Priority Communication

Biological Psychiatry

Psychological Stress Activates the Inflammasome via Release of Adenosine Triphosphate and Stimulation of the Purinergic Type 2X7 Receptor

Masaaki Iwata, Kristie T. Ota, Xiao-Yuan Li, Fumika Sakaue, Nanxin Li, Sophie Dutheil, Mounira Banasr, Vanja Duric, Takehiko Yamanashi, Koichi Kaneko, Kurt Rasmussen, Andrew Glasebrook, Anja Koester, Dekun Song, Kenneth A. Jones, Stevin Zorn, Gennady Smagin, and Ronald S. Duman

ABSTRACT

BACKGROUND: The mechanisms underlying stress-induced inflammation that contribute to major depressive disorder are unknown. We examine the role of the adenosine triphosphate (ATP)/purinergic type 2X7 receptor (P2X7R) pathway and the NLRP3 (nucleotide-binding, leucine-rich repeat, pyrin domain containing 3) inflammasome in interleukin (IL)-1β and depressive behavioral responses to stress.

METHODS: The influence of acute restraint stress on extracellular ATP, glutamate, IL-1β, and tumor necrosis factor alpha in hippocampus was determined by microdialysis, and the influence of acute restraint stress on the NLRP3 inflammasome was determined by western blot analysis. The influence of P2X7R antagonist administration on IL-1β and tumor necrosis factor alpha and on anxiety and depressive behaviors was determined in the chronic unpredictable stress rodent model. The role of the NLRP3 inflammasome was determined by analysis of *Nlrp3* null mice.

RESULTS: Acute restraint stress rapidly increased extracellular ATP, an endogenous agonist of P2X7R; the inflammatory cytokine IL-1β; and the active form of the NLRP3 inflammasome in the hippocampus. Administration of a P2X7R antagonist completely blocked the release of IL-1β and tumor necrosis factor alpha, another stress-induced cytokine, and activated NLRP3. Moreover, P2X7R antagonist administration reversed the anhedonic and anxiety behaviors caused by chronic unpredictable stress exposure, and deletion of the *Nlrp3* gene rendered mice resistant to development of depressive behaviors caused by chronic unpredictable stress.

CONCLUSIONS: These findings demonstrate that psychological "stress" is sensed by the innate immune system in the brain via the ATP/P2X7R–NLRP3 inflammasome cascade, and they identify novel therapeutic targets for the treatment of stress-related mood disorders and comorbid illnesses.

Keywords: ATP, Depression, IL-1β, NLRP3 inflammasome, P2X7, Stress

http://dx.doi.org/10.1016/j.biopsych.2015.11.026

Approximately 17% of the population is affected by major depressive disorder (MDD), resulting in devastating personal, social, and economic consequences. Imaging studies demonstrate decreased volume of brain regions implicated in depression, including the hippocampus and prefrontal cortex (PFC), whereas atrophy of neurons and glia has been reported in postmortem samples from depressed subjects and rodent chronic stress models (1). Although the mechanisms underlying these effects are not well known, these findings indicate that protection against these damaging effects of stress and depression could have therapeutic benefit. There is strong evidence that MDD is associated with inflammation, demonstrated by high levels of proinflammatory cytokines in blood samples of patients with MDD (2). There are also high rates of comorbidity of MDD with systemic inflammatory diseases, including diabetes, cancer, stroke, and rheumatoid arthritis (3). Psychological stress can increase peripheral inflammation in humans (4,5), including elevated levels of interleukin (IL)-1β in blood of patients with MDD (6).

Preclinical studies have demonstrated that chronic stress leads to activation of the peripheral and central innate immune system and that increased inflammatory cytokines contribute to depressive behaviors (7–10). We have shown that stress and IL-1β decrease neurogenesis in the adult hippocampus, and this effect is associated with the development of anhedonia (11). Administration of an IL-1β receptor antagonist abolishes both the antineurogenic and the anhedonic behavioral effects of chronic unpredictable stress (CUS) (11,12), and IL-1 receptor deletion mutant mice display reduced anxiety (13). However, the mechanisms underlying the ability of stress

SEE COMMENTARY ON PAGE 4

 ISSN: 0006-3223

to increase IL-1β and inflammatory responses in brain have not been determined. Studies of peripheral immune cells demonstrate that the inflammatory response to danger substances involves two major steps: 1) toll-like receptor mediated induction of NLRP3 (nucleotide-binding, leucine-rich repeat, pyrin domain containing 3) expression and 2) adenosine triphosphate (ATP)/purinergic type 2X7 receptor (P2X7R)–induced oligomerization of NLRP3 with an adaptor protein (ASC [apoptosis-associated speck-like protein containing a CARD]) and pro-caspase-1 (14,15). The resulting multiprotein complex termed the inflammasome cleaves pro-caspase-1 to mature caspase-1, which cleaves pro-IL-1β to mature IL-1β (16); in brain, this occurs primarily in resident microglia as well as invasive macrophages. P2X7R is an ionotropic receptor located predominantly on microglia and macrophages and is activated in response to cellular danger signals, such as ATP (16). The *P2X7R* gene is located within a region on chromosome 12q24.31, which has been identified as a susceptibility locus for affective disorders (17).

In the present study, we used rodent models of stress and depression to identify the pathways by which psychological stress increases IL-1β and the resulting neurogenic and anhedonic responses. We show that stress rapidly increases glutamate, which releases ATP as a gliotransmitter from astrocytes. ATP activates P2X7R and releases IL-1β with subsequent induction of tumor necrosis factor alpha (TNFα) via activation of the NLRP3 inflammasome. We confirmed that these biological responses result in cellular and behavioral deficits by showing that a P2X7R antagonist, which blocks the release of IL-1β and TNFα, blocks the cellular and behavioral deficits caused by stress.

METHODS AND MATERIALS

Animals

Male Sprague Dawley rats (Charles River Laboratories, Wilmington, Massachusetts) and male *Nlrp3* deletion mutant mice (obtained from Dr. N. Fasel, University of Lausanne, Switzerland) (18) were used. All procedures were approved by the Yale University Animal Care and Use Committee.

Reagents

The P2X7R antagonist A-804598 (10 mg/kg, intraperitoneal) (provided by Lundbeck Research USA, Paramus, New Jersey) was dissolved in 10% dimethylsulfoxide and 90% polyethylene glycol 400; bromodeoxyuridine (BrdU) (150 mg/kg, intraperitoneal; Sigma-Aldrich, St. Louis, Missouri) was dissolved in saline. C28 is a mouse IgG1 anti-IL-1β antibody (provided by Eli Lilly and Company, San Diego, California) that neutralizes mouse IL-1β; C28 was administered subcutaneously at a dose of .2 mg/mouse every 4 days. Rats display mild transient discomfort after administration of 10% dimethylsulfoxide/90% polyethylene glycol 400.

Immobilization Stress

Rats were restrained in tapered plastic film tubes (DecapiCones; Braintree Scientific, Inc., Braintree, Massachusetts) for 1 hour.

CUS Model

The CUS model is a rodent model of depression in which rats (19) or mice (20) were exposed to a variable sequence of two mild and unpredictable stressors per day preventing habituation as previously described (19,20). The stressors include cage tilt, light off, light on, crowd, odor, cold stress, no bedding, wet bedding, isolation, food and water deprivation, stroboscope, forced swim, cage rotation, and immobilization (IMM) stress (Supplemental Tables S1 and S2). These stressors were adapted from our previous study. Control rats were handled every day.

Astrocyte Culture

Astrocyte primary cell cultures were prepared from neonatal rats, as described by Kajitani *et al.* (21).

Stereotactic Surgery and Microdialysis

Animals were anesthetized with isoflurane and held in a stereotactic frame, and a guide cannula was implanted into the hippocampus (coordinates: anteroposterior −5.3, dorsolateral −4.8 from bregma, ventral −4.0 from the skull surface) or PFC (coordinates: +3.4, −.8, −1.0). After 7 days of recovery, a microdialysis probe was inserted. We used a 4-mm-long microdialysis probe (polyarylethersulphone, 100 kDa molecular weight cutoff; BrainLink B.V., Groningen, The Netherlands) for collecting ATP and glutamate, and high cutoff probe (polyethylene, 3 MDa molecular weight cutoff) for collecting IL-1β and TNFα, equipped with an infusion syringe pump (PHD 2000; Harvard Apparatus, Holliston, Massachusetts) and refrigerated fraction collector (CMA 470; CMA Microdialysis AB, Solna, Sweden). Probes were attached to microbore tubing traveling through a microdialysis swivel and head tether assembly (Instech Laboratories, Inc., Plymouth Meeting, Pennsylvania) that allowed animal movement around the cage. The microdialysis perfusion fluid consisted of artificial central nervous system fluid (Harvard Apparatus), which was delivered at a rate of 3 μL/min, 15-minute intervals for ATP and glutamate analysis, and 1.5 μL/min, 60-minute intervals for IL-1β and TNFα analysis.

