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The neuroprotection of progesterone against Aβ-induced NLRP3-Caspase-1 inflammasome activation via enhancing autophagy in astrocytes

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ABSTRACT

Neuroinflammation and autophagy dysfunction are known to be involved in the pathological procession of Alzheimer's disease (AD). Progesterone (PG), neuroactive steroids, exerts a characteristic neuroprotective function in improving AD syndrome. The NOD-like receptor pyrin 3 (NLRP3)-Caspase-1 inflammasome has specific relevance to AD pathological procession. However, the exact role of PG in regulating NLRP3-Caspase-1 inflammasome remains to be elucidated. We demonstrated Aβ up-regulated IL-1β expression in astrocytes by activating NLRP3-Caspase-1 inflammasome. However, pharmacological activation of autophagy by Rapamycin (RAPA) efficiently suppressed Aβ-, lipopolysaccharides (LPS)-induced IL-1β expression via regulating NLRP3-Caspase-1 inflammasome in astrocytes. Remarkably, PG significantly inhibited Aβ-induced NLRP3-Caspase-1 inflammasome activation. Autophagy inhibitor 3-MA blocked the protective effects of PG in mediating NLRP3 inflammasome and IL-1β processing. Taken together, our observations suggest that autophagy-lysosome pathway is one specific molecular mechanism in regulating Aβ-induced NLRP3-Caspase-1 inflammasome activation in astrocytes, particularly uncover the potential neuroprotection of PG in regulating upstream signaling leading to the sequence events of neuroinflammation. That neuroprotective mechanism of PG in regulating NLRP3-Caspase-1 inflammasome can be a potential therapeutic target for ameliorating the pathological procession of AD.

1. Introduction

Alzheimer's disease (AD), the most common cognitive dysfunction illness, is characterized by β-amyloid deposition, neurofibrillary tangles, excessive neuroinflammation and neuronal loss [1]. Neuroinflammation is recognized as a chronic factor in neurodegenerative disease, especially the pathological procession of AD [2]. Studies show that comprehensive inhibition of neuroinflammation can reduce the formation of brain plaques and ameliorate the progression of AD, suggesting that pharmacological interventions in the inflammatory signaling pathway can be expected to improve the progression of AD [3,4].

Astrocytes widely reside in the brain, participate in the formation of synapses and other aspects of function in central nervous system [5]. However, a large number of reactive astrocytes are found in AD brain, especially surrounding senile plaques [6]. In this state, reactive astrocytes can synthesize and secret high levels of pro-inflammatory cytokines, such as tumor necrosis factor (TNF)-α, interferon (IFN)-γ, interleukin (IL)-1β. Although the neuroinflammatory response plays a vital role in the development of AD, the mechanism of the regulation of the inflammatory response of astrocytes has not been fully elucidated. The neuron-centric vision of neurodegenerative disorders has undergone considerable changes. Can astrocytes become the target for new AD drugs?

Interleukin (IL)-1β, a member of the IL-1 cytokine family, is produced as the inactive precursor pro-IL-1β in the cytoplasm in response to a wide variety of stimuli [7,8]. Studies confirm that blocking IL-1β signaling pathway can significantly ameliorate the status of cognitive dysfunction [9] and effectively regulate the pathological symptoms of AD [10]. In order to exert IL-1β functions, pro-IL-1β must be processed into its mature active form by the Caspase-1, which itself is activated by cytosolic multiprotein complexes named inflammasome [11]. NLRP3, a member of NLRs family inflammasomes, is a complex composed of multiple proteins, including the core protein NLRP3, regulatory molecule Caspase-1 and ASC. It is often seen as an upstream protein that

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activates the pro-inflammatory cytokines IL-1β and IL-18 [11,12]. Although the molecular mechanism of NLRP3 inflammasome has not been fully elucidated, it can be confirmed that intracellular NLRP3 synthesis is activated under stress state, such as lysosomal injury [13], mitochondrial ROS oxidative stress [14,15].

Progesterone (PG), endogenous neurosteroid compounds, exerts neuroprotective neurorestorative characteristics in several models of neurodegenerative disorders [16–18]. Our previous results have found neurosteriod PG represented a characteristic neuroprotective function against AD cognitive abilities dysfunction [19,20]. In order to elucidate the molecular mechanism underlying PG neuroprotection, we treated astrocytes with PG and discussed the potential modulation effect in neuroinflammation. Early findings demonstrated PG significantly suppressed Aβ-induced neuroinflammatory responses, and regulated astrocytic function by inhibiting endoplasmic reticulum stress [21] and activating autophagy [22]. However, the upstream regulatory mechanism of neuroinflammation in response to PG treatment is still unclear, and the molecular connection between autophagy dysfunction and NLRP3-Caspase-1 inflammasome activation also needs further investigation. In the present study, we determined whether PG represented a resulting regulation to NLRP3-Caspase-1 inflammasome, then investigated the potential upstream signaling leading to the sequence events of Aβ-induced neuroinflammation in astrocytes.

2. Experimental procedures

2.1. Reagents and chemicals

$A\beta_{1-42}$ fragment, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Lipopolysaccharide (LPS), 3-methyladenine (3-MA) were purchased from Sigma Inc. (St. Louis, MO, USA). Z-VAD-FMK was purchased from PromoKine Inc. (Huissen, Netherlands). Rapamycin (RAPA), Antibodies to IL-1β, LC3, P62, NLRP3, Caspase-1 and β-actin were purchased from Cell Signaling Technology Inc. (Beverly, MA, USA). Goat anti-rabbit lgG/FITC and Rabbit anti-rat lgG/Red were purchased from Bioss Inc. (Beijing, China).

2.2. Preparation of $A\beta_{1-42}$ oligomers

$A\beta_{1-42}$ oligomers were prepared as previously described [22]. $A\beta_{1-42}$ monomer was dissolved in sterile H_2O (2 mM), and evaporated with N_2 gas at 37 °C for 30 min. $A\beta_{1-42}$ monomer dissolved in DMEM, and further incubated at 37 °C for another 24 h to prepare oligomers. $A\beta_{1-42}$ were used its oligomer form for experiments.

2.3. Primary astrocyte culture and treatment

We have established an experimental method for primary astrocyte culture. Primary cortical astrocytes were isolated and cultured as previously described [22]. New born mice were sacrificed, and the cortico-hippocampal regions were dissected in DMEM. The cells were seeded in culture flasks and incubated in humidified 5% CO_2 at 37 °C. The medium was changed every 5 days. At day 15, non-astroglial cells were removed by shaking overnight at 200 rpm/37 °C. The remaining adherent cells were evaluated by immunofluorescence assay using antibody against GFAP. More than 90% of the incubated cells were astrocytes.

