t = 9.701, $P < 0.001$) (Fig. 5E, 5F and 5G).

Ori suppressed CUMS-induced autophagy impairment in the hippocampus

A strong association among autophagy, neuroinflammation and depression has been reported[43–45]. 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 (Fig. 6A). As shown in Fig. 6B, relative protein level of Beclin-1 were significantly downregulated in both CUMS (t = 6.472, $P < 0.05$) and CUMS + Veh (t = 6.932, $P < 0.05$) groups compared with Control group, while Ori treatment (t = 3.608, $P < 0.05$) increased expression level of Beclin-1 compared with CUMS + Veh group. Further, both Ori (t = 6.045, $P < 0.05$) and FLX (t = 4.207, $P < 0.05$) treatments upregulated expression levels of P62 compared with CUMS + Veh group respectively (Fig. 6C). As shown in Fig. 6D, relative protein level of Atg5 were significantly downregulated in both CUMS (t = 4.664, $P < 0.05$) and CUMS + Veh (t = 5.104, $P < 0.05$) groups compared with Control group, while both Ori (t = 6.108, $P < 0.05$) and FLX treatments (t = 4.175, $P < 0.05$) 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, $P < 0.05$) and FLX (t = 4.707, $P < 0.05$) treatments respectively (Fig. 6E).

Ori dose-dependently blocked the interaction between NLRP3 and NEK7

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) (Fig. 7A). According to immunoprecipitation and subsequent immunoblotting analyses, Ori treatment dose-dependently blocked the interaction between NLRP3 and NEK7 (Fig. 7B). Co-immunoprecipitation of NEK7 and NLRP3 was then detected (Fig. 7C, 5 mg/kg group, t = 21.86, $P < 0.001$; 10 mg/kg group, t = 32.2, $P < 0.001$. Figure 7E, 5 mg/kg group, t = 22.39, $P < 0.001$; 10 mg/kg group, t = 55.6, $P < 0.001$). As shown in Fig. 7D and 7F, furthermore, western blotting analysis showed that no alteration in expressions of NEK7 or NLRP3 with Ori treatment, suggesting that Ori could not decrease endogenous levels of NEK7 or NLRP3.

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 (Fig. 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 umol/L). As shown in Fig. 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 umol/L (t = 8.362, $P < 0.001$). Ori was not cytotoxic at concentrations below 20 umol/L, but it decreased cell viability to 93.62% and 92.27% at 40 umol/L in DMSO group and LPS group respectively (t = 3.889, $P < 0.05$; t = 3.015, $P < 0.05$. vs. DMSO group) (Fig. 8C). Due to Ori at 40umol/L has certain toxicity to cells, the final concentration of ≦ 20 umol/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 < 0.001; t = 8.586, P < 0.001). In addition, treatment with Ori 10 umol/L decreased the expression levels of IL-1β and IL-18 compared with DMSO group (respectively, t = 7.4, P < 0.05; t = 3.932, P < 0.05). Furthermore, treatment with Ori 20 umol/L has stronger inhibition of IL-1β and IL-18 expressions compared with DMSO group (respectively, t = 11.77, P < 0.001; t = 5.782, P < 0.001) (Fig. 8D and 8E). Above results showed that Ori dose-dependently inhibited inflammatory cytokines in BV2 cells induced by LPS.

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 Fig. 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 was significantly decreased in DMSO group (t = 9.61, P < 0.001) compared with Control group, while Ori treatment increased expression level of Beclin-1 compared with DMSO group at 5 umol/L, 10 umol/L and 20 umol/L (respectively, t = 5.093, $P < 0.05$; t = 3.865, $P < 0.05$; t = 7.903, $P < 0.05$) (Fig. 9B). Further, 5 umol/L, 10 umol/L and 20 umol/L Ori treatments increased expression levels of P62 compared with DMSO group respectively (t = 3.973, $P < 0.05$; t = 5.313, $P < 0.05$; t = 4.802, $P < 0.05$) (Fig. 9C). And both 10 umol/L and 20 umol/L Ori treatments increased expression levels of Atg5 compared with DMSO group respectively (t = 4.615, $P < 0.05$; t = 5.444, $P < 0.05$) (Fig. 9D). As shown in Fig. 9E, 5 umol/L, 10 umol/L and 20 umol/L Ori treatments increased expression levels of LC3BII compared with DMSO group respectively (t = 3.818, P < 0.05; t = 4.243, P < 0.05; t = 4.069, P < 0.05).

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) [46, 47]. 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[48, 49]. Thus, this study aimed to determine whether autophagy and neuroinflammation mediate the antidepressant effects of Ori.

In the present study, CUMS induced neuroinflammation and depressive-like behaviors, accompanied by autophagy impairment. And 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 neuroinflammation 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. Importantly, our results demonstrated that Ori modulated not only neuroinflammation associated with microglial activation but also autophagy impairment induced by chronic inflammatory response. Furthermore, it is also confirmed that Ori is a potent adjuvant to increase the anti-depressive effects of FLX. Our results underpinning these points are as follows: (1) In prophylactic and therapeutic administration, Ori can prevent and reverse the depressive-like behavior induced by CUMS respectively, but there is ceiling effect in the treatment process; (2) The 10mg/kg dose of Ori has a stronger and longer lasting antidepressant effect than the 10mg/kg dose of fluoxetine; (3) The antidepressant effect of Ori in combination with fluoxetine was greater than that of high-dose fluoxetine alone; (4) The activity of microglia cells was inhibited in a dose-dependent manner by Ori in vitro and in vivo; (5) By blocking the interaction between NLRP3 and NEK7, the expression of IL-1β, IL-18 and Caspase-1 in the hippocampus was reduced; (6) Meanwhile, Ori increased the downregulated expression of nearly all autophagy-related proteins (Beclin-1, p62, Atg5 and LC3B) induced by CUMS.

To the best of our knowledge, this is the first report to demonstrate an anti-depressive function of Ori, although this drug has recently been recommended as a potential drug for the treatment of tumors[50–52] and neurodegenerative diseases such as AD and PD[26, 27, 53]. In this study, both prophylactic and therapeutic treatments with Ori (5 mg/kg and 10mg/kg) 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. Previous study demonstrated that Ori did not attenuate the memory impairment in AD mice at 20 mg/kg[26], 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 one week, although we applied Ori for the first three 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[54]. Furthermore, it is also frequently employed in animal experiments to explore the efficacy of other new drugs as a reference[55, 56]. In this study, we chose a relatively common dose (10 mg/kg) and maximum effective dose (18 mg/kg) of FLX to assess the potency of Ori in rats subjected to CUMS. 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 at 5mg/kg or 10mg/kg with FLX dose-dependently enhanced the anti-depressive of FLX alone, with the most prominent effect of Ori observed at 10mg/kg, which even better than maximum effective dose of FLX alone.

