

## RESEARCH ARTICLE

# NLRP3 Inflammasome Mediates Chronic Mild Stress-Induced Depression in Mice via Neuroinflammation

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## Abstract

**Background:** Evidence from both clinical and experimental research indicates that the immune-brain interaction plays a pivotal role in the pathophysiology of depression. A multi-protein complex of the innate immune system, the NLRP3 inflammasome regulates cleavage and secretion of proinflammatory cytokine interleukin-1 $\beta$ . The inflammasome detects various pathogen-associated molecule patterns and damage-associated molecule patterns, which then leads to a series of immune-inflammatory reactions.

**Methods:** To explore the role of inflammasome activation in the underlying biological mechanisms of depression, we established a mouse model of depression with unpredictable chronic mild stress.

**Results:** Mice subjected to chronic mild stress for 4 weeks had significantly higher serum corticosterone levels, serum interleukin-1 $\beta$  levels, and hippocampal active interleukin-1 $\beta$  protein levels. They also displayed depressive-like symptoms, including decreased sucrose preference and increased immobility time. Moreover, the hippocampi of chronic mild stress-exposed mice had significantly higher activity of caspase-1, which accompanied by higher protein levels of NLRP3 and the apoptotic speck-containing protein with a card. Pretreatment with the NLRP3 inflammasome inhibitor VX-765 decreased serum and hippocampal levels of interleukin-1 $\beta$  protein and significantly moderated the depressive-like behaviors induced by chronic mild stress.

**Conclusions:** These data suggest the NLRP3 inflammasome mediates stress-induced depression via immune activation. Future procedures targeting the NLRP3 inflammasome may have promising effects in the prevention and treatment of depression.

**Keywords:** NLRP3 inflammasome, interleukin-1 $\beta$ , depression, stress, inflammation

## Introduction

Depression, as a pervasive mental disorder and a significant contributor to the global burden of disease, was characterized by enduring sadness, loss of interest or pleasure, decreased energy or feelings of tiredness, feelings of low self-worth, disturbed sleep or appetite, and poor concentration ([World Health](#)

[Organization, 2012](#)). As the main cause of mental and behavioral disorders, depression affects 350 million people across the world and increased from 15th in 1990 to 11th rank (37% increases) of leading causes of global disability-adjusted life years in 2010 ([World Health Organization, 2008](#); [Murray et al.,](#)

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2012). A systematic review showed that the overall estimation of lifetime prevalence of depression was 3.3% in mainland China, and rural residents seemed to have a greater risk of depression than urban residents (Gu et al., 2013).

Although studies of antidepressants, depression-specific psychotherapies, and somatic treatments have made clear advances, there are no fully satisfactory treatments available yet for depression (Kupfer et al., 2012). Further studies are needed to comprehensively understand the pathophysiologic mechanisms of depression and to develop new compounds for its treatment.

Research in recent decades has suggested that overproduction of proinflammatory cytokines constituted key phenomena underlying the various aspects of immune-brain interaction in depression (Maes, 1995; Dowlati et al., 2010). Proinflammatory cytokine interleukin (IL)-1 $\beta$  was the first cytokine known to enhance hypothalamic-pituitary-adrenal (HPA) axis activity during an immune response (Bernton et al., 1987; Sapolsky et al., 1987). Abundant evidence demonstrated that depression is characterized by HPA axis hyperactivity and accompanied by increased brain or peripheral IL-1 $\beta$  production (Maes et al., 1993; Goshen et al., 2008). IL-1 $\beta$  signaling mediated the development of depressive symptoms partially via suppression of hippocampal neurogenesis after exposure to stress (Goshen and Yirmiya, 2009). A meta-analysis concluded that pharmacological antidepressant treatment reduced depressive symptoms, while it reduced serum levels of IL-1 $\beta$  (Hannestad et al., 2011). This further confirmed the key role of IL-1 $\beta$  in the pathogenesis of depression.

The NLRP3 inflammasome assembled as a multi-molecular platform and activated upon a bunch of divergent invading pathogens and cellular damage, which then regulated the cleavage and maturation of IL-1 $\beta$  (Lamkanfi and Kanneganti, 2010; Schroder and Tschoop, 2010). The intracellular pattern recognition receptor NLRP3, the apoptotic speck-containing protein with a card (ASC) and the effector protein caspase-1 constituted the NLRP3 inflammasome (Petrilli et al., 2007).

Research has indicated that inflammasome signaling contributes to some autoinflammatory disorders such as colitis, multiple sclerosis, and diabetes (Schroder et al., 2010; Lamkanfi et al., 2011; Menu and Vince, 2011). The NLRP3 inflammasomes have been implicated as a new target for therapy in diverse diseases (Cook et al., 2010).

As our previous study demonstrated the involvement of NLRP3 inflammasome in lipopolysaccharide (LPS) induced depression, we postulated the NLRP3 inflammasome might be a central mediator of stress-induced depression and a possible new target for depression therapy (Iwata et al., 2013; Zhang et al., 2014). Therefore, we used the chronic mild stress (CMS)-induced depression mouse model to explore the potential role of NLRP3 inflammasome in stress-induced depression.