Astrocytes cultures were used at 4 day incubation. The cells were pretreated with RAPA or Z-YVAD-FMK for 1 h, washed with fresh medium and then treated with 10 μg/mL LPS for 6 h or 5 μM $A\beta_{1-42}$ for indicated times, while PG was added at the time of LPS or $A\beta_{1-42}$ exposure for co-treatment. Autophagy inhibitor 3-MA was added 1 h prior to LPS or $A\beta_{1-42}$ treatment.

2.4. Immunofluorescence assay

In present experiment, the astrocytes were treated according to the experimental design. Immunofluorescence assay was performed as staining protocol described [22]. Briefly, the cells were fixed with 4% paraformaldehyde for 30 min, permeabilized with 0.05% Triton X-100 and then blocked with 3% bovine serum albumin (BSA) in PBS for 1 h. The primary antibodies were anti-NLRP3 (1:400 dilution) and LC3 (1:300 dilution). After the blocking step, the cells were incubated with primary antibody at 4 °C overnight. The secondary antibodies were FITC-conjugated goat anti-rabbit lgG (1:100 dilution), and Red-conjugated rabbit anti-rat lgG (1:100 dilution). The cells were then washed with PBS and incubated for 1 h with the secondary antibody. The nuclei were subsequently stained with DAPI for 5 min. The astrocytes were measured by fluorescence microscope (IX-81, Olympus).

2.5. Western blotting assay

Western blotting assay was performed as previously described [22]. The astrocytes were treated according to the experimental design. The astrocyte lysates was collected for western blotting assay. Total protein had been quantified by a BCA protein assay kit, and electrotransferred to PVDF membrane (Millipore, Burlington, MA), which incubated with primary antibodies (anti-NLRP3, Caspase-1, LC3, IL-1β and P62 1:1000) at 4 °C overnight. Subsequently, membranes were incubated with secondary antibody for another 2 h. All blots were detected using ECL method, and the scanned band images were quantified by ImageJ software.

2.6. ELISA assay

ELISA assay was performed as previously described [22]. In present experiment, the supernatants of the treated astrocytes were assayed for pro-inflammatory cytokines IL-1β and TNF-α by ELISA (Blue Gene, Shanghai China). The standard curve was prepared according to the instruction protocol. The range of analysis was between 0 and 1000 pg/mL for IL-1β and TNF-α. ELISA assay was performed as analysis protocol described previously. Briefly, the supernatants were added into the appropriate well of anti-IL-1 β or TNF-α pre-coated plates. After 2 h of incubation, the substrate A and substrate B added into each wells according to the analysis protocol. Finally, the stop solution was added to terminate reaction. Detection and quantification of the plates were performed with a microplate reader (Molecular Device Corporation, Sunnyvale, CA, USA) at 450 nm.

2.7. Assessment of cell viability

MTT assay was used to quantify the cell viability, which was performed as previously described [20]. In current experiment, the astrocytes were treated in accordance with the experimental design. Meanwhile, 20 μL of MTT solution (5 mg/mL) was added to each well and incubated for 4 h at 37 °C. Then, the MTT medium was deserted and replaced with 200 μL DMSO to dissolve the formazan crystals. Detection and quantification of the plates were performed with a microplate reader at 570 nm. The absorbance of the formazan formed in control cells was taken as 100% viability.

2.8. Statistical analysis

Each experiment was performed at least 3 times. Data are expressed as the means ± SD. The significance differences between experiments groups were determined by student's *t*-test or one-way ANOVA followed by the Dunnett's test for multiple comparisons. p-Values < 0.05 or less were considered to be statistically significant.