The NLRP3 is an intracellular signalling 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 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 neuropathic states, which can be ameliorated by their pharmacological antagonisms[42, 57]. In the present study, prophylactic application of Ori significantly reduced neuroinflammation in the prefrontal cortex and hippocampus, as manifested by suppression of microglial activation, reversal of cytokine levels and blocking the interaction between NEK7 and NLRP3. NEK7 is an important component of the NLRP3 inflammasome in macrophages. However, it is not known that whether NEK7 also expressed in brain cells. In this study, our finding showed that it was highly expressed in microglia but not in astrocytes and neuron-like cells. Shi et al.[58] demonstrated that NEK7 serves non-redundant functions in NLRP3 inflammasome activation and mitosis that can not occur simultaneously. Furthermore, Wu et al.[6] 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.

Although the association between neuroinflammation and depression has been demonstrated, the detailed mechanisms are unknown. Previous studies on the role of autophagy in depression yielded inconclusive results[21, 59, 60]. However, some recent animal experiments have shown that CUMS reduced the expression of autophagy-related proteins[61, 62]. Therefore, one of our aims was to investigate the mechanistic relationship between neuroinflammation, depression, and autophagy function after Ori treatment. In this study, our results also indicated that CUMS decreased the expression of autophagy-related proteins. However, Ori, as well as fluoxetine treatment, significantly reversed the expression of these proteins, suggesting that Ori plays a key role in improving autophagy under stress conditions. The mechanism regulating autophagy might be involved in tissue or cell types. Furthermore, there are also studies of NLRP3-dependent regulation of autophagy, such as in experimental models of Parkinson's disease where NLRP3 inflammasome activation induces impaired autophagy[63]. In this study, our results demonstrated that CUMS-induced depressive-like behavior and neuroinflammation are associated with autophagy impairment via NLRP3 inflammasome activation. In addition, our results showed that Ori significantly abolished the effects of CUMS on autophagy impairment and NLRP3 inflammasome activation in microglia cells.

Currently, there are many inhibitors of NLRP3 inflammasome, but the mechanism of action is different. BAY11–7082, as NF-κB inhibitor, indirectly inhibited NLRP3 infammasome activation to alleviate neuroinfammation[64]. In addition, MCC950, another small-molecule inhibitor, has been used to treat NLRP3-associated autoinfammatory and autoimmune diseases[65]. It directly interacts with the Walker B motif within the NLRP3 NACHT domain, thereby inhibiting NLRP3 activation and inflammasome formation[66]. 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[29]. Thus, MCC950 and Ori may have similar pharmacological mechanism. Several observations demonstrated that MCC950 completely abrogated neuroinflammation[67–69]. Ori is a commonly used traditional Chinese medicine for treatment of inflammatory diseases and a high-lipophilic small molecule diterpene compound that passes through the

blood-brain barrier through passive diffusion. Therefore, as shown in Fig. 10, we believed that the anti-depressive effect of Ori might be due to inhibition of neuroinflammation and autophagy impairment. Our finding indicated that blocking the interaction between NLRP3 and NEK7 by Ori-mediated is common cause for the anti-depressive effect.

Conclusion

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 were associated with autophagy impairment under CUMS-induced stress conditions. Ori acts as an antidepressant by attenuating neuroinflammation. Furthermore, importantly Ori abolished CUMS effects on 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, but it may has the dose ceiling effect in anti-depressive treatment.

Abbreviations

Ori: Oridonin; FST: Forced swim test; SPT: Sucrose preference test; IL-1β: interleukin 1β; IL-18: interleukin 18; NLRP3: Nod-like receptor pyrin containing 3 inflammasome; FLX: fluoxetine; CUMS: Chronic unpredictable mild stress; LPS: Lipopolysaccharide

Declarations

Acknowledgements

Not applicable.

Authors' contributions

Liang Liang and Gaohua Wang performed design and wrote the manuscript, Hui Wang, Hetao Bian and Ling Xiao managed the literature searches and figure drawing. The authors read and approved the final manuscript.

Funding

This study was supported by the National Natural Science Foundation of China (No.81571325 and NO.81401117)

Availability of data and materials

The datasets and materials supporting the conclusions of this article are included within the article.

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

Competing interests

The authors declare that they have no competing interests.

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Figures

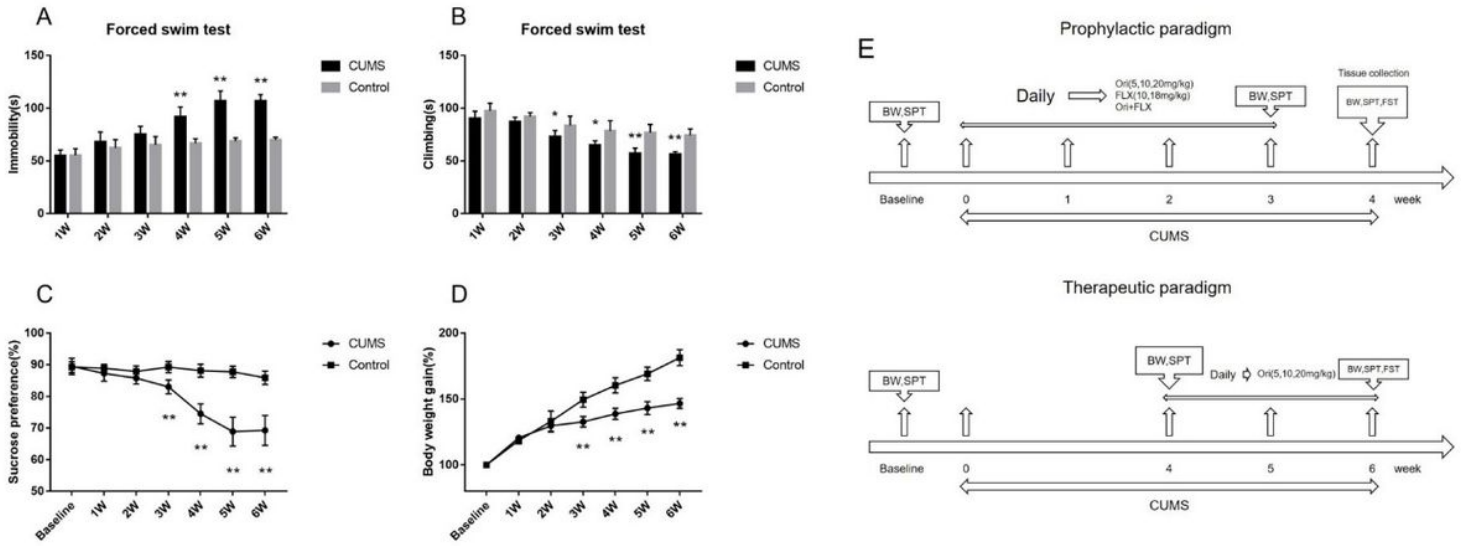

Figure 1

CUMS induced depression-like behaviors and experimental programs. (A) Immobility time in the FST. (B) Climbing time in the FST. (C) Consumption of sucrose water in the SPT. (D) Body weight gain. (E) Experimental programs. The following groups were used: CUMS and Control. The data (mean ± SEM)

were analyzed by Student's t test for two-group comparisons, n = 8. * P<0.05, ** P<0.001 versus Control group.