## Materials and Methods

### Subjects

Male 8-week old BALB/c mice (Animal Centre, Second Military Medical University, China) were housed in a standard animal room (22  $\pm$  2°C, lights on from 7 AM to 7 PM), with food and water ad libitum except specified otherwise. Animals were housed in individual cages and randomly assigned to 5 groups, including Control, Vehicle (5% dimethyl sulphoxide in saline solution), VX-765, Stress, and VX-765 + Stress groups. Mice were adapted to their new environment and 1% sucrose solution (wt/vol) for 2 weeks before the CMS procedures. All the experiment

procedures were approved by the Second Military Medical University Animal Care Committee and carried out in accordance with related regulations and laws.

### CMS

The unpredictable CMS protocol in this experiment was adapted from other researchers (Goshen et al., 2008; Peng et al., 2012; Lu et al., 2014). Briefly, Stress group and VX-765 + Stress group mice were administered the following mild stressors randomly for 4 weeks: 45° cage tilting for 14 hours; cage shaking for 10 minutes; damp bedding for 16 hours; swimming in 4°C cold water for 5 minutes; confinement in a tube for 2 hours; continuous illumination during the dark cycle; 45°C oven for 5 minutes; and food and water deprivation for 12 hours during the light cycle. Body weight and sucrose preference were assessed on each Sunday.

### VX-765 Administration

The novel investigational antiinflammatory agent VX-765 could inhibit IL-1 $\beta$  secretion as a potent and selective caspase-1 inhibitor (Wannamaker et al., 2007). VX-765 (product number: BCP001020, CAS number: 273404-37-8, purity: 98%) was purchased from Biochempartner Co. Ltd (Shanghai, China). The VX-765 and VX-765 + Stress groups were i.p. injected VX-765 (50 mg/kg body weight dissolved in 200  $\mu$ L vehicle) daily for 4 weeks. This dose was shown to significantly reduce LPS-induced serum IL-1 $\beta$  production and block seizure-induced production of IL-1 $\beta$  in the hippocampus (Ravizza et al., 2006; Wannamaker et al., 2007). The Control and Stress groups were injected with 200- $\mu$ L saline solutions daily for 4 weeks, while the Vehicle group was injected with 200- $\mu$ L vehicles.

### Behavioral Measurements

Sucrose preference test was performed at the dark phase as we described in our previous papers: each mouse was presented with 2 bottles for 1 hour after 12 hours of water and food deprivation, one containing tap water and the other containing 1% sucrose solution (Peng et al., 2012; Zhang et al., 2014). Sucrose preference was calculated as the percentage of sucrose solution consumption out of the total water intake volume (Goshen et al., 2008; Koo and Duman, 2008).

Tail suspension test is a different test widely used for assessing depressive-like behaviors via measuring immobility time of animals (Lu et al., 2014). We performed the tail suspension test after the sucrose preference test during the dark cycle at the end of the CMS procedures. Each mouse was suspended individually in a PHM-300 tail suspension chamber of the MED Associates' tail suspension hardware for 1 minute to adapt to the new environment, and then immobility time (time below the lower threshold value 0.05) during the next 5 minutes suspension was detected and analyzed with the tail suspension software (Med Associates Inc., St. Albans, VT).

### Corticosterone Enzyme-Linked Immunosorbent Assay

To measure serum corticosterone levels, we purchased an enzyme-linked immunosorbent assay kit (product no: ADI-900-097) from Enzo Life Sciences, Inc. (Farmingdale, NY). The kit's sensitivity was 27 pg/mL (range 32-20000 pg/mL) and had low cross-reactivity with related steroids. On the next morning (from 9 to 12 AM) immediately after the fourth week of CMS,

mice were anesthetized with pentobarbital (40 mg/kg in a volume of 10 mL/kg body weight) and decapitated to collect blood and brain tissue. The hippocampi were dissected, flash frozen in liquid nitrogen, and stored at  $-80^{\circ}\text{C}$  until later analysis. Serum samples were handled and stored at  $-80^{\circ}\text{C}$  for further detection. Detection of serum corticosterone levels was performed according to the manufacturer's established protocol.

### Western Blot

The frozen mouse hippocampi were homogenized in ice-cold radio immunoprecipitation assay (RIPA) buffer containing 1 mM phenylmethanesulfonyl fluoride (PMSF) protease inhibitor (Beyotime Institute of Biotechnology, Nantong, Jiangsu, China). The samples were then centrifuged at 10000 *g* for 5 minutes at  $4^{\circ}\text{C}$  before the lysate supernatants were collected. Total protein concentrations were determined with the BCA assay kit (product no.: P0010) from Beyotime Institute of Biotechnology (Nantong, Jiangsu, China).

Protein samples were separated by 6 to 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to nitrocellulose membranes, and incubated with primary antibodies (dilution: 1:1000) at  $4^{\circ}\text{C}$  overnight. After washing 3 times, membranes were incubated with second antibodies (dilution: 1:5000) at room temperature for 1 hour. Then the membranes were scanned, and the densitometry of bands was quantified with Odyssey Infrared Imaging System (LI-COR, Inc., Lincoln, NE).