ATP Bioluminescence Assay and Liquid Chromatography/Tandem Mass Spectrometry for Glutamate

The ATP levels were measured by the ATP Bioluminescence Assay Kit CLS II (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instruction. Concentrations of glutamate were determined by high-performance liquid chromatography combined with tandem mass spectrometry detection using an internal standard.

Multiplex Electrochemiluminescence Assay

A multiplex electrochemiluminescence assay was performed using custom multiplex plates for detection of IL-1β and TNFα (Meso Scale Discovery, Rockville, Maryland) according to the manufacturer's standard protocol. The signal was detected by a SECTOR Imager 6000 (Meso Scale Discovery) with DISCOVERY WORKBENCH software (Meso Scale Discovery).

Analysis of BrdU Incorporation: DNA Dot Blot Assay

DNA dot blot assay is an approach described by Wu and Castren (22) to detect the concentration of DNA that incorporates BrdU as a marker of cell proliferation.

Co-immunoprecipitation

The NLRP3 inflammasome was co-immunoprecipitated using Dynabeads Co-Immunoprecipitation Kit (Life Technologies Corporation, Grand Island, New York) according to the manufacturer's instructions. Anti-ASC antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, California) or control IgG (Santa Cruz Biotechnology, Inc.) was incubated with Dynabeads M-270 Streptavidin (Life Technologies Corporation). Anti-ASC antibody was supposed to immunoprecipitate ASC, NLRP3, and caspase-1.

Western Blotting

Western blotting was conducted in our laboratory as previously described (23). The positive and negative controls for NLRP3 were, respectively, HEK293 cell lysate transfected and untransfected with mouse NLRP3 (Novus Biologicals, LLC, Littleton, Colorado). Immunoblotting was performed with anti-NLRP3 antibody (1:1000; Adipogen Corporation, San Diego, California).

Behavioral Tests

Animals were subjected to behavioral testing using vehicle-controlled experiments and were analyzed by experimenters blinded to the identity of the groups. The sucrose preference test (24), novelty suppressed feeding test (NSFT) (24), elevated plus maze (EPM) (25), open field test (OFT) (23), sucrose consumption test (20), and forced swim test (FST) (24) were conducted as previously described in our laboratory. Briefly, in the sucrose preference test, animals were habituated for 48 hours to 1% sucrose; following a 4-hour deprivation period, preference for sucrose or water was determined for 1 hour. The NSFT was performed after 24 hours of food deprivation. Rats were placed in an open field (76.5 cm × 76.5 cm) with a small amount of food in the center, and latency to feed was determined. The EPM consisted of two open arms (50 cm × 10 cm for rats, 30 cm × 5 cm for mice) and two closed arms (50 cm × 10 cm × 40 cm for rats, 30 cm × 5 cm × 30 cm for mice). The frequency and time spent in the open arms and closed arms were measured. In the OFT, animals were placed in the open field (76.5 cm × 76.5 cm for rats, 50 cm × 50 cm for mice) and allowed to explore for 10 minutes. Time and distance in the center zone were measured using the ANY-maze tracking system (Stoelting Co., Wood Dale, Illinois). In the sucrose consumption test, mice were habituated to 1% sucrose for 48 hours, then overnight sucrose intake was measured. In the FST, rats were placed for 10 minutes in a clear cylinder with water (24°C, 45 cm deep). Video-recorded sessions were scored for immobility time.

Statistical Analyses

Statistical differences were determined by analysis of variance (StatView 5.0; SAS Institute, Inc., Cary, North Carolina) followed by Fisher's protected least significant difference post hoc analysis (three or more groups) or Student *t* test (two groups). Using pre-established guidelines, values were excluded from analysis if they were >2 SD from the mean. Statistical significance was set at $p < .05$. For microdialysis results, GraphPad Prism version 4.02 (GraphPad Software, Inc., San Diego, California) or MATLAB (The Mathworks, Inc., Natick, Massachusetts) was used for statistical analysis. All data sets were tested for normality using the Lilliefors test. In cases where data were normally distributed, outliers were eliminated using Peirce's criterion (26). In cases where data were not normally distributed, significance testing was conducted using the Kruskal-Wallis nonparametric analysis of variance with Holm-Bonferroni corrected Mann-Whitney *U* post hoc tests where appropriate.

RESULTS

Psychological Stress Increases Extracellular ATP in Hippocampus: Role of Glutamate

One of the main sources of ATP acting on microglial P2X7R is damaged or dying cells, but ATP is also a neurotransmitter (27). However, it is unknown whether psychological stress increases extracellular ATP in the brain. To address this question, we examined the influence of IMM stress (1 hour) on extracellular ATP in the hippocampus using in vivo microdialysis (see Supplemental Figure S1A for location of probe). The results show a biphasic ATP response with extracellular ATP rapidly increasing within the first 15 minutes of stress, falling, and then increasing again at the end of stress exposure (Figure 1A). The release of ATP from glia could be controlled by the excitatory neurotransmitter glutamate, which is stimulated by stress (28–30). In vivo microdialysis demonstrates that IMM stress rapidly increases glutamate in the hippocampus (Figure 1B). To directly test the ability of glutamate to stimulate astrocytes to release ATP, we used rat astrocyte primary cell cultures. The results demonstrate that glutamate (100 μmol/L) rapidly stimulates ATP release into the media (20–60 seconds) (Figure 1C), consistent with our hypothesis that astrocytes respond to glutamate by stimulating ATP release.

Psychological Stress Increases Extracellular IL-1β and TNFα: Blockade by ATP/P2X7R Antagonist

We established an in vivo microdialysis approach to assess extracellular levels of IL-1β and TNFα (Supplemental Figure S1A) and analyzed cytokine levels using a multiplex electrochemiluminescence assay (31) (Supplemental Figure S2A, B). The results demonstrate that IMM stress significantly increases IL-1β release in the hippocampus, peaking at 2 hours, a delayed time course relative to ATP (Figure 1D). The return to basal levels could be due to negative feedback mechanisms (32) or to depletion of IL-1β (16). In vitro studies demonstrate a similar rapid ATP induction of IL-1β release that is maximal within 10–20 minutes and no further release with continuous ATP exposure (33). The role of ATP and P2X7R in the regulation of IL-1β was directly tested using a potent and selective P2X7R antagonist, A-804598 (2-cyano-1-[(1S)-1-phenylethyl]-3-quinolin-5-ylguanidine) (34). A-804598 penetrates the brain, and pharmacokinetic studies show that drug concentrations are sufficiently high in both blood and brain tissue to significantly block P2X7R (Supplemental Figure S3A, B).