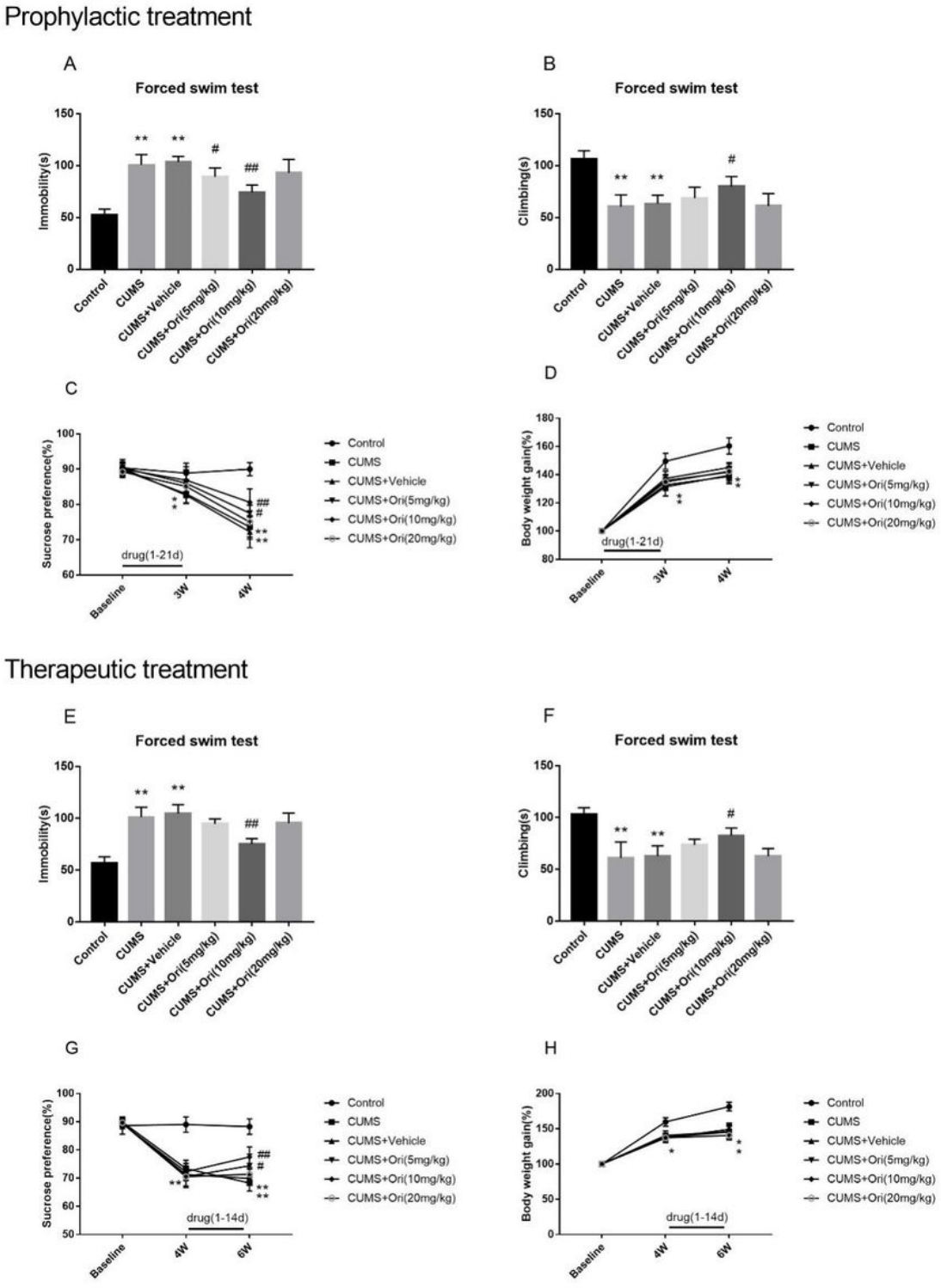

Figure 2

Prophylactic treatment with Ori at various doses. (A) Immobility time in the FST. (B) Climbing time in the FST. (C) Consumption of sucrose water in the SPT. (D) Body weight gain. Therapeutic treatment with Ori at various doses. (E) Immobility time in the FST. (F) Climbing time in the FST. (G) Consumption of

sucrose water in the SPT. (H) Body weight gain. The following groups were used: Control, CUMS, CUMS+Vehicle, CUMS+Ori (5mg/kg), CUMS+Ori (10mg/kg) and CUMS+Ori (20mg/kg). The data (mean ± SEM) were analyzed by Student's t test for two-group comparisons, n = 8. * P<0.05, ** P<0.001 versus Control group; # P<0.05, ## P<0.001 versus CUMS + Veh group.