Primary antibodies  $\beta$ -actin (ab133626), IL-1 $\beta$  (ab9722), NALP3/NLRP3 (ab4207), and TMS1/ASC (ab175449) were purchased from Abcam (Hong Kong) Ltd. (HKSP, New Territories, HK). IRDye 800CW secondary antibodies (product part no.: 926-32214, 926-32211) were purchased from LI-COR Biosciences.

### Caspase-1 Activity Assay

Caspase-1 activity of the hippocampus lysate supernatants was determined with a colorimetric assay kit (product no.: C1101) from Beyotime Institute of Biotechnology (Nantong, Jiangsu, China). The caspase-1 activity analysis was based on the cleavage of the Ac-YVAD-pNA (acetyl-Tyr-Val-Ala-Asp p-nitroanilide) substrate into pNA. The frozen hippocampi were homogenized in ice-cold RIPA buffer containing 1 mM PMSF protease inhibitor. The hippocampus lysates were centrifuged at 20000 *g* for 10 minutes at  $4^{\circ}\text{C}$  and supernatants were collected. The total protein concentrations were determined with the Bradford assay kit (product no.: P0006) from Beyotime Institute of Biotechnology (Nantong, Jiangsu, China). The hippocampus supernatants were incubated in a 96-well microplate with 10  $\mu\text{L}$  acetyl-Tyr-Val-Ala-Asp p-nitroanilide (2 mM) for 2 hours at  $37^{\circ}\text{C}$  according to the manufacturer's instructions. The absorbance values of pNA at 405 nm ( $\text{OD}_{405}$ ), which indicated activation of caspase-1, were detected by Synergy H1 Hybrid Microplate Reader (BioTek Instruments, Inc., Winooski, VT). The activity of caspase-1 was determined by interpolation of the standard curve derived from serial dilutions of the standard pNA supplied with the assay kit. Final results were normalized to the total protein accordingly. Data were presented as relative activity of caspase-1 of the Control group.

### Detection of IL-1 $\beta$ Levels

IL-1 $\beta$  levels in serum and hippocampus supernatant were determined with Bio-Plex Pro Mouse Cytokine Assay (Bio-Rad Laboratories Inc., Hercules, CA). This assay was a well-validated fluorescent magnetic bead-based immunoassay with a

sensitivity of 0.2 pg/mL (range 0.2-3200 pg/mL) for serum and of 2.0 pg/mL (range 2-32000 pg/mL) for tissue supernatant (Juul et al., 2008; Mukherjee et al., 2011; Jafarinaveh et al., 2014). The assay was carried out according to the manufacturer's instructions. Briefly, the frozen hippocampi were homogenized in ice-cold RIPA buffer containing 1 mM PMSF protease inhibitor (Beyotime Institute of Biotechnology, Nantong, Jiangsu, China). The supernatants were collected after the hippocampus lysates were centrifuged at 10000 *g* for 15 minutes at  $4^{\circ}\text{C}$ . The total protein concentrations of supernatants were determined with the Bradford assay kit (Beyotime Institute of Biotechnology, Nantong, Jiangsu, China). Premixed coupled magnetic beads were transferred to the 96-cell filter plate and washed twice with Bio-Plex wash buffer. The hippocampus supernatant samples were diluted 1:2 in the Bio-Plex sample diluent, while the serum samples were undiluted. Diluted standards and samples were added to each well of the filter plate containing coupled beads. The plate was incubated at room temperature for 30 minutes on shaker at low speed (300 rpm). After incubating and washing, prepared detection antibodies were added to each well. The plate was incubated at room temperature for 30 minutes on shaker at low speed (300 rpm). After incubating and washing, prepared streptavidin-PE was added to each well. The plate was incubated at room temperature for 10 minutes on shaker at low speed (300 rpm). After washing 3 times, the beads were suspended in 125  $\mu\text{L}$  of Bio-Plex assay buffer. Beads were read on the Bio-Plex system, and the data were analyzed using Bio-Plex Manager software 6.0 with the help of an experienced technician from Bio-Rad Laboratories (Shanghai) Co., Ltd. (Shanghai, China). The final results of hippocampal IL-1 $\beta$  levels were normalized to the total protein of each hippocampal supernatant sample accordingly.

### Statistical Analysis

The data were presented as mean  $\pm$  SEM and differences considered statistically significant at  $P < .05$ . Data were analyzed with SPSS 17.0 for Windows (SPSS Science, Chicago, IL). Kolmogorov-Smirnov test and Levene's test were performed before comparisons. Statistical significance was determined with the unpaired Student's *t* test for 2 sets of data comparison. Multiple groups' data were analyzed with 1-way ANOVA test and followed by Fisher's least significant difference test for posthoc comparisons.