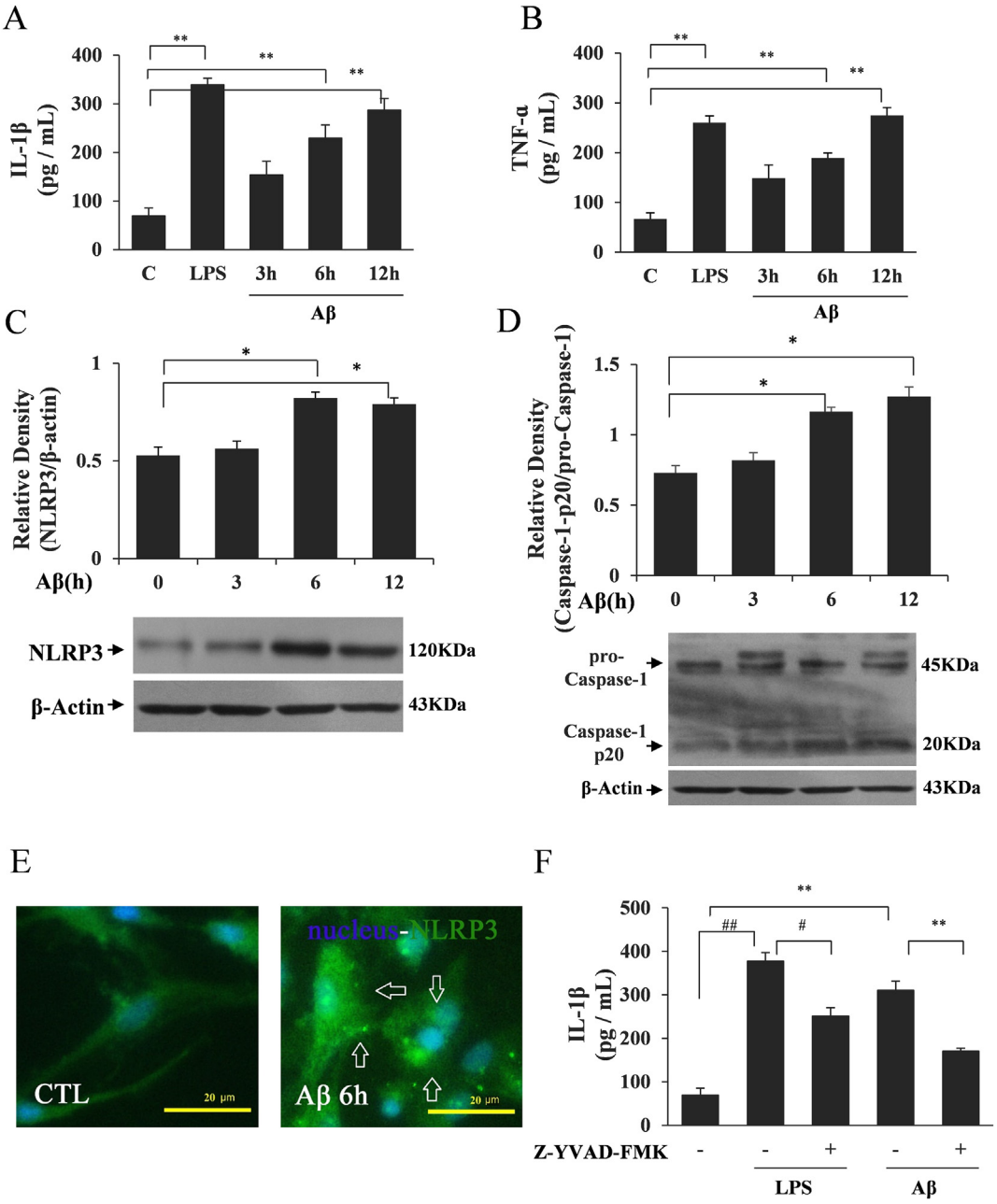

Fig. 1. Aβ induced NLRP3 inflammasome activation in astrocytes.
A, B. Aβ, LPS increases IL-1β and TNF-α release in astrocytes. ELISA assay was used to detect the level of IL-1β (A) and TNF-α (B) in the medium of treated astrocytes following Aβ or LPS treatment for indicated time (**$p < 0.01$, compared with untreated group, statistically analysis was carried out by student's *t*-test). C, D. Aβ induces NLRP3-Caspase-1 inflammasome activation in astrocytes. Western blotting was used to detect NLRP3, pro-Caspase-1, Caspase-1 p20 expression in astrocytes (*$p < 0.05$, compared with untreated group, statistically analysis was carried out by student's *t*-test). E. Aβ improves NLRP3 inflammasome expression in astrocytes. Astrocytes were treated with Aβ for indicated time, and then the cells were immunostained against antibodies NLRP3 (green), DAIP was used to stain nuclei (blue). F. Aβ-induced NLRP3-Caspase-1 inflammasome activation triggers IL-1β production in astrocytes. ELISA assay was used to detect the level of IL-1β in the supernatant of treated astrocytes (**$p < 0.01$, compared with Aβ-treated group, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, compared with LPS-treated group, statistically analysis was carried out by student's *t*-test). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3. Result

3.1. Aβ activated NLRP3-Caspase-1 inflammasome in astrocytes

Neuroinflammation has been known to be involved in the pathological procession of AD. In order to evaluate whether Aβ affects NLRP3 inflammasome activation in astrocytes, we investigate the level of proinflammatory cytokines in the culture supernatant of astrocytes. The results showed that Aβ activated astrocytes after 6 h treatment, characterized by an increase of IL-1β (Fig. 1A) and TNF-α (Fig. 1B) release. To further determine the role of Aβ in activating the NLRP3 inflammasome in astrocytes, the expression of NLRP3, Caspase-1 P20, pro-Caspase-1 were evaluated in response to Aβ treatment. After 6 h Aβ treatment, NLRP3 expression and Caspase-1 activation (assessed by the appearance of Caspase-1 p20/pro-Caspase-1) manifested a significantly increase in astrocytes (Fig. 1C and D). Additionally, immunofluorescence results showed a mass of NLRP3 expression was positively stained in Aβ-treated group (Fig. 1E). In order to investigate whether