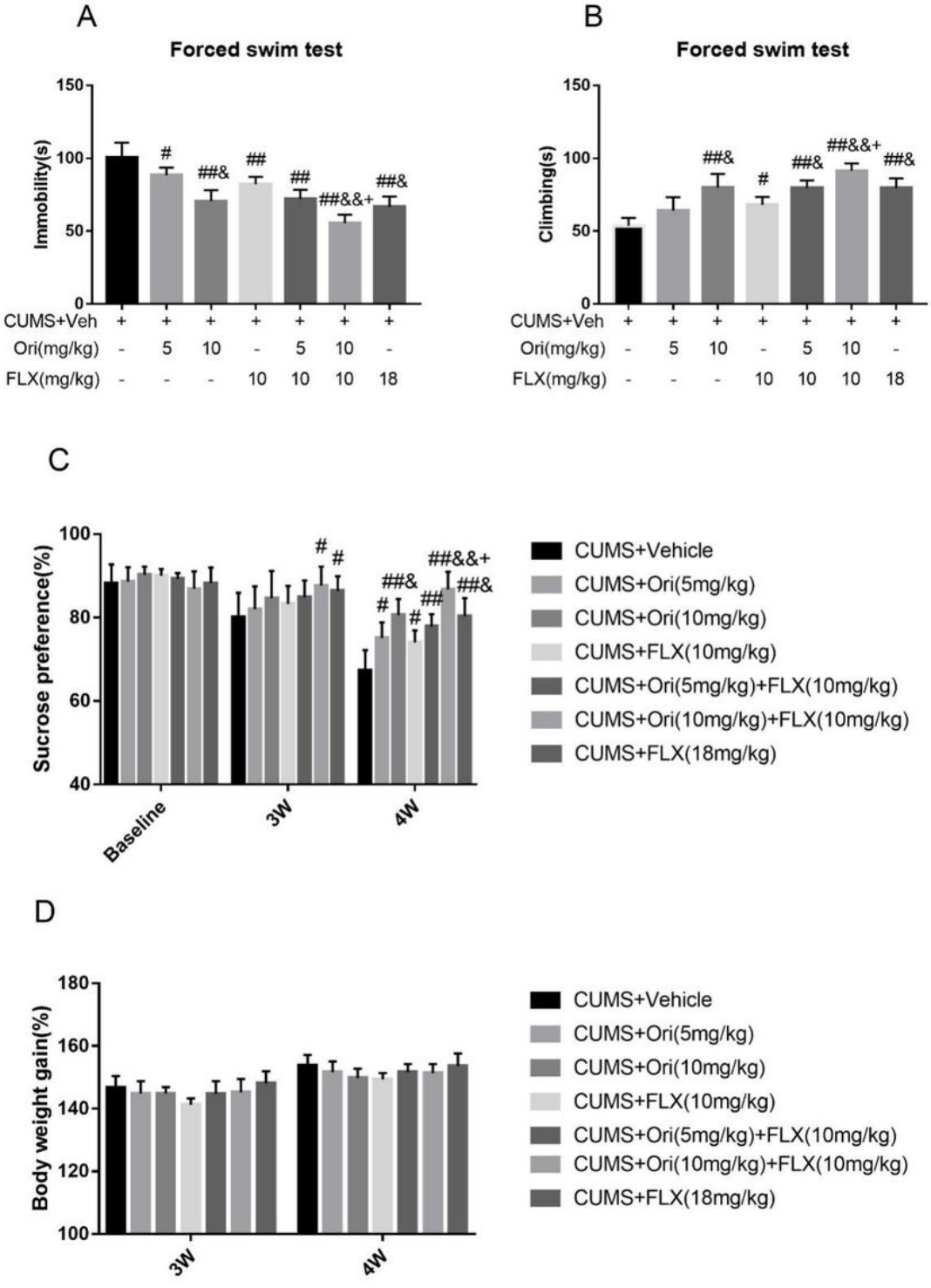

Figure 3

Comparison of the anti-depressive actions between Ori and FLX and the total effects of these two drugs. (A) Immobility time in the FST. (B) Climbing time in the FST. (C) Consumption of sucrose water in the SPT. (D) Body weight gain. The following groups were used: CUMS+Vehicle, CUMS+Ori (5mg/kg), CUMS+Ori (10mg/kg), CUMS+FLX (10mg/kg), CUMS+Ori (10mg/kg)+FLX (10mg/kg), CUMS+Ori (5mg/kg)+ FLX (10mg/kg) and CUMS+FLX (18mg/kg). The data (mean ± SEM) were analyzed by one-way analysis of variance (ANOVA), n = 8. # P<0.05, ## P<0.001 versus CUMS + Veh group; & P<0.05, && P<0.001 versus FLX 10 mg/kg group; + P<0.05 versus FLX 18 mg/kg.