## Results

### CMS Increased Serum Corticosterone Levels and Induced Depressive-Like Behaviors

Serum levels of stress hormone corticosterone increased significantly after mice were subjected to 4 weeks CMS exposure ( $t = 2.612$ ,  $P < .05$ ) (Figure 1a), which marked activation of the HPA axis and implied depressive symptoms. We performed a sucrose preference test and tail suspension test to determine depressive status of mice. Mice that were exposed to CMS displayed depressive-like behaviors, such as lower sucrose preference percentage ( $t = 3.179$ ,  $P < .01$ ; Figure 1b) and longer immobility time in tail suspension test ( $t = -2.482$ ,  $P < .05$ ; Figure 1c) compared with nontreated Control group mice.

### CMS Increased Active IL-1 $\beta$ Levels in Hippocampi

IL-1 $\beta$  had critical roles in both hippocampal neurogenesis suppression and neuroinflammation, which were 2 important

pathophysiological mechanisms of depression. Expression of the inactive pro-IL-1 $\beta$  could be induced by proinflammatory stimuli like TLRs, while maturation and release of active IL-1 $\beta$  were regulated by the inflammasomes. We tested protein levels of pro-IL-1 $\beta$  and biologically active IL-1 $\beta$  in mice hippocampi with Western blot. The Stress group showed a significant decrease of pro-IL-1 $\beta$  protein levels ( $t = 7.369, P < .01$ ) and a significant increase of active IL-1 $\beta$  protein levels ( $t = 5.214, P < .05$ ) compared with their respective controls (Figure 1d-e). Maturation and secretion of active IL-1 $\beta$  in mice hippocampi were regarded as a result of activation of caspase-1, which was tightly regulated by the NLRP3 inflammasome.

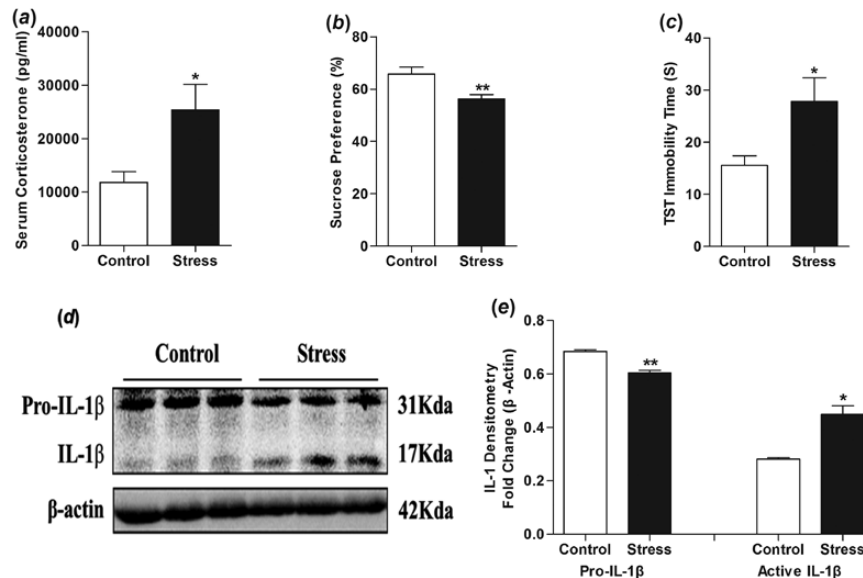
### CMS Activated Hippocampal NLRP3 Inflammasome

A significant increase of active IL-1 $\beta$  protein in the Stress group mice suggested activation and expression of the NLRP3 inflammasome in hippocampi. We performed a caspase-1 activity assay and determined protein levels of NLRP3 and ASC in hippocampi with Western blot. As we expected, there was a significant increase of caspase-1 activity in the hippocampi of

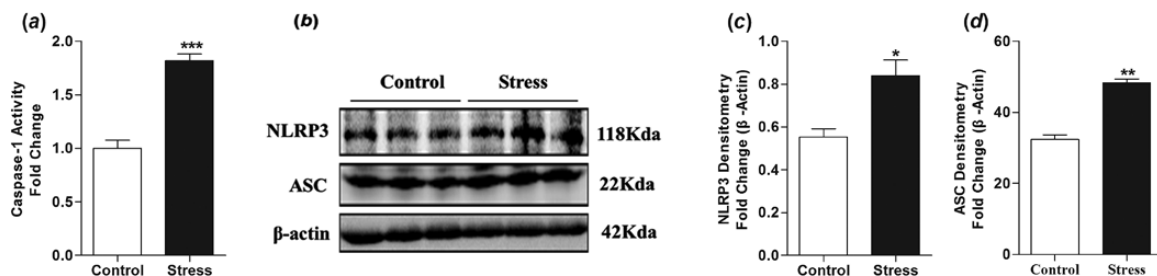
Stress group mice ( $t = 8.448, P < .001$ ) (Figure 2a). Increased caspase-1 catalytic activity indicated autoactivation of caspase-1 within the NLRP3 inflammasome, which was a result of activation of the multiprotein complex. The other 2 components of NLRP3 inflammasome including NLRP3 receptor ( $t = 3.454, P < .05$ ) (Figure 2b-c) and adapter protein ASC ( $t = 9.353, P < .01$ ) (Figure 2b, d) also had significant higher expression levels in the Stress group mice compared with nontreated Control group. Increased active IL-1 $\beta$  and caspase-1 catalytic activity in combination with higher expression of inflammasome components NLRP3 and ASC in mice hippocampi indicated that the NLRP3 inflammasome involved in CMS-induced depression.