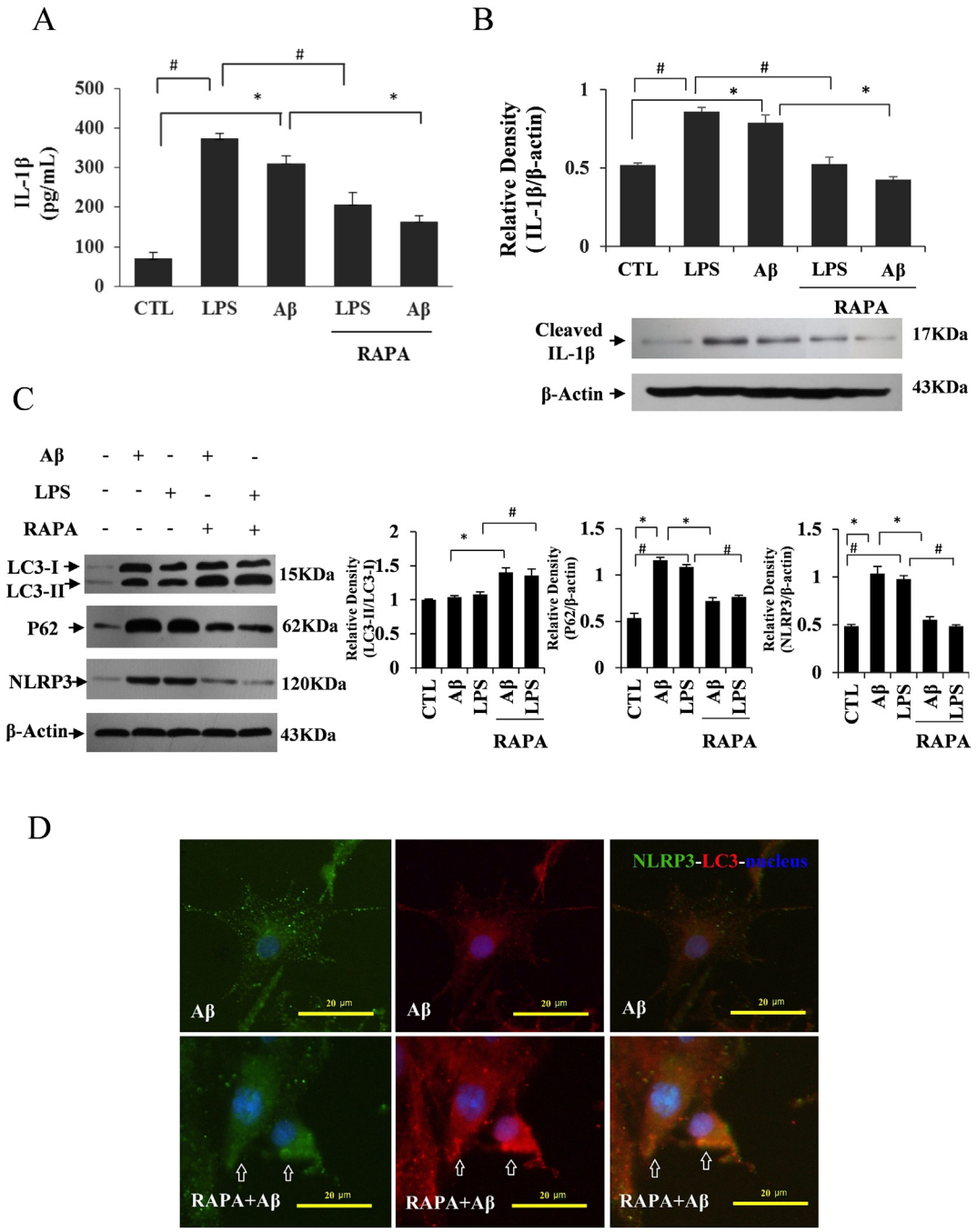

Fig. 2. Aβ-induced NLRP3-Caspase-1 activation subjected to the dysfunction of autophagy in astrocytes.
A, B. RAPA inhibits IL-1β expression in Aβ-treated astrocytes. ELISA assay was used to detect the level of IL-1β in the supernatant of treated astrocytes as well as western blotting was used to detect the protein level of mature cleaved IL-1β in the astrocyte lysates following Aβ or LPS treatment (*$p < 0.05$, compared with Aβ-treated group, #$p < 0.05$, compared with LPS-treated group, statistically analysis was carried out by student's *t*-test). C. RAPA inhibits Aβ-induced NLRP3 expression in astrocytes. Western blotting was used to detect NLRP3, LC3, P62 expression in astrocytes (*$p < 0.05$, compared with Aβ-treated group, #$p < 0.05$, compared with LPS-treated group, statistically analysis was carried out by Student's *t*-test). D. RAPA improves NLRP3 and LC3 co-localization in Aβ-treated astrocytes. Astrocytes were immunostained against antibodies NLRP3 (green), LC3 (red), DAIP was used to stain nuclei (blue). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

there is a relationship between NLRP3-Caspase-1 activation and IL-1β production. We treated astrocytes-enrich cultured pretreated with or without *Z*-YVAD-FMK (Caspase-1 inhibitor) 1 h, and then exposed to 5 μM Aβ for additional 6 h treatment. A statistical analysis revealed that Z-YVAD-FMK (10 μM) significantly inhibited LPS or Aβ-induced IL-1β production in astrocytes (Fig. 1F).

3.2. Aβ-induced NLRP3-Caspase-1 activation subjected to autophagy deficiency in astrocytes

Autophagy has been implicated in the process of several neurodegenerative disorder [23]. Our previous findings indicated Aβ induced astrocytes dysfunction via inhibiting autophagic activation [22]. In order to verify whether Aβ-induced IL-1β processing subjected to autophagy deficiency in astrocytes. Firstly, we detected the level of IL-1β

in the supernatant of treated astrocytes. The ELISA results showed that RAPA significantly inhibited IL-1β secretion in Aβ or LPS-induced astrocytes (Fig. 2A). Meanwhile, we also detected the level of mature cleaved IL1β in the astrocyte lysates. The western blotting results showed RAPA down-regulated cleaved IL1β expression in Aβ or LPS-induced astrocytes (Fig. 2B). Additionally, a massive increase in protein levels of P62 and decreasing LC3 processing indicated that autophagic lysosomal degradation activity was impaired by Aβ treatment. RAPA represented a resulting improvement in LC3 processing, and suppression in autophagic adaptor P62 aggregation following Aβ or LPS treatment in astrocytes (Fig. 2C). However, Aβ or LPS-induced NLRP3 expression was significantly down-regulated after RAPA treatment in astrocytes. The immunofluorescence results showed RAPA substantially increased the co-localization of LC3-positive autophagic vacuoles and NLRP3 inflammasome (Yellow spots, Fig. 2D). The findings suggest that autophagy activation represents a resulting regulation to NLRP3 inflammasome in astrocytes.

3.3. Progesterone inhibited Aβ-induced IL-1β secretion in astrocytes via regulating NLRP3-Caspase-1 activation

Our previous study has found PG exhibited a neuroprotective function by inhibiting Aβ-induced mitochondrial apoptosis in neurons [20]. Initial assessment, we investigated whether PG represented directly toxic to astrocytes with concentrations of 0.25–2 μM for 24 h. MTT assay showed no significant toxicity in these treatments with 0.25, 0.5 or 1 μM PG (Fig. 3A). Based on these results, we treated astrocytes with 1 μM PG for 3 h, 6 h, 12 h following Aβ 6 h treatment. The ELISA results indicated that PG significantly inhibited Aβ-induced IL-1β production following 6 h treatment (Fig. 3B). To determine whether neuroprotective effect of PG in inhibiting Aβ-induced IL-1β processing is dependent on NLRP3-Caspase-1 inflammasome activation. We also performed an assessment of intracellular pathological changes of astrocytes following PG treatment. The western blotting results showed PG significantly inhibited Aβ-induced NLRP3 expression (Fig. 3C). Caspase-1 activation was assessed by appearance of Caspase-1 p20. The expression of Pro-Caspase-1 protein was detected in all groups, but the expression of active Caspase-1 (p20) was suppressed following 6 h PG treatment in Aβ-induced astrocytes (Fig. 3D).

3.4. Progesterone suppressed Aβ-induced NLRP3-Caspase-1 activation by up-regulating autophagy in astrocytes

Previously, we have found the potential role of PG in accelerating autophagy activation [22]. To determine whether neuroprotective effect of PG in inhibiting Aβ or LPS-induced IL-1β processing is dependent on autophagy activation. Firstly, we detected the level of IL-1β in the supernatant of treated astrocytes. The ELISA results showed 3-MA also blocked the suppression of PG on IL-1β secretion in Aβ or LPS-induced astrocytes (Fig. 4A). Meanwhile, we also detected the level of mature cleaved IL1β in the astrocyte lysates. The results showed 3-MA apparently blocked the function of PG on cleaved IL1β expression in Aβ or LPS-induced astrocytes (Fig. 4B). We further examine whether dysfunction of autophagy was involved in the activation of the NLRP3-Caspase-1 inflammasome. The western blotting result revealed that 3-MA significantly suppressed the effect of PG on regulating NLRP3 expression in astrocytes (Fig. 4C). Additionally, we also evaluated the potential influence of PG on Caspase-1 activation in astrocytes following Aβ treatment. The expression of Pro-Caspase-1 protein was detected in all groups, but 3-MA blocked the inhibition of PG in the expression of active Caspase-1 p 20 (Fig. 4D). These date suggested that PG represented a resulting regulation to NLRP3-Caspase-1 inflammasome by initiating autophagy in astrocytes.