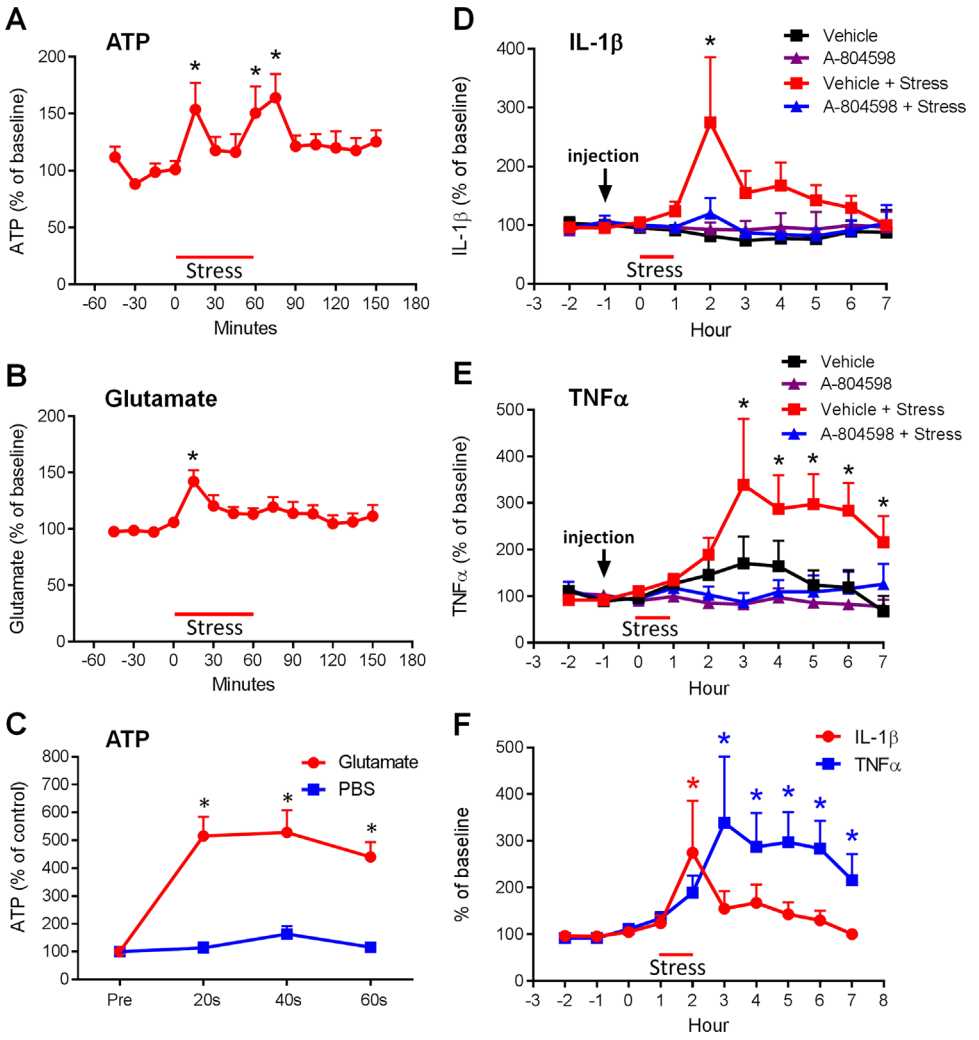

Figure 1. **(A–F)** Immobilization stress increases levels of glutamate, adenosine triphosphate (ATP), interleukin (IL)-1β, and tumor necrosis factor alpha (TNFα) in hippocampus microdialysates. Blockade by administration of a P2X7 receptor antagonist. **(A)** Rats were exposed to immobilization stress for 60 minutes as indicated (red line), and samples were collected by microdialysis and analyzed for levels of ATP ($n = 18$), **(B)** glutamate ($n = 19$), **(D)** IL-1β (Vehicle, $n = 7$; A-804598, $n = 3$; Vehicle + Stress, $n = 24$; A-804598 + Stress, $n = 9$), and **(E)** TNFα (Vehicle, $n = 7$, A-804598, $n = 3$; Vehicle + Stress, $n = 24$; A-804598 + Stress, $n = 9$) using a multiplex electrochemiluminescence assay, bioluminescence, and high-performance liquid chromatography combined with tandem mass spectrometry as described in Methods and Materials. Arrows indicate administration time of vehicle (polyethylene glycol 400 + dimethylsulfoxide) or P2X7 receptor antagonist, A-804598. The mean of three pretreatment microdialysis samples was used to define the baseline for each animal. **(F)** Immobilization stress increases levels of IL-1β and TNFα in microdialysates from hippocampus. The combined time course for each cytokine demonstrates that the increase in IL-1β precedes the increase in TNFα. In addition, levels of IL-1β immediately returned to baseline, whereas the increased TNFα levels persisted longer ($n = 24$ per group). **(C)** ATP levels in astrocyte cell culture stimulated by glutamate ($n = 9$ per group). Results are expressed as mean ± SEM. *$p < .05$ compared with control (vehicle) (Kruskal-Wallis nonparametric analysis of variance with Holm-Bonferroni corrected Mann-Whitney *U* post hoc tests where appropriate).

The results demonstrate that A-804598 (10 mg/kg) pretreatment (1 hour) completely suppresses the release of IL-1β (Figure 1D), confirming that IL-1β release in response to IMM stress occurs via ATP/P2X7R activation.

We also found that IMM stress significantly increases hippocampal levels of TNFα, another proinflammatory cytokine implicated in depression (7,35,36), and this effect is also blocked by pretreatment with A-804598 (Figure 1E). TNFα is not directly controlled by ATP/P2X7R, although it is possible that the blockade by A-804598 occurs indirectly via inhibition of IL-1β, which can stimulate the release of TNFα (37). Comparison of the time courses for levels of these two cytokines demonstrates that the release of IL-1β precedes the release of TNFα (Figure 1F).

The IMM stress also caused similar effects in the PFC, another region implicated in depression, including increased levels of ATP, IL-1β, and TNFα (Supplemental Figures S1B and S4A–C). The results are similar to the hippocampus, although there is a time lag in the release of ATP, suggesting that the induction of ATP and IL-1β in PFC may be secondary to the effects in hippocampus (Supplemental Figure S4A). The induction of IL-1β and TNFα in PFC is also blocked by pretreatment with A-804598 (Supplemental Figure S4B, C).

P2X7R Antagonist Treatment Blocks Cellular and Behavioral Effects of Stress

We next determined the influence of P2X7R antagonist administration on depression and anxiety behaviors, first in naïve, nonstressed rats. Administration of A-804598 did not significantly influence behavioral immobility in the FST or anxiety in the OFT and EPM (Supplemental Figure S5A–C). The lack of effect of P2X7R blockade could be due to the short-term, mild stress in these behavioral models, suggesting that more long-term stress activation of the inflammasome is required to cause depressive and anxiety behaviors. To address this issue, we used a CUS model (Figure 2A), which causes anhedonia and anxiety, core symptoms of MDD that are reversed by long-term administration of a typical