### VX-765 Inhibited Activation and Expression of the NLRP3 Inflammasome

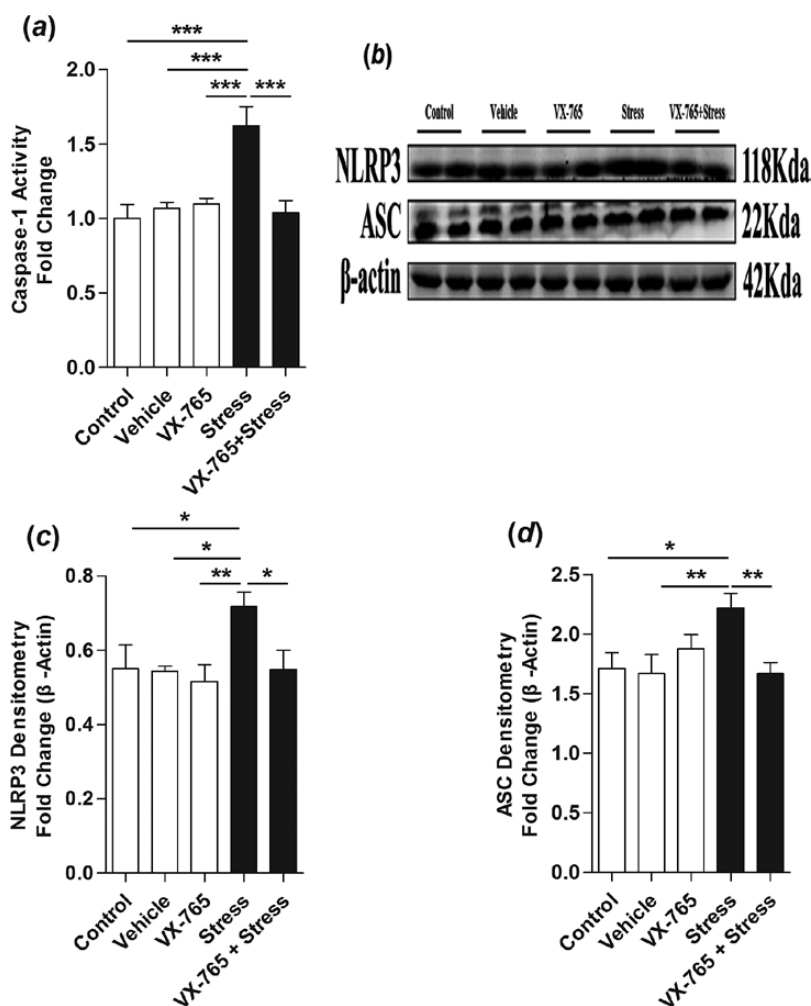
To further investigate the functional role of NLRP3 inflammasome in depression, we used VX-765 as the specific and potent inflammasome inhibitor. The activation of caspase-1 in the hippocampi of Stress group mice was successfully inhibited by VX-765 ( $F = 9.369, P < .001$ ) (Figure 3a) compared with



**Figure 1.** Measurement of serum corticosterone levels, depressive-like behaviors, hippocampal pro-interleukin (IL)-1 $\beta$ , and active IL-1 $\beta$  levels. (a) Chronic mild stress (CMS) increased serum corticosterone levels ( $n = 6, *P < .05$  vs Control). (b) The stressed mice had lower sucrose preference percentage in sucrose preference test ( $n = 10-11, **P < .01$  vs Control). (c) The stressed mice showed longer immobility time in tail suspension test ( $n = 6-8, *P < .05$  vs Control). (d) Western blot was used to determine protein levels of pro-IL-1 $\beta$  and active IL-1 $\beta$ . (e) CMS decreased pro-IL-1 $\beta$  levels and increased hippocampal active IL-1 $\beta$  levels ( $n = 3, *P < .05, **P < .01$  vs Control). Data were presented as mean  $\pm$  SEM.



**Figure 2.** Activation and expression of the NLRP3 inflammasome after chronic mild stress (CMS) exposure. (a) The activity of hippocampal caspase-1 significantly increased after mice were subjected to 4 weeks of CMS ( $n = 4, ***P < .001$  vs Control). (b) Western blot was used to determine protein levels of NLRP3 and apoptotic speck-containing protein with a card (ASC) in the hippocampi. (c) Protein expression levels of NLRP3 increased significantly in the hippocampi of CMS-exposed mice ( $n = 3, *P < .05$  vs Control). (d) Protein expression levels of ASC also increased significantly in the hippocampi of CMS-exposed mice ( $n = 3, **P < .01$  vs Control). Data were presented as mean  $\pm$  SEM.