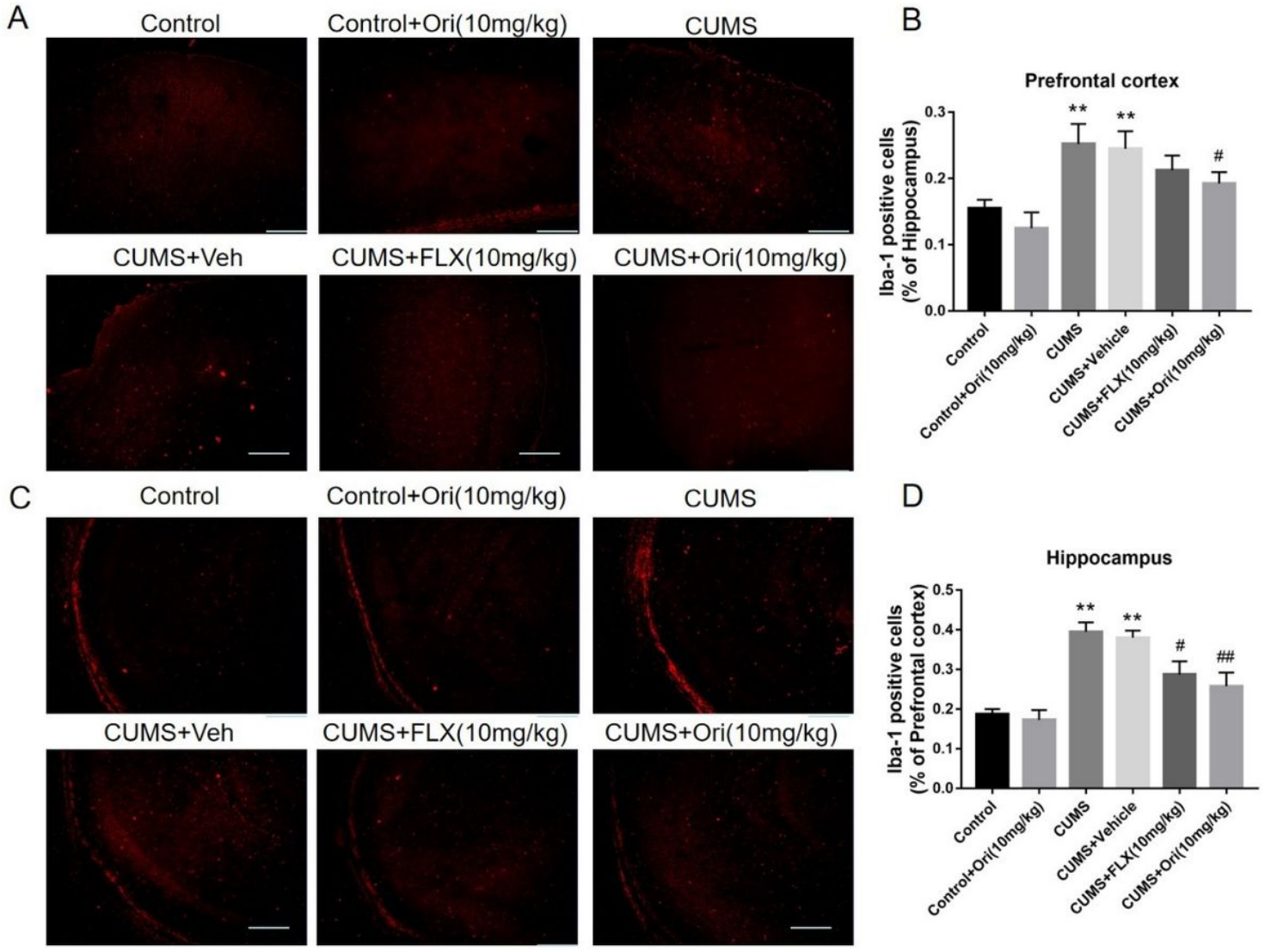

Figure 4

The immunostaining and morphometric analysis of Iba-1 in the prefrontal cortex and hippocampus. (A) Immunostaining of Iba-1 in prefrontal cortex. (B) Expression level of Iba-1 in prefrontal cortex. (C) Immunostaining of Iba-1 in hippocampus. (D) Expression level of Iba-1 in hippocampus. The following groups were used: Control, Control+Ori (10mg/kg), CUMS, CUMS+FLX (10mg/kg) and CUMS+Ori (10mg/kg). The data (mean ± SEM) were analyzed by Student's t test for two-group comparisons, n = 3-4. ** P<0.001 versus Control group; # P<0.05, ## P<0.001 versus CUMS + Veh group.

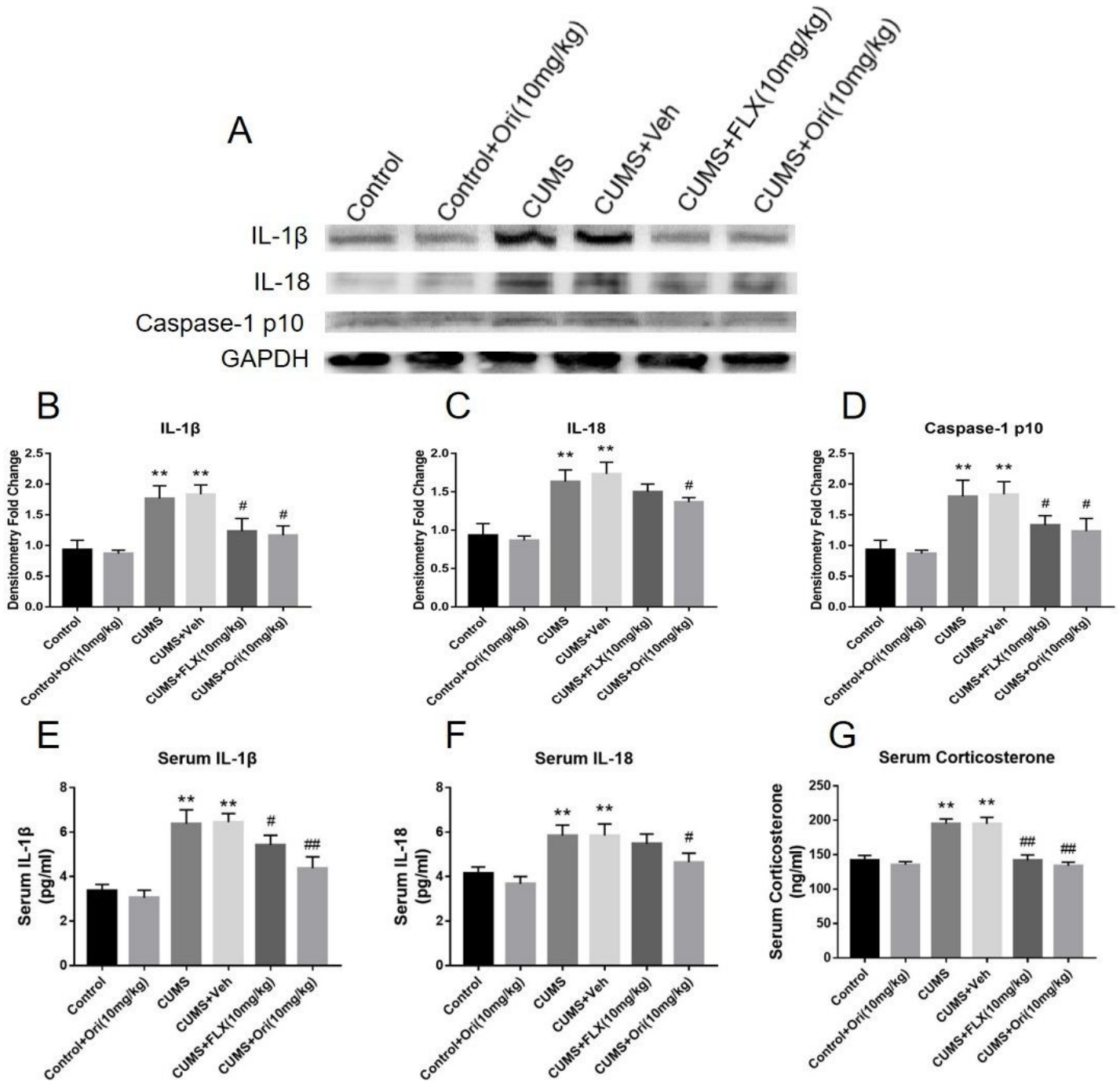

Figure 5

Detection of cytokine productions in hippocampus and serum. (A) Densitometry analyses of the bands. (B-D) Western blot analysis of IL-1β (B), IL-18 (C) and Caspase-1 (D) protein expression. (E-G) ELISA analysis of serum IL-1β (E), IL-18 (F) and Corticosterone (G) expression. The following groups were used: Control, Control+Ori (10mg/kg), CUMS, CUMS+FLX (10mg/kg) and CUMS+Ori (10mg/kg). The data (mean ± SEM) were analyzed by Student's t test for two-group comparisons, n = 3-4. ** P<0.001 versus Control group; # P<0.05, ## P<0.001 versus CUMS + Veh group.