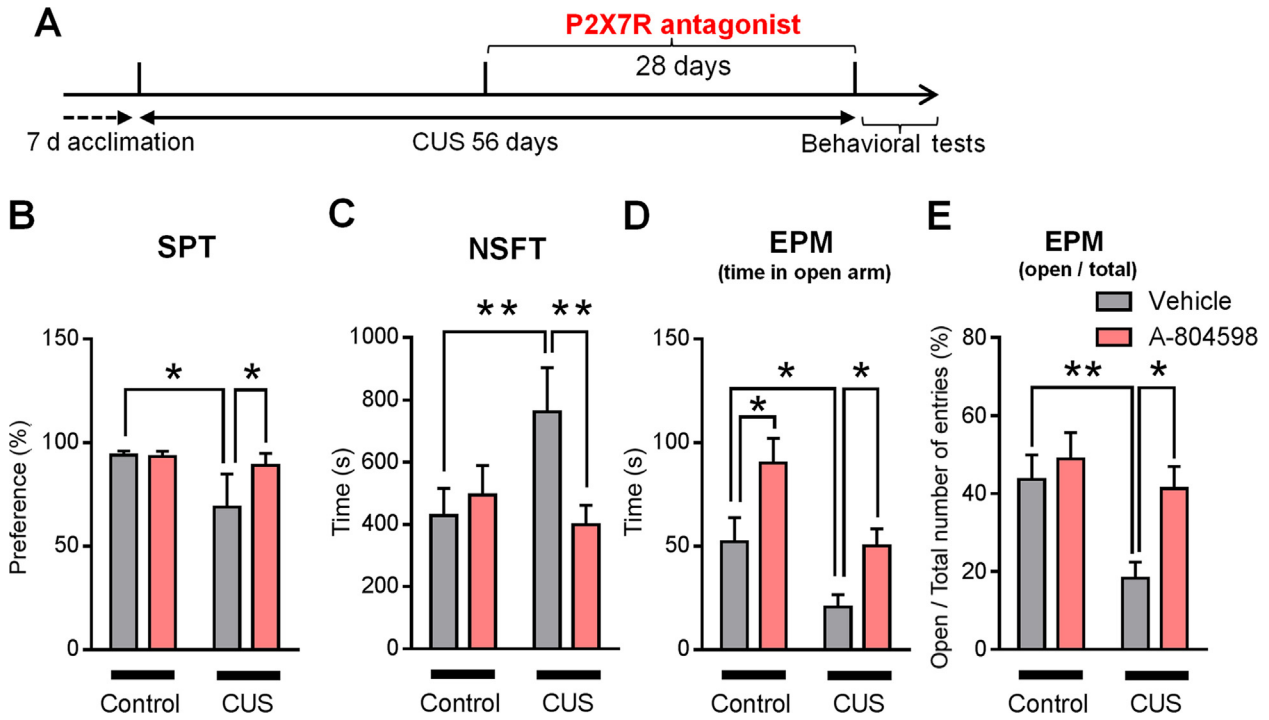

Figure 2. Influence of P2X7 receptor (P2X7R) antagonist administration on anxiety and depressive behaviors. **(A–E)** Influence of A-804598 on behavioral deficits caused by chronic unpredictable stress (CUS). **(A)** Experimental paradigm. Rats were exposed to CUS, two stressors per day for 28 days, and were then administered A-804598 (5 mg/kg, twice daily) for an additional 28 days. Behavioral tests were then conducted, including **(B)** sucrose preference test (SPT) ($F_{3,25} = 3.162$, $p < .05$; $n = 7–8$ per group), **(C)** novelty suppressed feeding test (NSFT) ($F_{3,25} = 4.544$, $p < .05$; $n = 7–8$ per group), and **(D, E)** elevated plus maze (EPM), time in open arm **(D)** and open/total entries **(E)** (time in open arm [$F_{3,26} = 8.522$, $p < .01$]; open/total entries [$F_{3,27} = 4.971$, $p < .01$]; $n = 7–8$ per group for both). All data are expressed as mean ± SEM. *$p < .05$ and **$p < .01$.

antidepressant (19,24). In this study, we found that CUS increased anhedonia, measured in the sucrose preference test, increased latency to feed in the NSFT, and reduced time in the open arms in the EPM (Figure 2B–E). Long-term (28 days) administration of A-804598 significantly reversed these effects of CUS on anhedonia in the sucrose preference test and anxiety in the NSFT (decreased latency) and the EPM (increased open arm time) (Figure 2B–E).

Stress causes a rapid downregulation of neurogenesis in the adult hippocampus (38) that is blocked by administration of an IL-1β receptor antagonist (11). To test the role of ATP/P2X7R, A-804598 was administered 1 hour before IMM stress, and the levels of hippocampal neurogenesis were assessed. Immediately after IMM stress, BrdU was injected to label newborn neurons, and a hippocampus sample was collected 2 hours later for analysis of BrdU incorporation (22) (Supplemental Figures S6A, C and S7A–C). Pretreatment with A-804598 resulted in a dose-dependent blockade of the BrdU reduction caused by IMM stress (Supplemental Figure S6B).

Stress Activates NLRP3 Inflammasome: *Nlrp3* Null Mice Are Resilient to Chronic Stress

The NLRP3 inflammasome complex, which consists of NLRP3, ASC, and pro-caspase-1, can detect diverse danger signals and produce the accompanying immune-inflammatory reactions, most importantly, the processing and release of IL-1β (8). NLRP3 has attracted particular attention because it can detect not only pathogen-associated molecules, but also cell damage–associated molecules such as ATP. This ability led us to hypothesize that the NLRP3 inflammasome may be a central mediator by which psychological and physical stressors can contribute to the development of depression as well as systemic diseases (8). ATP/P2X7R activation triggers NLRP3 oligomerization to form an active inflammasome complex. We investigated whether IMM stress increases the active form of NLRP3, using a combination of co-immunoprecipitation with an anti-ASC antibody and NLRP3 western blotting methods (Figure 3A, B). Co-immunoprecipitated NLRP3 was significantly increased in the stress group relative to the control group, whereas total NLRP3 levels were unchanged (Figure 3C, D), and this effect was reversed by A-804598 administration (Figure 3D). The results indicate that stress increases levels of the active form of the NLRP3 inflammasome complex via P2X7R stimulation.

The NOD-like receptors serve as sensors for internal and external danger signals, and the NLRP3 inflammasome has been implicated in diverse systemic illnesses (39–41). Based on our findings, we hypothesized that the NLRP3 inflammasome is a key mediator of the stress response in brain. This hypothesis was directly tested in *Nlrp3* null mutant mice (Supplemental Figure S8A, B) (18). We found that under basal nonstressed conditions, the *Nlrp3* null mice exhibit decreased anxiety and depressive-like behaviors in the EPM and OFT (Figure 3E–H). In addition, the *Nlrp3* null mice are resilient to CUS compared with their wild-type littermates. We found that