**Figure 3.** Inhibition of the NLRP3 inflammasome by VX-765. (a) The NLRP3 inflammasome inhibitor VX-765 significantly inhibited activation of hippocampal caspase-1 in the VX-765 + Stress group mice ( $n = 4$ ,  $***P < .001$  vs Stress). There were no significant differences among Control, Vehicle, VX-765, and VX-765 + Stress groups. (b) Western blot was used to determine protein levels of NLRP3 and apoptotic speck-containing protein with a card (ASC) in the hippocampus. (c) The protein expression levels of NLRP3 decreased significantly in the hippocampi of VX-765 + Stress group mice ( $n = 4$ ,  $*P < .05$  vs Stress). There were no significant differences among Control, Vehicle, VX-765, and VX-765 + Stress groups. (d) The protein expression levels of ASC also decreased significantly in the hippocampi of VX-765 + Stress group mice ( $n = 4$ ,  $**P < .01$  vs Stress). There were no significant differences among Control, Vehicle, VX-765, and VX-765 + Stress groups. Data were presented as mean  $\pm$  SEM.

VX-765 + Stress group mice. Meanwhile, the NLRP3 ( $F = 3.109$ ,  $P < .05$ ) (Figure 3b-c) and ASC ( $F = 3.474$ ,  $P < .05$ ) (Figure 3b, d) protein expression levels also decreased significantly in the hippocampi of VX-765 + Stress group mice compared with their respective Stress group. There were no significant differences among Control, Vehicle, VX-765, and VX-765 + Stress group mice (Figure 3).

### VX-765 Decreased Protein Levels of Serum and Hippocampal IL-1 $\beta$

We measured serum and hippocampal IL-1 $\beta$  levels to determine if the inflammasome IL-1 $\beta$  signaling pathway was successfully blocked by VX-765 administration. First, we observed that mice with 4 weeks of CMS exposure exhibited a significant increase of IL-1 $\beta$  protein levels in their serum ( $F = 6.987$ ,  $P < .001$ ) (Figure 4a) and hippocampus ( $F = 2.058$ ,  $P < .05$ ) (Figure 4b) when compared with their respective Control group. Next, we found that the inflammasome inhibitor VX-765 significantly decreased serum ( $F = 6.987$ ,  $P < .001$ ) (Figure 4a) and hippocampal ( $F = 2.058$ ,  $P < .05$ ) (Figure 4b) IL-1 $\beta$  levels in the VX-765 + Stress group mice when compared with their respective Stress group. There were

no significant differences among Control, Vehicle, VX-765, and VX-765 + Stress group mice (Figure 4a-b).

### VX-765 Improved CMS-Induced Depressive-Like Behaviors

If the NLRP3 inflammasome mediated CMS-induced depression, the depressive symptoms should be mitigated when the inflammasome-IL-1 $\beta$  pathway was successfully blocked. Indeed, the VX-765 + Stress group mice had significantly higher sucrose preference percentage ( $F = 5.215$ ,  $P < .001$ ) (Figure 4c) and shorter immobility time in the tail suspension test ( $F = 3.673$ ,  $P < .01$ ) (Figure 4d) compared with their respective Stress group. Mice exhibited no significant differences among Control, Vehicle, VX-765, and VX-765 + Stress group mice in the sucrose preference test and tail suspension test (Figure 4c-d).

## Discussion

We first found that the NLRP3 inflammasome was involved in LPS-induced depression in our previous study (Zhang et al., 2014). The present study further demonstrated the central role