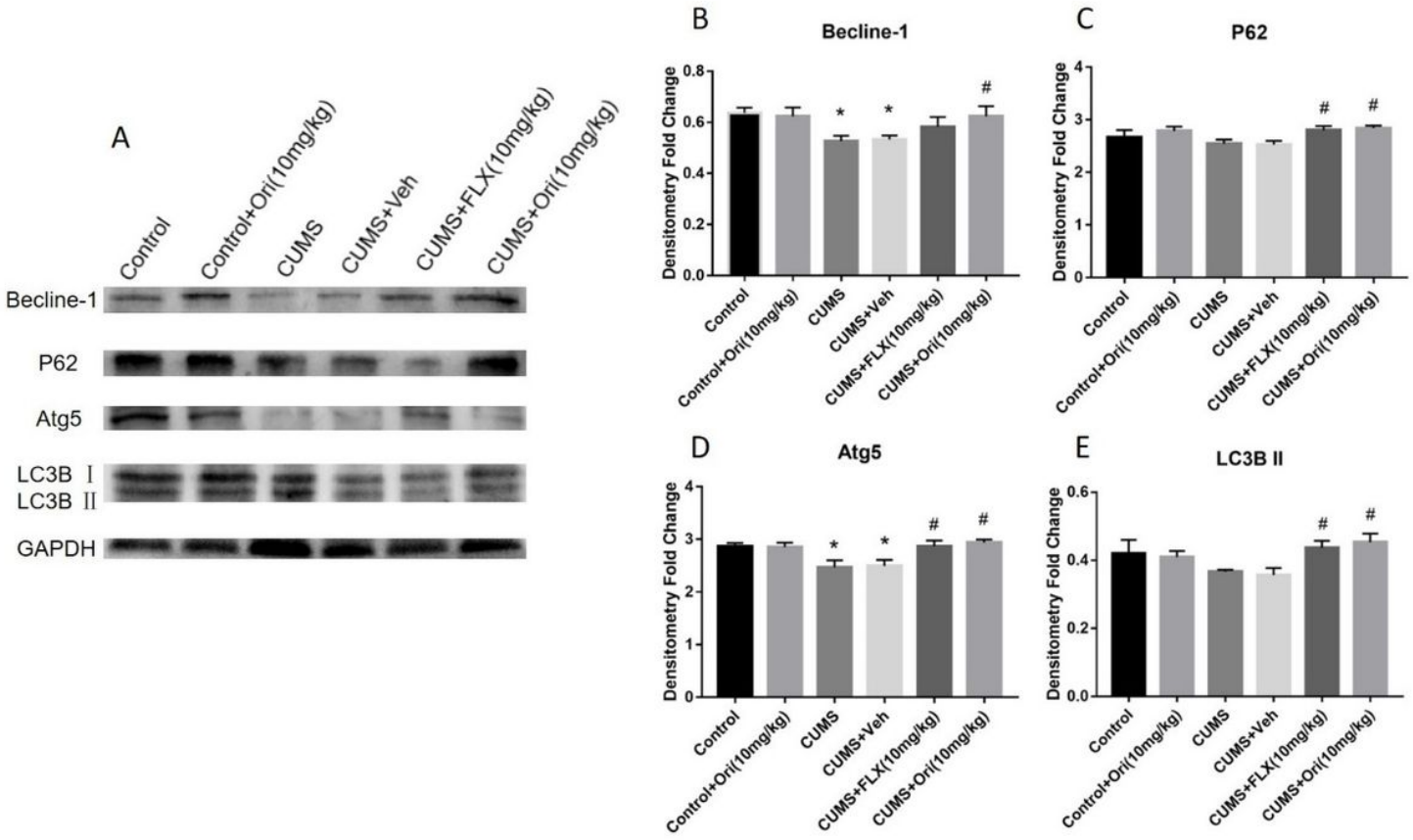

Figure 6

Detection of related protein levels of autophagy-related proteins in hippocampus. (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, Control+Ori (10mg/kg), CUMS, CUMS+FLX (10mg/kg) and CUMS+Ori (10mg/kg). The data (mean ± SEM) were analyzed by Student's t test for two-group comparisons, n = 3-4. * P<0.05 versus Control group; # P<0.05 versus CUMS + Veh group.