4. Discussion

Neuroinflammation is a characteristic factor in the pathological procession of AD. Several researches have focused on seeking for effective strategies to ameliorate AD-related neuroinflammation [6]. Indeed, astrocytic activation and subsequent production of neurotoxic pro-inflammatory molecules have a pivotal role in the progression of AD. The neuron-centric vision of pathological procession of AD has undergone considerable changes. Can astrocytes become the target for new AD drugs? Our previous results have found neurosteriod PG exhibited a characteristic neuroprotective function against astrocytic neuroinflammation by inhibiting endoplasmic reticulum stress [21] and activating autophagy [22]. However, the upstream regulatory mechanism of neuroinflammation in response to PG treatment is still unclear. IL-1β, known to play a role in the “sickness factor,” exerts sustaining influence on the hippocampal dependent memory function [9,24]. Maturation and release of IL1β requires the activation of the NLRP3 inflammasome. The NLRP3 inflammasome, which comprises the core protein NLRP3, regulatory molecule Caspase-1 and ASC, is involved in a range of chronic neurodegenerative disease, including AD [25]. Therefore, we performed an initial assessment of intracellular pathological changes of astrocytes following lipopolysaccharides (LPS) or Aβ treatment. LPS set as a positive control, which was a potential stimulator of NLRP3 inflammasome [26]. Results showed that Aβ or LPS treatment significantly induced astrocytes activation with an increase of IL-1β and TNF-α production, respectively. Additionally, we evaluated whether NLRP3 activation was involved in the early stage of Aβ-treated astrocytes activation. Results showed that NLRP3 expression and Caspase-1 activation, assessed by the appearance of Caspase-1 p20, manifested a significantly increase in astrocytes after 6 h Aβ treatment. However, pharmacological blocking NLRP3-Caspase-1 activation by Caspase-1 inhibitor Z-YVAD-FMK significantly suppressed Aβ or LPS-induced IL-1β expression in astrocytes, suggesting that a potential connection between IL-1β processing and NLRP3 inflammasome activation in astrocytes following Aβ treatment. Therefore, we speculated that the increase of IL-1β from astrocytes may be attributed to 2 reasons: 1) the increase of IL-1β may be the result of upstream neuroinflammation activation in response to direct Aβ treatment; 2) Aβ accumulation may result in structural injury to self-defensive mechanism, like autophagy, leading to release of IL-1β.

Autophagy, a self-defensive mechanism process, plays a vital regulatory role in maintaining intracellular homeostasis and metabolic balance. Several studies have shown that autophagy dysfunction is associated with various neurological diseases, including cancer, heart diseases, and neurodegenerative disorders [27]. In our previous studies, we found that Aβ made autophagy dysfunction, which represented a number of autophagic related protein P62 aggregation, and LC3-II reduction in astrocytes [22]. Herein, we explored the potential function of autophagy in regulating IL-1β processing. Results showed that RAPA efficiently inhibited the level of IL-1β secretion and cleaved IL-1β in lysates of astrocytes. Given the critical role of NLRP3 inflammasome in the maturation and release of proinflammatory cytokines, we evaluated whether autophagy was involved in regulation of the NLRP3 inflammasome activation in Aβ-treated astrocytes. Next, we explored the possible relationships between autophagy and the expression of NLRP3 inflammasome in response to Aβ treatment. Results showed RAPA resulted in a significant increase of Aβ or LPS-induced LC3 processing and inhibition of autophagic adaptor P62 aggregation, indicating RAPA induced the formation of autophagy, but illustrated by cytoplasmic accumulation of autophagosomes along with lysosomes containing degradation products. The results showed the presence of NLRP3 inflammasome in the cytoplasm and found that the NLRP3 inflammasome was widely co-localization with LC3-II upon Aβ treatment in astrocytes. Therefore, we speculate NLRP3 inflammasome complexes are one of those products to be degraded. Our observations indicated that autophagic lysosomal degradation activity was impaired by Aβ treatment,