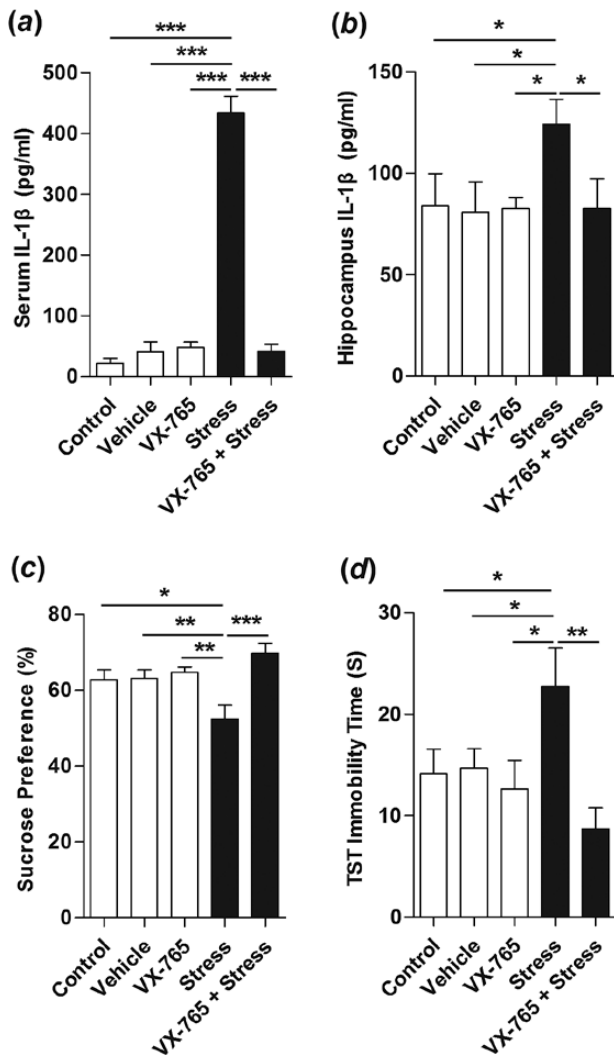


Figure 4. NLRP3 inflammasome inhibitor VX-765 decreased serum and hippocampal interleukin (IL)-1 $\beta$  levels and improved chronic mild stress (CMS)-induced depressive-like behaviors. (a) The protein levels of IL-1 $\beta$  in serum of VX-765 + Stress group mice were significantly reduced ( $n = 5$ ,  $***P < .001$  vs Stress). There were no significant differences among Control, Vehicle, VX-765, and VX-765 + Stress groups. (b) The protein levels of IL-1 $\beta$  in the hippocampi of VX-765 + Stress group mice were also significantly reduced ( $n = 7$ ,  $*P < .05$  vs Stress). There were no significant differences among Control, Vehicle, VX-765, and VX-765 + Stress groups. (c) The VX-765 + Stress group mice had higher sucrose preference percentage ( $n = 7-9$ ,  $***P < .001$  vs Stress). There were no significant differences among Control, Vehicle, VX-765, and VX-765 + Stress groups. (d) The VX-765 + Stress group mice displayed shorter immobility time in the tail suspension test ( $n = 8-10$ ,  $**P < .01$  vs Stress). There were no significant differences among Control, Vehicle, VX-765, and VX-765 + Stress groups. Data were presented as mean  $\pm$  SEM.

of NLRP3 inflammasome in stress-induced depressive behaviors and its underlying mechanism.

Stress had long been considered as an important contributing factor in the pathology of depression. In the recent decades, the increased stress levels of our daily lives have resulted in a growing prevalence of depression. Our findings that after 4 weeks of unpredictable CMS mice exhibited depressive-like behaviors (Figure 1b-c) along with significantly increased serum corticosterone levels (Figure 1a) were consistent with other studies in rodents (Goshen et al., 2008; Peng et al., 2012; Lu et al., 2014).

There is evidence that psychological and physical stressors could activate immune and inflammation processes, contributing to the development of depression (Iwata et al., 2013). Proinflammatory cytokine IL-1 $\beta$  in prefrontal cortex of depressive rats was implicated in the pathophysiology of depression (Kupfer et al., 2012; Pan et al., 2014). The HPA axis dysregulation was suggested as the underlying mechanism of the involvement of IL-1 $\beta$  in depression, while IL-1 $\beta$  was indicated to influence all levels of the HPA axis (Goshen et al., 2008).

Hippocampal neurogenesis was a critical biological and cellular pathogenesis of depression. Lu and colleagues (2014) found microglia activation and elevation of IL-6, IL-1 $\beta$ , and TNF- $\alpha$  levels in the hippocampus of CMS-exposed mice. Antidepressant drugs could increase hippocampal neurogenesis, whereas IL-1 $\beta$  could mediate CMS-induced depression via inhibiting hippocampal neurogenesis (Goshen et al., 2008; Kheirbek et al., 2012). Therefore, we focused on examining the hippocampus instead of other brain regions such as the prefrontal cortex. We found that CMS exposure could induce elevation of IL-1 $\beta$  protein levels in mouse serum (Figure 4a) and hippocampi (Figure 4b), which was in line with the increase of peripheral IL-1 $\beta$  levels in individuals who were stressed and depressed, as well as other rodents depression studies (Goshen et al., 2008; Lu et al., 2014). More specifically, we illustrated there was a significant decrease of pro-IL-1 $\beta$  protein levels, accompanied with significantly increased active IL-1 $\beta$  protein levels in the hippocampi of CMS-exposed mice (Figure 1d-e).

Pro-IL-1 $\beta$  was induced by priming signals such as TLR agonist LPS or proinflammatory cytokine TNF, which could activate the NF- $\kappa$ B transcription factor leading to activation of the IL-1 $\beta$  promoter (Schroder and Tschopp, 2010). The priming signals could also potentiate NLRP3 inflammasome activity through NF- $\kappa$ B-dependent induction of NLRP3 expression, which was a necessary but not sufficient checkpoint, because a second stimulus such as adenosine triphosphate was required for NLRP3 inflammasome activation (Bauernfeind et al., 2009; Juliana et al., 2010). Pro-IL-1 $\beta$  was processed to the mature, active form of IL-1 $\beta$  by the NLRP3 inflammasome (Jin and Flavell, 2010). Based on several lines of evidence, the NLRP3 inflammasome was postulated as a bridge between stress and depression (Iwata et al., 2013). First, stress led to generation of reactive oxygen species and adenosine triphosphate, which were key activators of the NLRP3 inflammasome (Schroder and Tschopp, 2010; Iwata et al., 2013). Second, IL-1 $\beta$  exposure was widely reported to cause depressive behaviors, including anhedonia (a core symptom of depression) and suppression of locomotor activity and social exploration (Goshen et al., 2008; Iwata et al., 2013). Moreover, the present study found that the NLRP3 inflammasome was expressed and activated after 4 weeks of CMS exposure (Figure 2), which was consistent with our previous study and other reports (Iwata et al., 2013; Lu et al., 2014; Pan et al., 2014; Zhang et al., 2014).