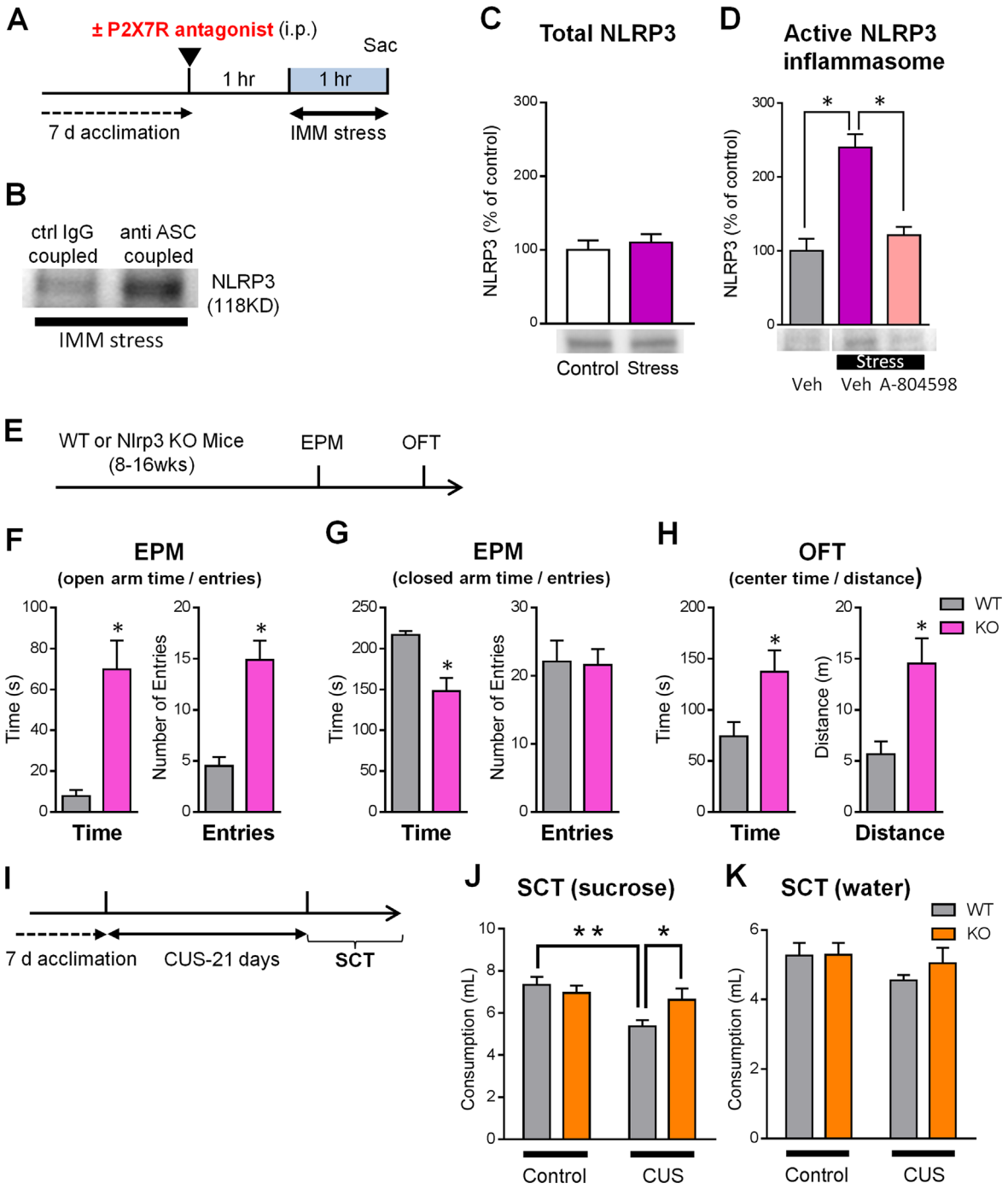

Figure 3. Immobilization (IMM) stress activates the NLRP3 inflammasome. *Nlrp3* deletion mutant mice exhibit behavioral resilience to chronic unpredictable stress (CUS). **(A)** Experimental paradigm for IMM stress showing that rats were sacrificed after 1 hour of stress. **(B)** Co-immunoprecipitation of NLRP3 using anti-ASC antibody coupled beads or IgG control and western blotting; the anti-ASC enriched fraction represents the activated form of NLRP3. Influence of IMM stress on **(C)** total protein levels of NLRP3 and **(D)** anti-ASC co-immunoprecipitated NLRP3; reversal by administration of A-804598 (10 mg/kg, i.p.). Results are expressed as mean ± SEM. *$p < .05$ compared with control mice. **(E)** Experimental paradigm for anxiety behavioral studies in nonstressed *Nlrp3* null mice. Results are shown for the elevated plus maze (EPM), including **(F)** open arm time ($t_{18} = 4.322$, $p < .01$; $n = 10$ per group), open arm entries ($t_{18} = 5.013$, $p < .01$; $n = 10$ per group), **(G)** closed arm time ($t_{18} = 4.026$, $p < .01$; $n = 10$ per group), and closed arm entries ($t_{18} = .306$, $p =$ not significant; $n = 10$ per group). Results in the open field test (OFT) include **(H)** time in center ($t_{18} = 3.444$, $p < .01$; $n = 10$ per group) and center distance traveled ($t_{18} = 3.209$, $p < .01$; $n = 10$ per group). **(I)** Experimental paradigm for CUS in mice. After 7 days of acclimation, mice are exposed to two stressors per day for 21 days, and anhedonia-like deficits are then measured in the **(J)** sucrose consumption test (SCT) ($F_{3,31} = 5.418$, $p < .01$; $n = 8$–10 per group) and **(K)** water consumption that served as a control ($F_{3,31} = 1.226$, $p =$ not significant; $n = 8$–10 per group). *$p < .05$ and **$p < .01$. Ctrl, control; i.p., intraperitoneal; KO, knockout; Sac, sacrifice; WT, wild-type; Veh, vehicle.

wild-type control mice that have been exposed to CUS show a reduction in the consumption of a sweetened solution, whereas there is no effect of CUS in the *Nlrp3* null mice (Figure 3I, J); there was no effect on water consumed (Figure 3K). There is a small, nonsignificant difference in locomotor activity and no difference in body weight between wild-type and knockout mice (Supplemental Figure S8C, D).

Blockade of Peripheral IL-1β Blocks Behavioral Deficits Caused by Chronic Stress

Previous studies demonstrated that blockade of IL-1β in the central nervous system (via intracerebroventricular infusion of IL-1β receptor antagonist) blocks the effects of CUS on anhedonic behavior, consistent with our studies of the P2X7 antagonist and *Nlrp3* null mice. However, whether blockade of peripheral IL-1β can produce an antidepressant response, as reported for peripheral blockade of TNFα (42,43), is unknown. This possibility was directly examined by peripheral administration of an IL-1β neutralizing antibody (Figure 4A–D). An anti-IL-1β antibody (.2 mg/mouse) was administered subcutaneously every 4 days, the approximate half-life of the antibody, and behavioral responses to CUS were examined. The IL-1β antibody administration completely blocked the deficit in sucrose consumption as well as increased anxiety behavior in the EPM caused by CUS exposure relative to the control IgG group.

We also found that subacute peripheral administration of the IL-1β antibody (1–3 days after infusion) was sufficient to produce antidepressant and anxiolytic behavioral effects in nonstressed mice (Figure 4E–H). A single dose of IL-1β antibody decreased immobility time in the FST, particularly during the last 5 minutes of the test, and increased the ratio of distance in open field relative to total distance. There was no significant difference in latency to feed in the NSFT. A single dose of the IL-1β antibody, but not the P2X7R antagonist, may produce an antidepressant/anxiolytic response because of the more long-term blockade of IL-1β by the antibody during the 2- to 3-day period of behavioral testing or because there is P2X7R-independent peripheral IL-1β.

DISCUSSION

In the present study, we show that psychological stress causes a rapid increase in extracellular ATP and glutamate that precedes the release of extracellular IL-1β in the hippocampus. The release of IL-1β is blocked by pretreatment with a P2X7R antagonist, confirming that ATP activates P2X7R to stimulate the processing and release of IL-1β (Figure 5). Astrocytes express glutamate receptors that regulate intracellular Ca^{2+} (44,45), which could then stimulate the release of ATP, a "warning molecule" for microglial activation (44–48). Extracellular ATP from astrocytes is essential for both the rapid baseline dynamics and the injury-induced responses of microglia (49). We found that glutamate stimulates release of ATP from astrocytes, consistent with the hypothesis that these cells are the source of stress/glutamate-induced ATP release. Further studies are needed to test this hypothesis, as there is also evidence of neuronal release of ATP (50), and to identify the glutamate receptor subtype that mediates this response. In addition, studies to identify other danger substances that facilitate the transcription of pro-IL-1β, NLRP3, and other components of the inflammasome in response to psychological stress are needed.

These results appear to conflict with a recent study reporting that chronic social defeat stress decreases basal levels of ATP in the hippocampus and that ATP administration produces an antidepressant response in rodents (51), but there are several major differences from the present study. First, we analyzed real-time levels of ATP during stress exposure, whereas the previous study analyzed samples 24 hours after social defeat stress. Second, we perfused the inserted probe for 18 hours before sample collection, whereas the previous study started collection 1 hour after probe insertion, and analysis could be confounded by acute tissue damage, activated microglia, and reactive astrocytes around the insertion site (52,53). In addition, the dose of ATP used in the previous report would selectively activate the P2X2R subtype, not P2X7R, based on receptor affinities (P2X2 is 100-fold > P2X7). These and other differences explain the results of these studies and indicate that both P2X2R and P2X7R are potential targets for drug development.

We also found that IMM stress increases the release of TNFα with a time course that is slightly delayed relative to IL-1β. Pretreatment with the P2X7R antagonist also blocked the release of extracellular TNFα in the hippocampus. Release of TNFα is not directly regulated by ATP/P2X7R and inflammasome activation (54–57) [however, see Hide *et al.* (58)], but it is possible that the elevation of IL-1β stimulates TNFα release. Although the mechanisms are unclear, the results indicate that P2X7R blockade produces a more generalized inhibition of the stress-induced inflammatory responses.

Although P2X7R antagonist rapidly blocks the induction of IL-1β by acute stress, it did not produce a response in acute behavioral models. This finding is consistent with a report that a nonselective P2X7R antagonist decreases immobility in the FST only after repeated testing (59). Similar effects have been reported in *P2x7r* null mice (60), although there is an earlier report of decreased immobility in *P2x7r* null mice during the first FST (61). These studies indicate that P2X7R blockade is more effective under conditions of repeated stress exposure, which we tested using the CUS model of depression (19,24). We found that P2X7R antagonist treatment completely blocked the anhedonic and anxiety behaviors resulting from CUS. These findings indicate that chronic stress causes greater, sustained activation of the inflammasome consistent with previous reports that chronic stress serves as a "priming" stimulus for inflammatory agents (62,63). However, additional studies are needed to determine the relationship between ATP, the NLRP3 inflammasome, and behavioral responses to P2X7R blockade under chronic stress conditions.