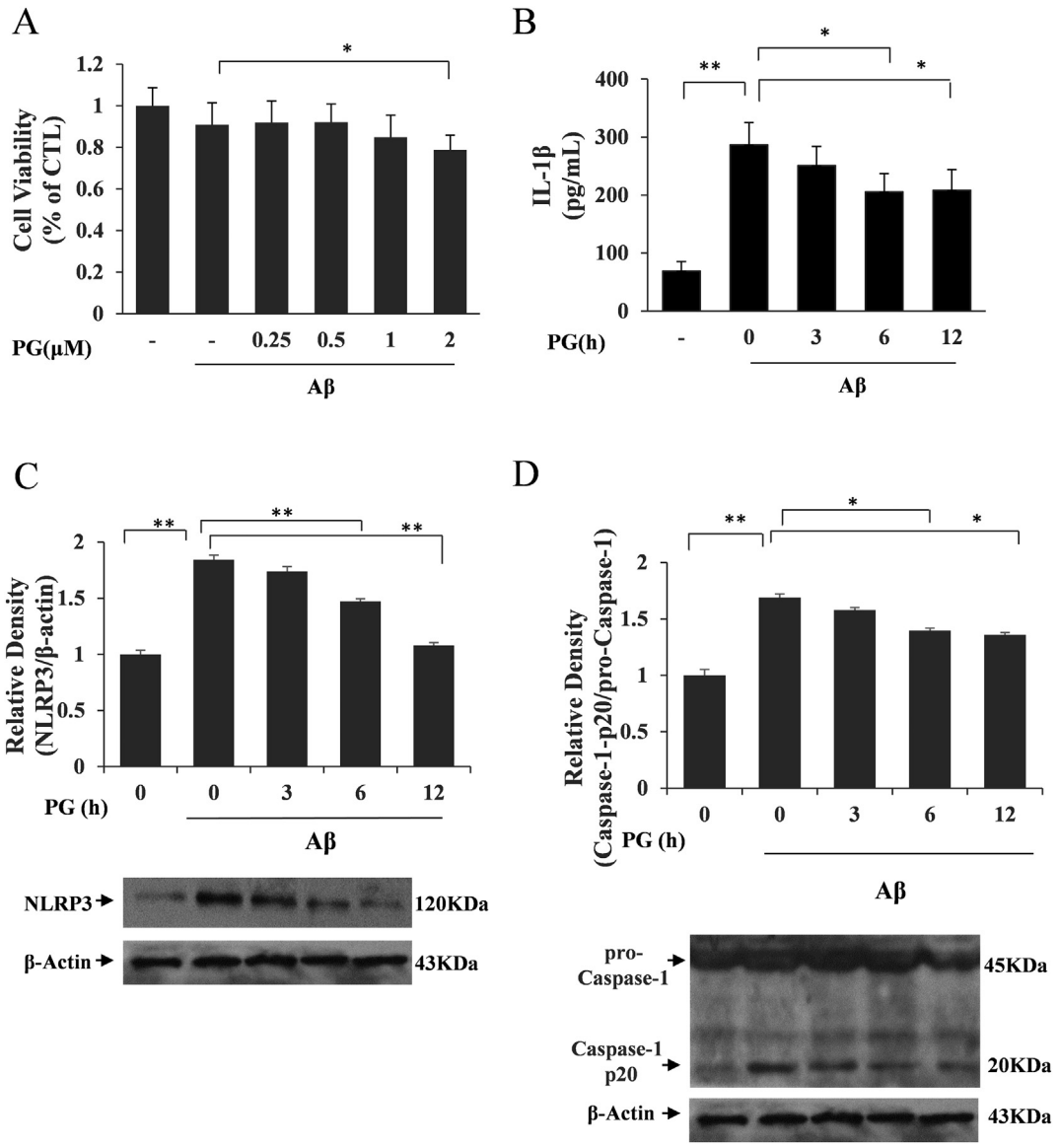

Fig. 3. Progesterone inhibited Aβ-induced IL-1β secretion in astrocytes via regulating NLRP3-Caspase-1 activation.
A. Protective effect of PG on Aβ-induced cytotoxicity. Cytotoxicity of progesterone on primary astrocytes is determined by using MTT assay after 24 h treatment with PG at the indicated concentrations (*$p < 0.05$, compared with Aβ-treated group, statistically analysis was carried out by Student's *t*-test). B. PG suppresses Aβ, LPS-induced release of IL-1β in a time-dependent manner. ELISA assay was used to detect the level of IL-1β in the supernatant of treated astrocytes (*$p < 0.05$, **$p < 0.01$, compared with Aβ-treated group, statistically analysis was carried out by Student's *t*-test). C, D. PG suppresses Aβ-induced NLRP3-Caspase-1 activation in astrocytes. Western blotting was used to detect NLRP3, pro-Caspase-1, Caspase-1 p20 expression in astrocytes (*$p < 0.05$, **$p < 0.01$, compared with Aβ-treated group, statistically analysis was carried out by Student's *t*-test).

but RAPA represented a resulting improvement in inhibiting NLRP3 inflammasome expression by up-regulating autophagy degradation activity in astrocytes. Autophagy has been suggested to restrict the activation of NLRP3 inflammasomes by targeting inflammasome complexes for degradation in the autophagosome. In consistent with our studies, conditional knockouts of autophagy-related genes Becn-1 lead to autophagy deficiency and generate high levels of pro-inflammatory cytokines IL-1β和IL-18 from microglia in Becn-1$^{+/-}$ mice [28]. This suggests the potential role of autophagy in inhibiting the maturation and secretion of inflammatory cytokines.

Early findings demonstrated a neuroprotective function of the neurosteroid PG, particularly its potential protective strategies against AD-related neuroinflammation by inhibiting endoplasmic reticulum stress and activating autophagy. However, the potential upstream regulatory mechanism of PG has not been demonstrated. Initial assessment, we investigated whether PG represented directly toxic to astrocytes. Results showed 1 μM PG had no significant toxicity in these treatments for 24 h and represented an improvement in inhibiting Aβ-induced IL-1β production after 6 h treatment. Above studies has found the possible relationships between autophagy activation and IL-1β processing in response to Aβ treatment. However, whether neuroprotective effect of PG in inhibiting Aβ or LPS-induced IL-1β processing is dependent on autophagy activation. Results showed autophagy inhibitor 3-MA suppressed the regulation of PG in IL-1β secretion and cleaved IL-1β in lysates of astrocytes. In order to further illuminate regulatory mechanism of PG, we performed an assessment of intracellular pathological changes of Aβ-treated astrocytes following PG exposure. Therefore, we evaluated autophagy pharmacological inhibitor 3-MA whether represented a decrease regulation of PG in Aβ-induced NLRP3-Caspase-1 activation. Results showed pre-exposure of astrocytes with 3-MA efficiently inhibited the neuroprotective function of PG on regulating NLRP3-Caspase-1 activation in Aβ-treated