This evidence suggested that the NLRP3 inflammasome was involved in stress-induced depression and probably had an important role between stress and depression. To further demonstrate this hypothesis and illustrate the underlying mechanism, we need to block the inflammasome signaling and explore its functional impact on stress-induced depression. Several clinical studies are exploring the modification of inflammasome signaling pathways: (1) those to target the upstream signaling, such as P2X7R antagonists, sulfonylurea drugs, antioxidants, and urate-lowering therapy; and (2) those to target the downstream signaling, including caspase-1 inhibitors, anti-IL-1 $\beta$  therapy, and recombinant IL-1Ra (Lopez-Castejon and Pelegrin, 2012).

We used a highly potent and selective caspase-1 inhibitor VX-765 to reduce production of IL-1 $\beta$  by blocking the NLRP3 inflammasome signaling (Maroso et al., 2011). Our study results confirmed that expression and activation of the NLRP3 inflammasome were effectively inhibited by the inflammasome inhibitor VX-765 (Figure 3). As a result of the inflammasome signaling inhibition, the stress-induced abundant production of serum and hippocampi IL-1 $\beta$  protein levels was successfully inhibited via VX-765 administration (Figure 4a-b).

The inhibitory effect of VX-765 on NLRP3 protein and ASC protein expression could be due to a negative feedback on the activation step of the NLRP3 inflammasome or might be due to other mechanisms such as interfering with signaling pathways involved in the priming step of NLRP3 inflammasome activation (Juliana et al., 2010). The underlying mechanisms of inhibitory effect of VX-765 on NLRP3 inflammasome protein expression need further research and clarification.

The underlying mechanisms of the CMS-induced increase of IL-1 $\beta$  levels in serum were unclear. It could be attributed to activation of the NLRP3 inflammasome in periphery, as Alcocer-Gomez et al. (2014) found activation of the NLRP3 inflammasome in mononuclear blood cells from major depressive disorder patients, or it might due to the bidirectional brain-immune interactions.

The bidirectional interactions between the brain and immune system could take place in different organs or on various levels (Denes and Miyan, 2014). There were several mechanisms by which peripheral proinflammatory cytokines such as IL-1 $\beta$  and TNF- $\alpha$  could affect the CNS and induce sickness response, including: 1) saturable transportation of cytokines into the brain, 2) leaking through cerebral endothelial or interacting with circumventricular organs lacking the blood-brain barrier, and 3) activation of afferent neurons of the vagus nerve (Hosoi et al., 2002). On the other hand, mood disorders, brain injuries, and stress were linked with development of systemic inflammatory conditions (Denes and Miyan, 2014). Matsunaga et al. (2013) suggested that the neural networks might regulate the secretion of peripheral proinflammatory cytokines.

Chronic life stressors caused elevation of brain IL-1 $\beta$  levels, which probably played a critical role in the induction of depression, because IL-1 $\beta$  was causally related to various aspects of depression (Goshen et al., 2008). If the NLRP3 inflammasome was a key mediator of stress and depression, mice with impaired inflammasome signaling would exhibit fewer or no effects after 4 weeks of stress exposure. As we predicted, mice with blocked inflammasome-IL-1 $\beta$  cascade by VX-765 displayed no depressive behaviors following 4 weeks of CMS regimen (Figure 4c-d). These results were in line with previous studies that demonstrated inhibition of the NLRP3 inflammasome could significantly ameliorate depressive symptoms (Lu et al., 2014; Zhang et al., 2014). The functional impact of NLRP3 inflammasome in depression could be further explored with NLRP3 knockout mice (Iwata et al., 2013).

In conclusion, the findings of our present study suggested that the NLRP3 inflammasome was involved in the stress-induced depression via regulating the production of IL-1 $\beta$  protein in serum and hippocampus, and the NLRP3 inflammasome-IL-1 $\beta$  axis had pivotal roles in the pathophysiology and behavioral symptomatology of stress-induced depression. The inflammasome inhibitor VX-765 effectively ameliorated depressive symptoms of mice and suggested novel therapeutic targets for treating depression. Our findings were consistent with the inflammasome hypothesis of depression that postulated stress could activate the NLRP3 inflammasome, leading to IL-1 $\beta$  release, and contribute to the development of depression. Moreover, our

findings further illustrated this inflammasome hypothesis of depression with explicit experiment results and suggested that interventions targeting the inflammasome-IL-1 $\beta$  signaling would provide promising strategies to alleviate depression in the future.

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## Interest Statement

None.

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