The possibility that the behavioral deficits caused by CUS occur via the NLRP3 inflammasome was tested in *Nlrp3* null mutant mice. Compared with wild-type littermates, we found that the *Nlrp3* null mice displayed decreased anxiety and anhedonic behaviors even under basal, unstressed conditions and were resilient to the behavioral deficits caused by CUS exposure. These results are consistent with our findings that stress activates the NLRP3 inflammasome and demonstrate

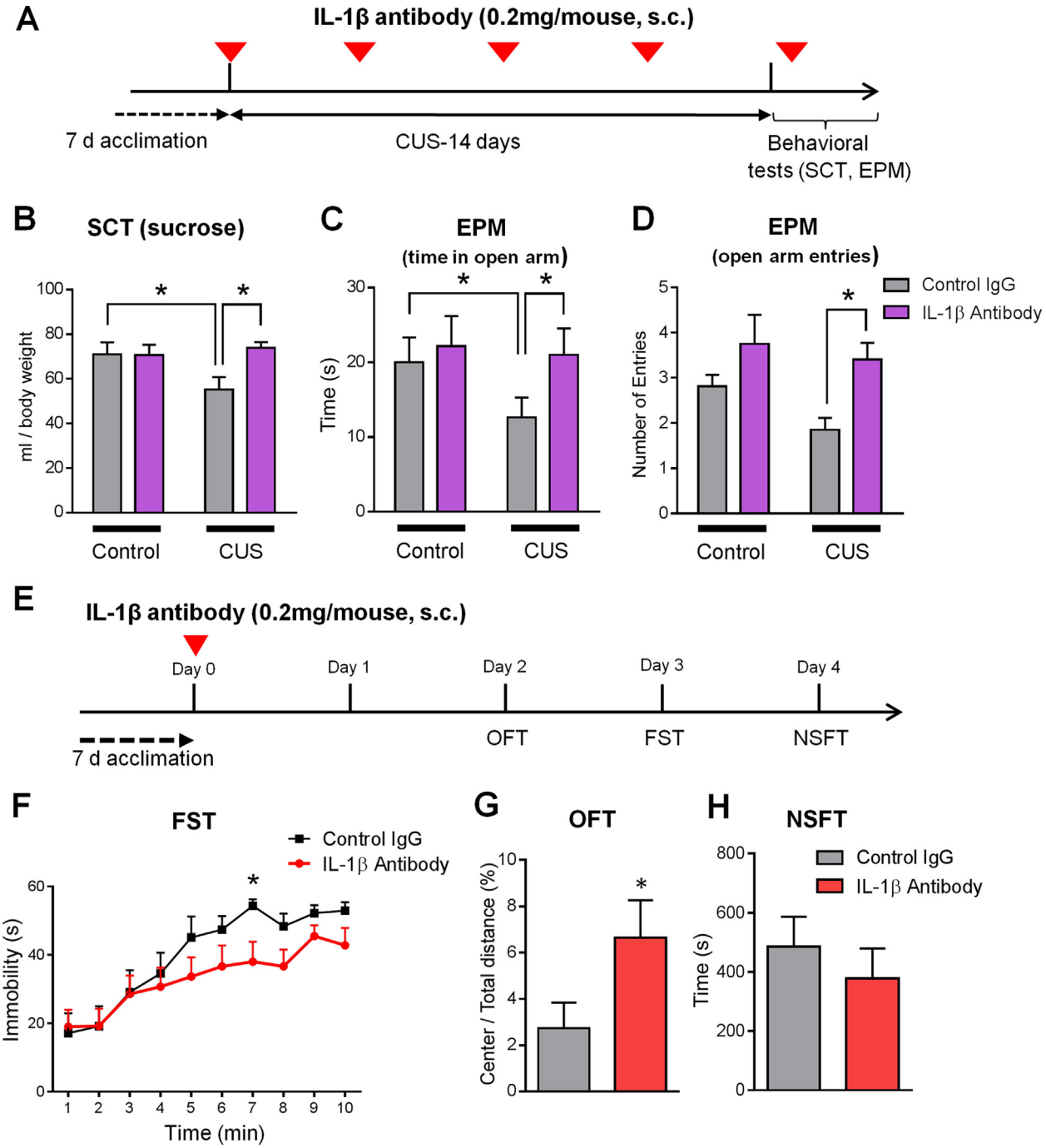

Figure 4. Peripheral administration of interleukin (IL)-1β antibody blocks the effects of chronic unpredictable stress (CUS). **(A)** Experimental paradigm of 14 days of CUS for mice. IL-1β antibody .2 mg/mouse is injected subcutaneously (s.c.) every 4 days. Behavioral tests included **(B)** sucrose consumption test (SCT) ($F_{3,25} = 2.996$, $p < .05$; $n = 21–22$ per group); **(C)** elevated plus maze (EPM), time in open arm ($F_{3,77} = 4.026$, $p < .05$; $n = 21–22$ per group); and **(D)** EPM, open arm entries ($F_{3,78} = 2.818$, $p < .05$; $n = 21–22$ per group). **(E)** Experimental paradigm of subacute administration of IL-1β antibody. **(F)** Immobilization time in the forced swim test (FST) ($t_{14} = 2.662$, $p < .05$; $n = 8$ per group). **(G)** The ratio of center to total distance in the open field test (OFT) ($t_{13} = 2.648$, $p < .05$, $n = 7–8$ per group). **(H)** The latency to feed in novelty suppressed feeding test (NSFT) ($t_{14} = .746$, $p =$ not significant; $n = 8$ per group).

that blockade of the inflammasome either by blockade of the P2X7R or null mutation blocks the behavioral effects of CUS.

We also demonstrate that peripheral neutralization of IL-1β is sufficient to block the behavioral deficits caused by CUS, extending previous studies of central IL-1β blockade (11). Stress can activate the peripheral immune system, including increased immune cell production and cytokine release (64), and can lead to increased monocyte trafficking to the brain (65). Administration of the IL-1β

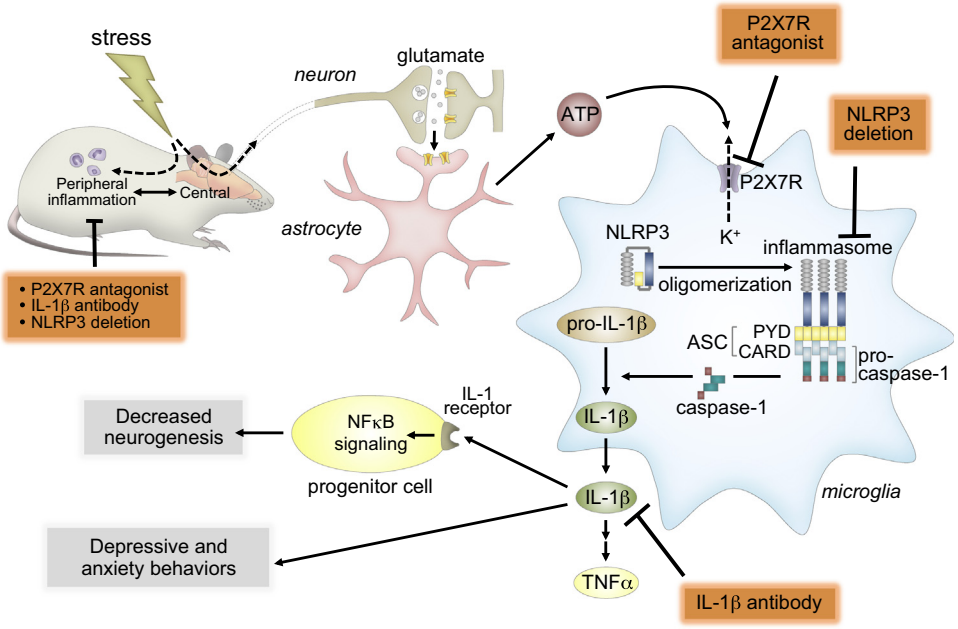

Figure 5. Scheme of the relationship between psychological stress and inflammation. Adenosine triphosphate (ATP) is released in response to psychological stress and binds to the P2X7 receptor (P2X7R). The efflux of K^+ caused by ATP induces NLRP3 activation and recruits adaptor protein ASC and pro-caspase-1, which makes a large multiprotein complex termed the inflammasome. Mature caspase-1 is cleaved from pro-caspase-1, which cleaves pro-interleukin (IL)-1β to make mature IL-1β. Released IL-1β is thought to induce tumor necrosis factor alpha (TNFα) release because P2X7R antagonist administration blocks not only IL-1β release but also TNFα release, which is not directly regulated by P2X7R. Evidence presented in this study demonstrates several approaches that block the behavioral deficits caused by chronic stress, including 1) P2X7R antagonist treatment, 2) *Nlrp3* deletion, and 3) IL-1β neutralization. These approaches could influence peripheral and central immune responses. Together, these studies provide evidence to support new therapeutic strategies that reduce excess release of IL-1β for the treatment of stress-related mood disorders such as depression. NFκB, nuclear factor kappa B.

antibody would neutralize these peripheral effects of stress as well as indirectly influence central nervous system immune responses (e.g., via the vagus nerve or prostaglandin diffusion). We would also expect that P2X7R antagonist administration or *Nlrp3* mutation would block peripheral and central immune responses (Figure 5). Together with previous reports of central IL-1β blockade (11), the results indicate that both the peripheral and the central IL-1β/immune responses contribute to depressive/anxiety behaviors. Further studies are needed to determine the contribution of the peripheral and central systems, including IL-1β and ATP, to the antidepressant actions of these treatments.