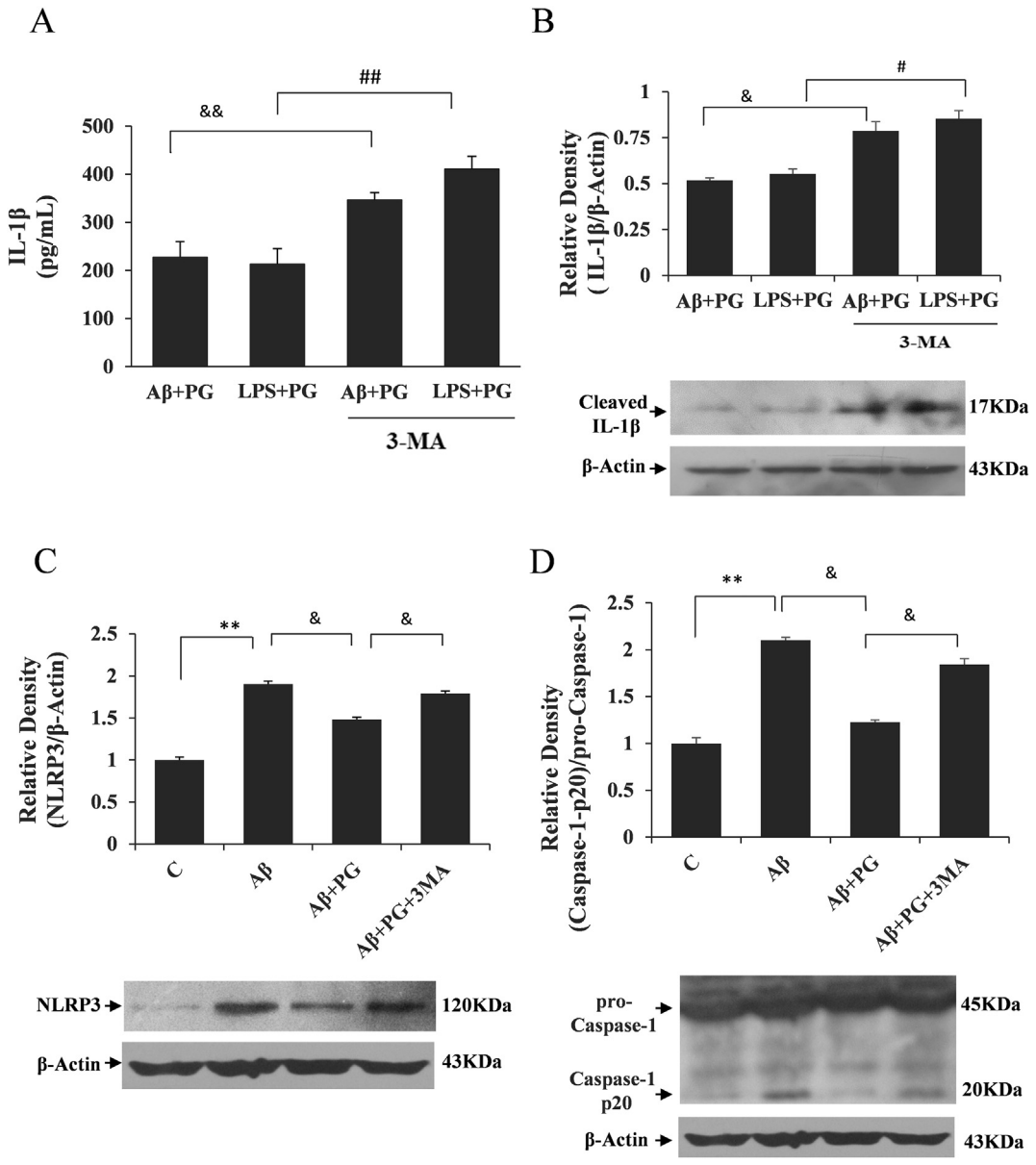

Fig. 4. Progesterone suppressed Aβ-induced NLRP3-Caspase-1 activation by up-regulating autophagy in astrocytes.
A, B. 3-MA suppresses the neuroprotection of PG in regulating IL-1β processing in Aβ-treated astrocytes. Western blotting was used to detect the protein level of mature cleaved IL-1β in the astrocyte lysates following Aβ or LPS treatment as well as ELISA assay was used to detect the level of IL-1β in the supernatant of treated astrocytes ($^{\&}p < 0.05$, $^{\&\&}p < 0.01$, compared with Aβ + PG treated group, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, compared with LPS + PG treated group, statistically analysis was carried out by Student's *t*-test). C, D. Autophagy regulates PG protection against Aβ-induced NLRP3-Caspase-1 activation in astrocytes. Western blotting was used to detect NLRP3, pro-Caspase-1, Caspase-1 p20 expression in astrocytes ($^{**}p < 0.01$, compared with Aβ-treated group, $^{\&}p < 0.05$, compared with Aβ + PG treated group, statistically analysis was carried out by Student's *t*-test).

astrocytes, which represented a number of NLRP3 aggregation and active Caspase-1 p20 expression in astrocytes. Consistent with these results, we speculate PG inhibited Aβ-induced IL-1β secretion is not simply dependent on upstream regulatory mechanism NLRP3 activation directly, but benefits from inducing autophagy activation in astrocytes. While investigating the hypothesis that PG has a potential influence on ameliorating Aβ-induced NLRP3 inflammasomes activation, we also illuminated that PG neuroprotection against Aβ-induced neurotoxicity was associated with autophagy activation in astrocytes.

In summary, the present results provide direct evidence that autophagy is found to play a vital role in inhibiting neuroinflammation from Aβ-treated astrocytes, and it mediates the neuroprotective mechanism of PG in suppressing IL-1β processing by directly targeting NLRP3 inflammasomes for degradation (Fig. 5). The results of this study provide a novel opinion into potential neuroprotective mechanism of PG in regulating astrocytes. Impressively, our observation established possible relationships between autophagy activation and NLRP3 inflammasome in response to PG treatment in astrocytes. The neuron-centric vision of pathological procession of AD has considerably changed to astrocytes. Astrocytes have become the target for new AD drugs. Hence, the neuroprotective mechanism of neurosteroid PG in regulating NLRP3 inflammasome activation can be a potential therapeutic target for inhibiting astrocytic neuroinflammation of AD.

Declaration of Competing Interest

There are no conflicts of interest.

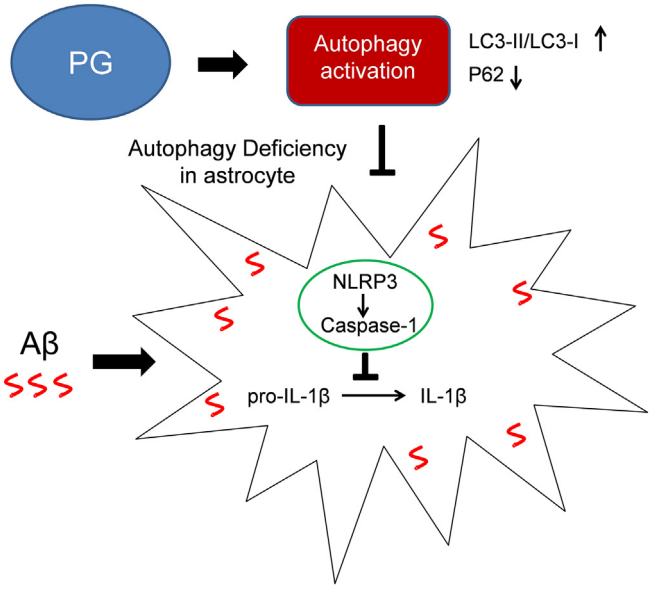

Fig. 5. The mechanism of PG in suppressing Aβ-induced IL-1β processing in astrocytes. Aβ induced astrocytes dysfunction, which exhibited NLRP3-Caspase-1 activation and IL-1β production. PG represented a resulting regulation to NLRP3-Caspase-1 inflammasome by initiating autophagy in astrocytes.

References

[1] D.J. Selkoe, Alzheimer's disease: genes, proteins, and therapy, Physiol. Rev. 81 (2001) 741–766.

[2] Lopez-Gonzalez I, Schluter A, Aso E, Garcia-Esparcia P, Ansoleaga B, F LL, et al. Neuroinflammatory signals in Alzheimer disease and APP/PS1 transgenic mice: correlations with plaques, tangles, and oligomeric species. J. Neuropathol. Exp. Neurol.. 2015; 74: 319–44.