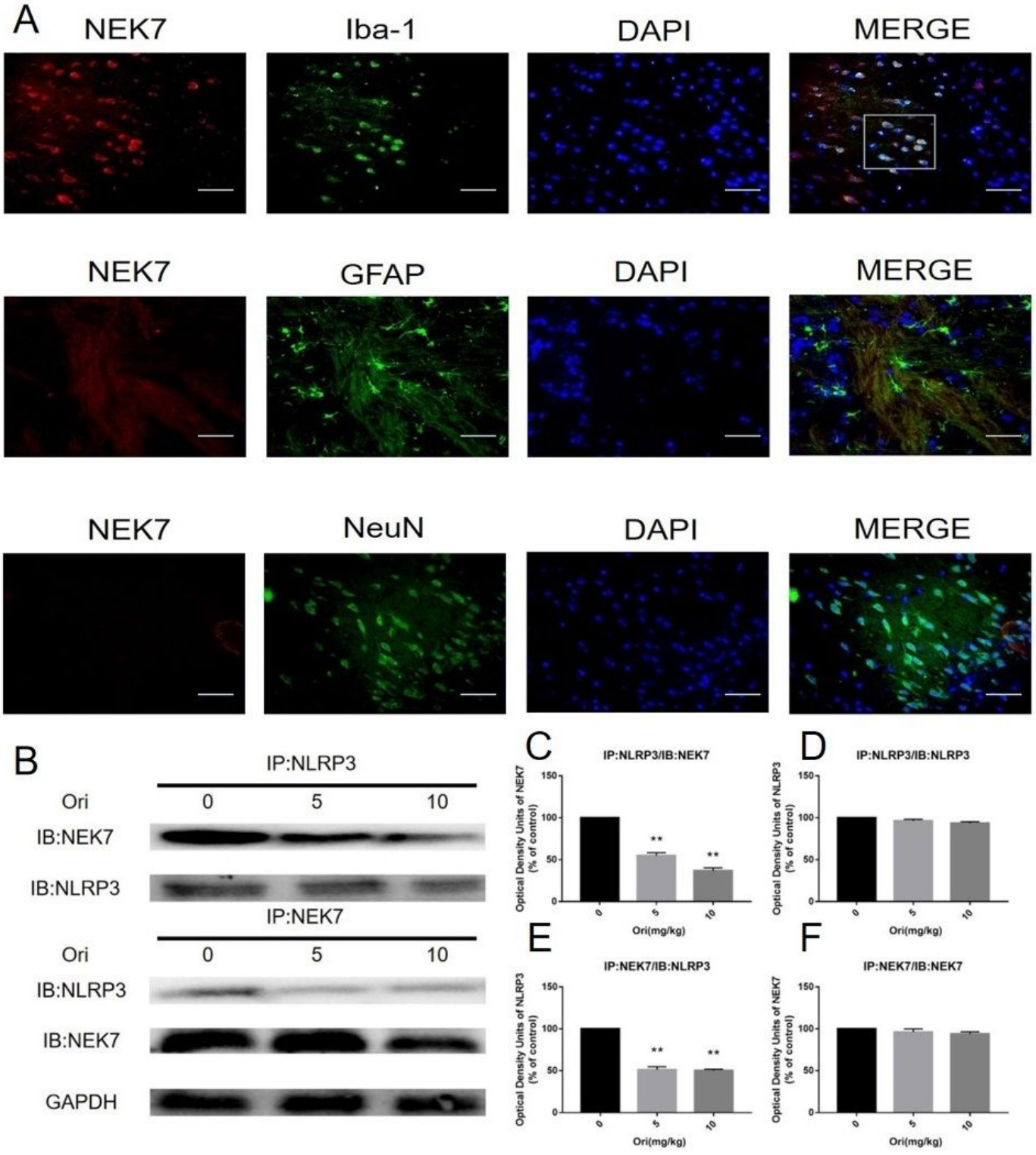

Figure 7

Detection of the interaction between NLRP3 and NEK7 in hippocampus. (A) Immunofluorescent double staining. (B) Co-immunoprecipitation of NEK7 and NLRP3. (C-F) Western blot analysis of NEK7 and NLRP3. The data (mean ± SEM) were analyzed by Student's t test for two-group comparisons, n = 3-4. ** P<0.001 versus Control group.

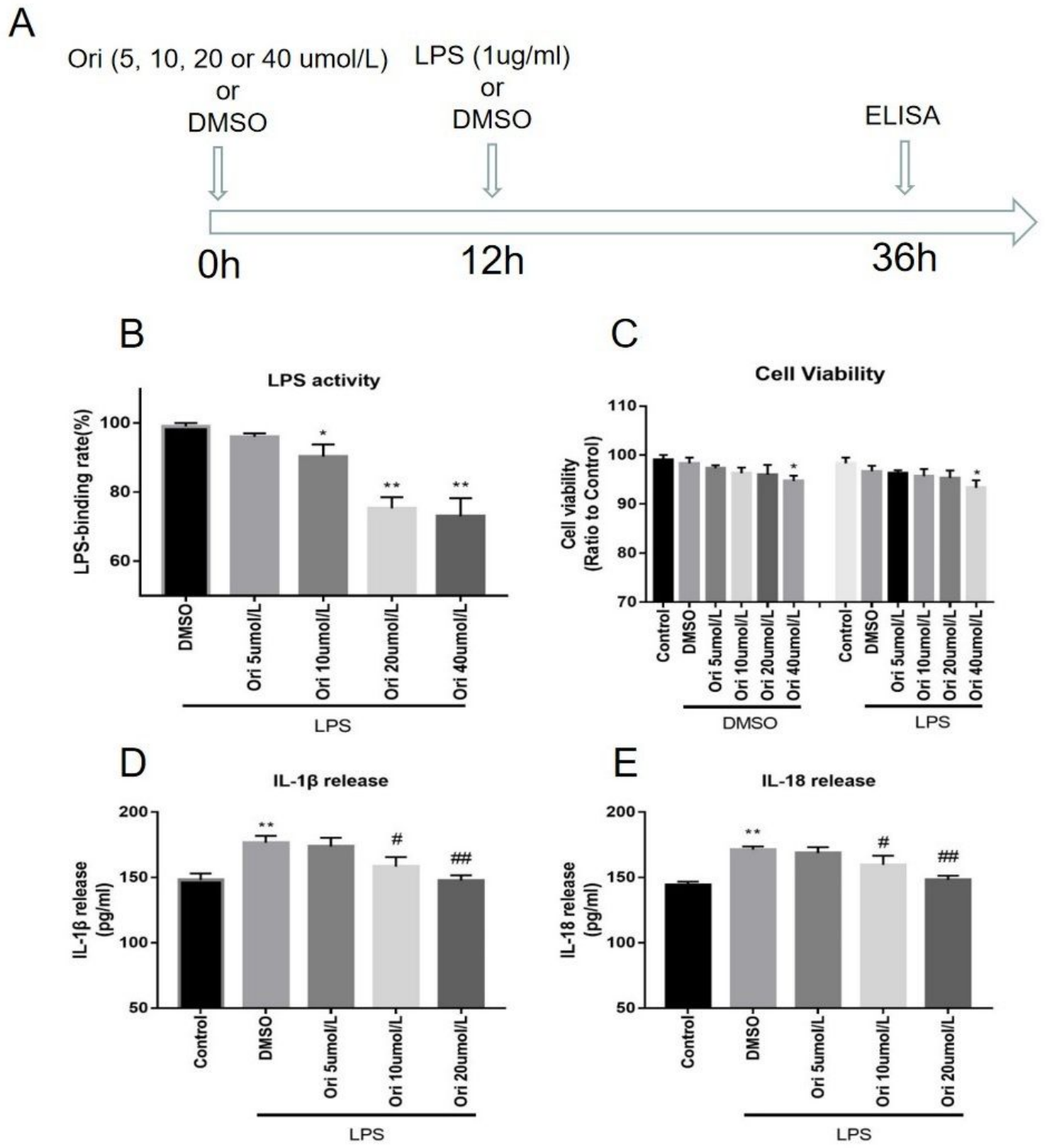

Figure 8

Detection of cytokine productions in LPS-activated BV2 cells. (A) Experimental paradigms illustrating the drug application. (B) LPS activity. (C) Cell Viability. * P<0.05, ** P<0.001 versus DMSO group. (D and E) ELISA analysis of IL-1β (D) and IL-18 (E) expression. The following groups were used: Control, DMSO, CUMS+Ori (5mg/kg), CUMS+Ori (10mg/kg) and CUMS+Ori (20mg/kg). The data (mean ± SEM) were

analyzed by Student's t test for two-group comparisons, n = 3-4. ** P<0.001 versus Control group; # P<0.05 versus DMSO group.