In conclusion, the inflammasome has become the focus of a wide range of medical research areas because it is a critical sensor for diverse diseases (e.g., diabetes, cardiovascular disorders, cancer), many of which have high rates of comorbidity with MDD (3). Because psychological stressors and systemic diseases cause elevation of inflammatory cytokines, notably IL-1β, NLRP3 may represent a common mediator for the development of depression by other systemic diseases as well as by chronic or traumatic psychological stress (8). The results also demonstrate several different approaches for blocking the deleterious effects of chronic stress on depressive and anxiety behaviors, including blockade of P2X7R, null mutation of *Nlrp3*, and IL-1β neutralization. Together, the characterization of these stress and disease signaling pathways and blocking strategies represent a rich area for development of novel therapeutics for the treatment of depression caused by stress as well as other comorbid illnesses.

ACKNOWLEDGMENTS AND DISCLOSURES

This work was supported by the State of Connecticut, a Yale University endowment (RSD), and an investigator initiated grant from Eli Lilly and Company.

We thank Dr. N. Fasel and Mr. A. Tardivel for providing *Nlrp3* knockout mice and Dr. A. Bhattacharya and Dr. M. Letavic for suggestions and helpful discussions.

The authors report no biomedical financial interests or potential conflicts of interest.

ARTICLE INFORMATION

From the Department of Psychiatry (MI, KTO, X-YL, FS, NL, SD, MB, VD, RSD), Yale University School of Medicine, New Haven, Connecticut; Department of Physiology and Pharmacology (VD), Des Moines University, Des Moines, Iowa; Division of Neuropsychiatry (TY, KK), Tottori University, Tottori, Japan; Lilly Research Laboratories (KR, AG, AK), Eli Lilly and Company, Indianapolis, Indiana; and Lundbeck Research USA (DS, KAJ, SZ, GS), Paramus, New Jersey.

Address correspondence to Ronald S. Duman, Ph.D., Department of Psychiatry, Yale University School of Medicine, 34 Park Street, New Haven, CT 06508; E-mail: ronald.duman@yale.edu.

Received Oct 20, 2015; revised and accepted Nov 24, 2015.

Supplementary material cited in this article is available online at http://dx.doi.org/10.1016/j.biopsych.2015.11.026.

REFERENCES

1. Duman RS (2009): Neuronal damage and protection in the pathophysiology and treatment of psychiatric illness: stress and depression. Dialogues Clin Neurosci 11:239–255.
2. Schiepers OJ, Wichers MC, Maes M (2005): Cytokines and major depression. Prog Neuropsychopharmacol Biol Psychiatry 29:201–217.
3. Evans DL, Charney DS, Lewis L, Golden RN, Gorman JM, Krishnan KR, *et al*. (2005): Mood disorders in the medically ill: scientific review and recommendations. Biol Psychiatry 58:175–189.
4. Bierhaus A, Wolf J, Andrassy M, Rohleder N, Humpert PM, Petrov D, *et al*. (2003): A mechanism converting psychosocial stress into mononuclear cell activation. Proceedings of the National Academy of Sciences of the United States of America 100:1920–1925.
5. Pace TW, Mletzko TC, Alagbe O, Musselman DL, Nemeroff CB, Miller AH, *et al*. (2006): Increased stress-induced inflammatory responses in

male patients with major depression and increased early life stress. Am J Psychiatry 163:1630–1633.
6. Howren MB, Lamkin DM, Suls J (2009): Associations of depression with C-reactive protein, IL-1, and IL-6: a meta-analysis. Psychosom Med 71:171–186.
7. Dantzer R, O'Connor JC, Freund GG, Johnson RW, Kelley KW (2008): From inflammation to sickness and depression: when the immune system subjugates the brain. Nat Rev Neurosci 9:46–56.
8. Iwata M, Ota KT, Duman RS (2013): The inflammasome: pathways linking psychological stress, depression, and systemic illnesses. Brain Behav Immun 31:105–114.
9. Frank MG, Watkins LR, Maier SF (2013): Stress-induced glucocorticoids as a neuroendocrine alarm signal of danger. Brain Behav Immun 33:1–6.
10. Yirmiya R, Rimmerman N, Reshef R (2015): Depression as a Microglial Disease. Trends Neurosci 38:637–658.
11. Koo JW, Duman RS (2008): IL-1beta is an essential mediator of the antineurogenic and anhedonic effects of stress. Proc Natl Acad Sci U S A 105:751–756.
12. Koo JW, Russo SJ, Ferguson D, Nestler EJ, Duman RS (2010): Nuclear factor-kappaB is a critical mediator of stress-impaired neurogenesis and depressive behavior. Proc Natl Acad Sci U S A 107: 2669–2674.
13. Koo JW, Duman RS (2009): Interleukin-1 receptor null mutant mice show decreased anxiety-like behavior and enhanced fear memory. Neurosci Lett 456:39–43.
14. Jin C, Flavell RA (2010): Molecular mechanism of NLRP3 inflammasome activation. J Clin Immunol 30:628–631.
15. Gross O, Thomas CJ, Guarda G, Tschopp J (2011): The inflammasome: an integrated view. Immunol Rev 243:136–151.
16. Ferrari D, Pizzirani C, Adinolfi E, Lemoli RM, Curti A, Idzko M, *et al*. (2006): The P2X7 receptor: a key player in IL-1 processing and release. J Immunol 176:3877–3883.
17. Lucae S, Salyakina D, Barden N, Harvey M, Gagne B, Labbe M, *et al*. (2006): P2RX7, a gene coding for a purinergic ligand-gated ion channel, is associated with major depressive disorder. Hum Mol Genet 15:2438–2445.
18. Martinon F, Petrilli V, Mayor A, Tardivel A, Tschopp J (2006): Gout-associated uric acid crystals activate the NALP3 inflammasome. Nature 440:237–241.
19. Banasr M, Valentine GW, Li X-Y, Gourley SL, Taylor JR, Duman RS (2007): Chronic unpredictable stress decreases cell proliferation in the cerebral cortex of the adult rat. Biol Psychiatry 62:496–504.
20. Duman CH, Schlesinger L, Terwilliger R, Russell DS, Newton SS, Duman RS (2009): Peripheral insulin-like growth factor-I produces antidepressant-like behavior and contributes to the effect of exercise. Behav Brain Res 198:366–371.
21. Kajitani N, Hisaoka-Nakashima K, Morioka N, Okada-Tsuchioka M, Kaneko M, Kasai M, *et al*. (2012): Antidepressant acts on astrocytes leading to an increase in the expression of neurotrophic/growth factors: differential regulation of FGF-2 by noradrenaline. PloS One 7:e51197.
22. Wu X, Castren E (2009): Co-treatment with diazepam prevents the effects of fluoxetine on the proliferation and survival of hippocampal dentate granule cells. Biol Psychiatry 66:5–8.
23. Duric V, Banasr M, Licznerski P, Schmidt HD, Stockmeier CA, Simen AA, *et al*. (2010): A negative regulator of MAP kinase causes depressive behavior. Nat Med 16:1328–1332.
24. Banasr M, Duman RS (2008): Glial loss in the prefrontal cortex is sufficient to induce depressive-like behaviors. Biol Psychiatry 64: 863–870.
25. Ota KT, Liu RJ, Voleti B, Maldonado-Aviles JG, Duric V, Iwata M, *et al*. (2014): REDD1 is essential for stress-induced synaptic loss and depressive behavior. Nat Med 20:531–535.
26. Ross SM (2003): Peirce's criterion for the elimination of suspect experimental data. Journal of engineering technology 20:38–41.
27. Dubyak GR, el-Moatassim C (1993): Signal transduction via P2-purinergic receptors for extracellular ATP and other nucleotides. The Am J Physiol 265:C577–606.
28. Musazzi L, Milanese M, Farisello P, Zappettini S, Tardito D, Barbiero VS, *et al*. (2010): Acute stress increases depolarization-evoked glutamate release in the rat prefrontal/frontal cortex: the dampening action of antidepressants. PloS One 5:e8566.
29. Bagley J, Moghaddam B (1997): Temporal dynamics of glutamate efflux in the prefrontal cortex and in the hippocampus following repeated stress: effects of pretreatment with saline or diazepam. Neuroscience 77:65–73.
30. Reznikov LR, Grillo CA, Piroli GG, Pasumarthi RK, Reagan LP, Fadel J (2007): Acute stress-mediated increases in extracellular glutamate levels in the rat amygdala: differential effects of antidepressant treatment. Eur J Neurosci 25:3109–3114.
31. Dabitao D, Margolick JB, Lopez J, Bream JH (2011): Multiplex measurement of proinflammatory cytokines in human serum: Comparison of the Meso Scale Discovery electrochemiluminescence assay and the Cytometric Bead Array. J Immunol Methods 372:71–77.
32. Goshen I, Yirmiya R (2009): Interleukin-1 (IL-1): a central regulator of stress responses. Front Neuroendocrinol 30:30–45.
33. Ferrari D, Chiozzi P, Falzoni S, Dal Susino M, Melchiorri L, Baricordi OR, *et al*. (1997): Extracellular ATP triggers IL-1 beta release by activating the purinergic P2Z receptor of human macrophages. J Immunol 159:1451–1458.
34. Donnelly-Roberts DL, Namovic MT, Surber B, Vaidyanathan SX, Perez-Medrano A, Wang Y, *et al*. (2009): [3H]A-804598 ([3H]2-cyano-1-[(1S)-1-phenylethyl]-3-quinolin-5-ylguanidine) is a novel, potent, and selective antagonist radioligand for P2X7 receptors. Neuropharmacology 56:223–229.
35. Raison CL, Capuron L, Miller AH (2006): Cytokines sing the blues: inflammation and the pathogenesis of depression. Trends Immunol 27:24–31.
36. Miller AH, Maletic V, Raison CL (2009): Inflammation and its discontents: the role of cytokines in the pathophysiology of major depression. Biol Psychiatry 65:732–741.
37. Takeuchi O, Akira S (2010): Pattern recognition receptors and inflammation. Cell 140:805–820.
38. Duman RS, Monteggia LM (2006): A neurotrophic model for stress-related mood disorders. Bio Psychiatry 59:1116–1127.
39. Vandanmagsar B, Youm YH, Ravussin A, Galgani JE, Stadler K, Mynatt RL, *et al*. (2011): The NLRP3 inflammasome instigates obesity-induced inflammation and insulin resistance. Nat Med 17:179–188.
40. Cook GP, Savic S, Wittmann M, McDermott MF (2010): The NLRP3 inflammasome, a target for therapy in diverse disease states. Eur J Immunol 40:631–634.
41. Lamkanfi M, Mueller JL, Vitari AC, Misaghi S, Fedorova A, Deshayes K, *et al*. (2009): Glyburide inhibits the Cryopyrin/Nalp3 inflammasome. J Cell Biol 187:61–70.
42. Tyring S, Gottlieb A, Papp K, Gordon K, Leonardi C, Wang A, *et al*. (2006): Etanercept and clinical outcomes, fatigue, and depression in psoriasis: double-blind placebo-controlled randomised phase III trial. Lancet 367:29–35.
43. Raison CL, Rutherford RE, Woolwine BJ, Shuo C, Schettler P, Drake DF, *et al*. (2013): A randomized controlled trial of the tumor necrosis factor antagonist infliximab for treatment-resistant depression: the role of baseline inflammatory biomarkers. JAMA Psychiatry 70:31–41.
44. Cornell-Bell AH, Finkbeiner SM, Cooper MS, Smith SJ (1990): Glutamate induces calcium waves in cultured astrocytes: long-range glial signaling. Science 247:470–473.
45. Inoue K, Koizumi S, Tsuda M (2007): The role of nucleotides in the neuron–glia communication responsible for the brain functions. J Neurochem 102:1447–1458.
46. Haydon PG (2001): GLIA: listening and talking to the synapse. Nature reviews Neuroscience 2:185–193.
47. Cotrina ML, Lin JH, Lopez-Garcia JC, Naus CC, Nedergaard M (2000): ATP-mediated glia signaling. J Neurosci 20:2835–2844.
48. Guthrie PB, Knappenberger J, Segal M, Bennett MV, Charles AC, Kater SB (1999): ATP released from astrocytes mediates glial calcium waves. J Neurosci 19:520–528.