[3] H.L. Weiner, D. Frenkel, Immunology and immunotherapy of Alzheimer's disease, Nat. Rev. Immunol. 6 (2006) 404–416.

[4] D. Schenk, R. Barbour, W. Dunn, G. Gordon, H. Grajeda, T. Guido, et al., Immunization with amyloid-beta attenuates Alzheimer-disease-like pathology in the PDAPP mouse, Nature 400 (1999) 173–177.

[5] D. Blackburn, S. Sargsyan, P.N. Monk, P.J. Shaw, Astrocyte function and role in motor neuron disease: a future therapeutic target? Glia 57 (2009) 1251–1264.

[6] A. Parachikova, M.G. Agadjanyan, D.H. Cribbs, M. Blurton-Jones, V. Perreau, J. Rogers, et al., Inflammatory changes parallel the early stages of Alzheimer disease, Neurobiol. Aging 28 (2007) 1821–1833.

[7] A. Weber, P. Wasiliew, M. Kracht, Interleukin-1beta (IL-1beta) processing pathway, Sci. Signal. 3 (2010) (cm2).

[8] M.G. Netea, A. Simon, F. van de Veerdonk, B.J. Kullberg, J.W. Van der Meer, L.A. Joosten, IL-1beta processing in host defense: beyond the inflammasomes, PLoS Pathog. 6 (2010) e1000661.

[9] M. Kitazawa, D. Cheng, M.R. Tsukamoto, M.A. Koike, P.D. Wes, V. Vasilevko, et al., Blocking IL-1 signaling rescues cognition, attenuates tau pathology, and restores neuronal beta-catenin pathway function in an Alzheimer's disease model, J. Immunol. 187 (2011) 6539–6549.

[10] A. Halle, V. Hornung, G.C. Petzold, C.R. Stewart, B.G. Monks, T. Reinheckel, et al., The NALP3 inflammasome is involved in the innate immune response to amyloid-beta, Nat. Immunol. 9 (2008) 857–865.

[11] V. Petrilli, C. Dostert, D.A. Muruve, J. Tschopp, The inflammasome: a danger sensing complex triggering innate immunity, Curr. Opin. Immunol. 19 (2007) 615–622.

[12] R. Zhou, A.S. Yazdi, P. Menu, J. Tschopp, A role for mitochondria in NLRP3 inflammasome activation, Nature 469 (2011) 221–225.

[13] V. Hornung, E. Latz, Critical functions of priming and lysosomal damage for NLRP3 activation, Eur. J. Immunol. 40 (2010) 620–623.

[14] K. Schroder, J. Tschopp, The inflammasomes, Cell 140 (2010) 821–832.

[15] K. Shimada, T.R. Crother, M. Arditi, Innate immune responses to Chlamydia pneumoniae infection: role of TLRs, NLRs, and the inflammasome, Microbes Infect. 14 (2012) 1301–1307.

[16] K.K. Borowicz, B. Piskorska, M. Banach, S.J. Czuczwar, Neuroprotective actions of neurosteroids, Front. Endocrinol. 2 (2011) 50.

[17] S.L. Gonzalez, F. Labombarda, M.C. Gonzalez Deniselle, A. Mougel, R. Guennoun, M. Schumacher, et al., Progesterone neuroprotection in spinal cord trauma involves up-regulation of brain-derived neurotrophic factor in motoneurons, J. Steroid Biochem. Mol. Biol. 94 (2005) 143–149.

[18] C. Espinosa-Garcia, A. Aguilar-Hernandez, M. Cervantes, G. Morali, Effects of progesterone on neurite growth inhibitors in the hippocampus following global cerebral ischemia, Brain Res. 1545 (2014) 23–34.

[19] S. Liu, H. Wu, G. Xue, X. Ma, J. Wu, Y. Qin, et al., Metabolic alteration of neuroactive steroids and protective effect of progesterone in Alzheimer's disease-like rats, Neural Regen. Res. 8 (2013) 2800–2810.

[20] Y. Qin, Z. Chen, X. Han, H. Wu, Y. Yu, J. Wu, et al., Progesterone attenuates Abeta (25-35)-induced neuronal toxicity via JNK inactivation and progesterone receptor membrane component 1-dependent inhibition of mitochondrial apoptotic pathway, J. Steroid Biochem. Mol. Biol. 154 (2015) 302–311.

[21] Y. Hong, X. Wang, S. Sun, G. Xue, J. Li, Y. Hou, Progesterone exerts neuroprotective effects against Abeta-induced neuroinflammation by attenuating ER stress in astrocytes, Int. Immunopharmacol. 33 (2016) 83–89.

[22] Y. Hong, Y. Liu, G. Zhang, H. Wu, Y. Hou, Progesterone suppresses Abeta42-induced neuroinflammation by enhancing autophagy in astrocytes, Int. Immunopharmacol. 54 (2018) 336–343.

[23] L.S. Whyte, A.A. Lau, K.M. Hemsley, J.J. Hopwood, T.J. Sargeant, Endo-lysosomal and autophagic dysfunction: a driving factor in Alzheimer's disease? J. Neurochem. 140 (2017) 703–717.

[24] W.S. Griffin, J.A. Nicoll, L.M. Grimaldi, J.G. Sheng, R.E. Mrak, The pervasiveness of interleukin-1 in alzheimer pathogenesis: a role for specific polymorphisms in disease risk, Exp. Gerontol. 35 (2000) 481–487.

[25] C. Dempsey, A. Rubio Araiz, K.J. Bryson, O. Finucane, C. Larkin, E.L. Mills, et al., Inhibiting the NLRP3 inflammasome with MCC950 promotes non-phlogistic clearance of amyloid-beta and cognitive function in APP/PS1 mice, Brain Behav. Immun. 61 (2017) 306–316.

[26] D. Wang, J. Zhang, W. Jiang, Z. Cao, F. Zhao, T. Cai, et al., The role of NLRP3-CASP1 in inflammasome-mediated neuroinflammation and autophagy dysfunction in manganese-induced, hippocampal-dependent impairment of learning and memory ability, Autophagy 13 (2017) 914–927.

[27] A.M. Choi, S.W. Ryter, B. Levine, Autophagy in human health and disease, N. Engl. J. Med. 368 (2013) 1845–1846.

[28] T. Saitoh, N. Fujita, M.H. Jang, S. Uematsu, B.G. Yang, T. Satoh, et al., Loss of the autophagy protein Atg 16L1 enhances endotoxin-induced IL-1beta production, Nature 456 (2008) 264–268.