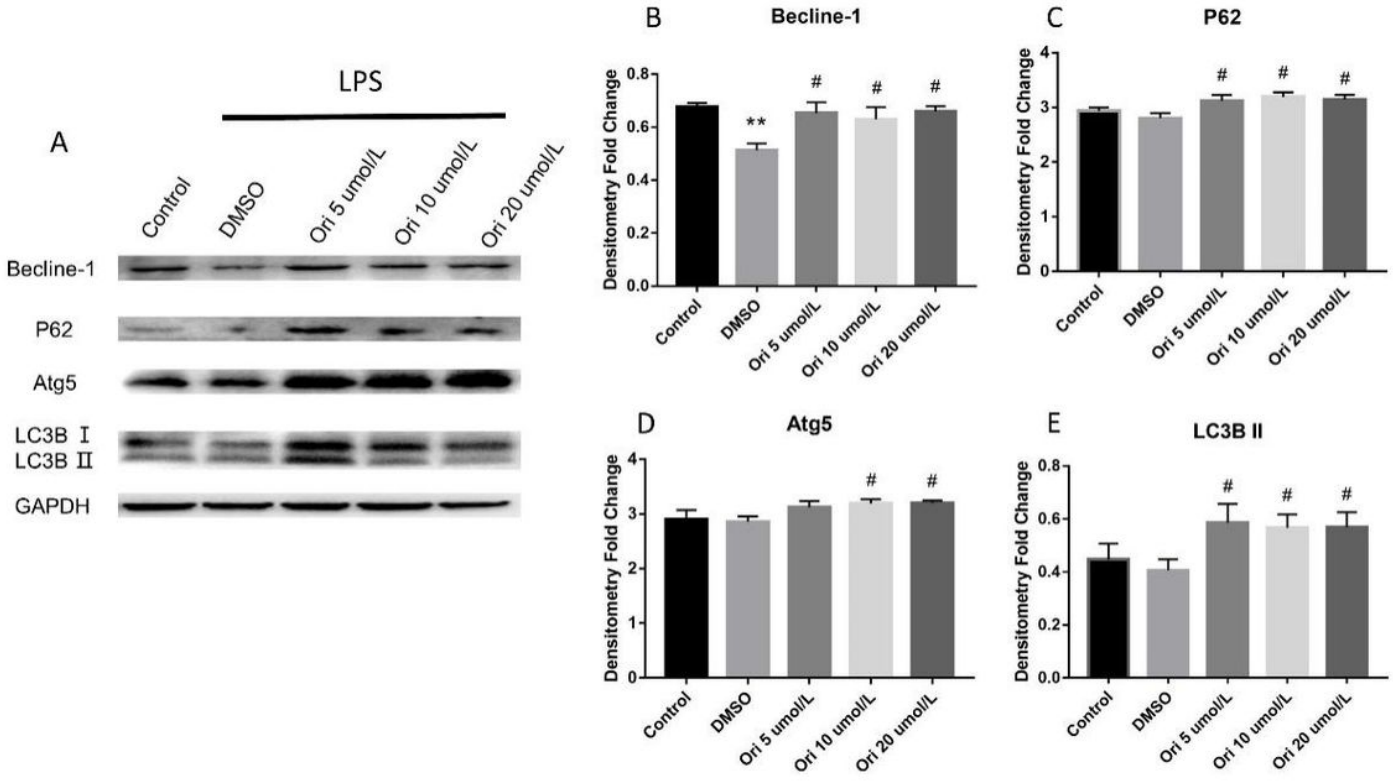

Figure 9

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 (5mg/kg), CUMS+Ori (10mg/kg) and CUMS+Ori (20mg/kg). The data (mean ± SEM) were analyzed by Student's t test for two-group comparisons, n = 3-4. * P<0.05, ** P<0.001 versus Control group; # P<0.05 versus DMSO group.

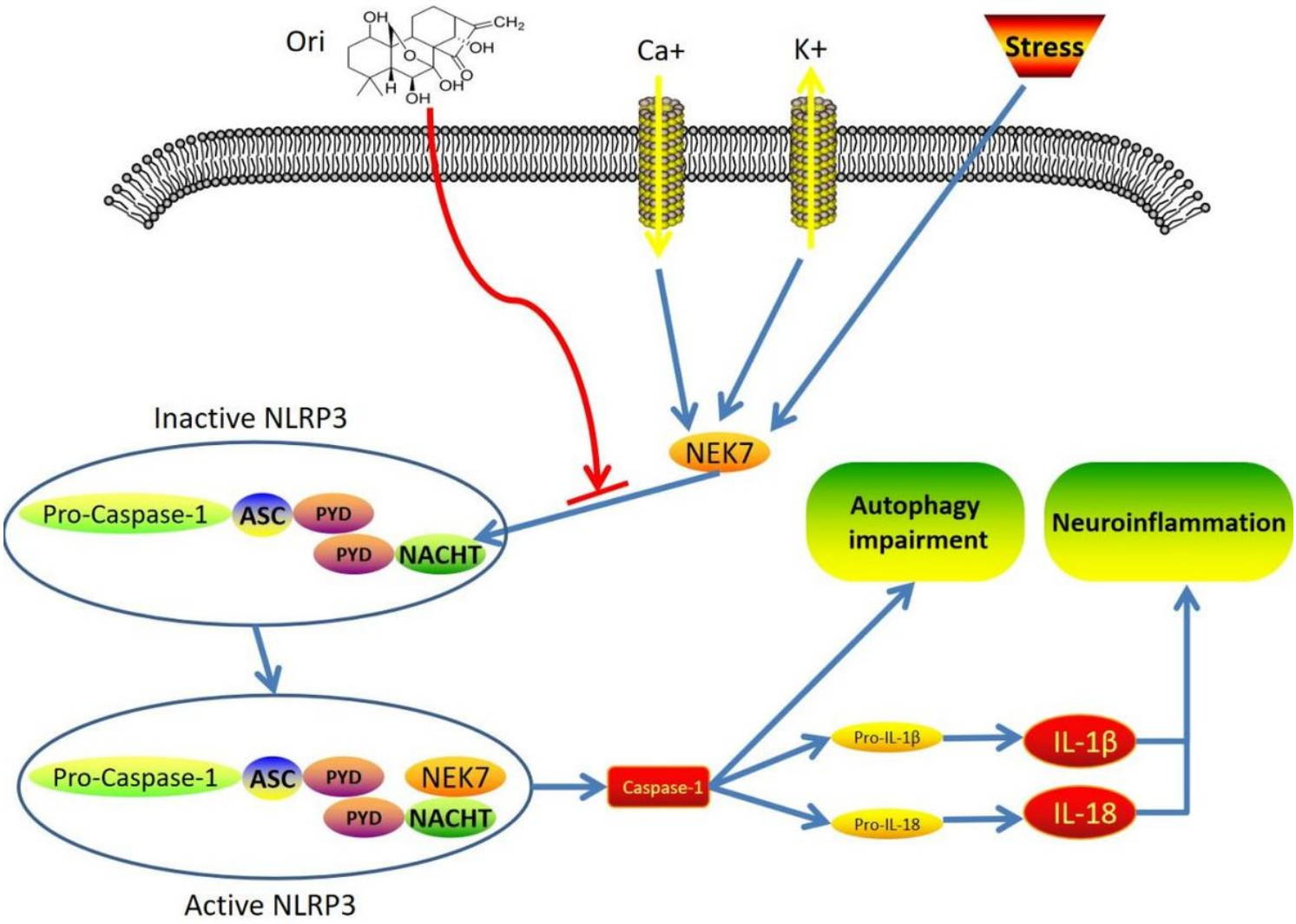

Figure 10

A schematic illustration of the proposed mechanism for Ori to restrain neuroinflammation and autophagy impairment.