49. Davalos D, Grutzendler J, Yang G, Kim JV, Zuo Y, Jung S, *et al*. (2005): ATP mediates rapid microglial response to local brain injury in vivo. Nat Neurosci 8:752–758.
50. North RA (2002): Molecular physiology of P2X receptors. Physiol Rev 82:1013–1067.
51. Cao X, Li LP, Wang Q, Wu Q, Hu HH, Zhang M, *et al*. (2013): Astrocyte-derived ATP modulates depressive-like behaviors. Nat Med 19:773–777.
52. Schwartz AB, Cui XT, Weber DJ, Moran DW (2006): Brain-controlled interfaces: movement restoration with neural prosthetics. Neuron 52: 205–220.
53. Kozai TD, Langhals NB, Patel PR, Deng X, Zhang H, Smith KL, *et al*. (2012): Ultrasmall implantable composite microelectrodes with bioactive surfaces for chronic neural interfaces. Nat Mater 11:1065–1073.
54. Mingam R, De Smedt V, Amedee T, Bluthe RM, Kelley KW, Dantzer R, *et al*. (2008): In vitro and in vivo evidence for a role of the P2X7 receptor in the release of IL-1 beta in the murine brain. Brain Behav Immun 22:234–244.
55. Murphy N, Cowley TR, Richardson JC, Virley D, Upton N, Walter D, *et al*. (2012): The neuroprotective effect of a specific P2X(7) receptor antagonist derives from its ability to inhibit assembly of the NLRP3 inflammasome in glial cells. Brain Pathol 22:295–306.
56. Niemi K, Teirila L, Lappalainen J, Rajamaki K, Baumann MH, Oorni K, *et al*. (2011): Serum amyloid A activates the NLRP3 inflammasome via P2X7 receptor and a cathepsin B-sensitive pathway. J Immunol 186: 6119–6128.
57. Laye S, Gheusi G, Cremona S, Combe C, Kelley K, Dantzer R, *et al*. (2000): Endogenous brain IL-1 mediates LPS-induced anorexia and hypothalamic cytokine expression. Am J Physiol Regul Integr Comp Physiol 279:R93–98.
58. Hide I, Tanaka M, Inoue A, Nakajima K, Kohsaka S, Inoue K, *et al*. (2000): Extracellular ATP triggers tumor necrosis factor-alpha release from rat microglia. Neurochem 75:965–972.
59. Csolle C, Ando RD, Kittel A, Goloncser F, Baranyi M, Soproni K, *et al*. (2013): The absence of P2X7 receptors (P2rx7) on non-haematopoietic cells leads to selective alteration in mood-related behaviour with dysregulated gene expression and stress reactivity in mice. Int J Neuropsychopharmacol 16:213–233.
60. Boucher AA, Arnold JC, Hunt GE, Spiro A, Spencer J, Brown C, *et al*. (2011): Resilience and reduced c-Fos expression in P2X7 receptor knockout mice exposed to repeated forced swim test. Neuroscience 189:170–177.
61. Basso AM, Bratcher NA, Harris RR, Jarvis MF, Decker MW, Rueter LE (2009): Behavioral profile of P2X7 receptor knockout mice in animal models of depression and anxiety: relevance for neuropsychiatric disorders. Behav Brain Res 198:83–90.
62. Munhoz CD, Lepsch LB, Kawamoto EM, Malta MB, Lima Lde S, Avellar MC, *et al*. (2006): Chronic unpredictable stress exacerbates lipopolysaccharide-induced activation of nuclear factor-kappaB in the frontal cortex and hippocampus via glucocorticoid secretion. Neurosci 26:3813–3820.
63. Espinosa-Oliva AM, de Pablos RM, Villaran RF, Arguelles S, Venero JL, Machado A, *et al*. (2011): Stress is critical for LPS-induced activation of microglia and damage in the rat hippocampus. Neurobiol Aging 32:85–102.
64. Heidt T, Sager HB, Courties G, Dutta P, Iwamoto Y, Zaltsman A, *et al*. (2014): Chronic variable stress activates hematopoietic stem cells. Nat Med 20:754–758.
65. Wohleb ES, Powell ND, Godbout JP, Sheridan JF (2013): Stress-induced recruitment of bone marrow-derived monocytes to the brain promotes anxiety-like behavior. J Neurosci 33:13820–13833.