ORIGINAL ARTICLE

NLRP3 Inflammasome Blockade Reduces Cocaine-Induced Microglial Activation and Neuroinflammation

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Abstract
Cocaine use disorder is a major health crisis that is associated with increased oxidative stress and neuroinflammation. While the role of NLRP3 inflammasome in mediating neuroinflammation is well-recognized, whether cocaine induces this response remains unexplored. Based on the premise that cocaine induces both reactive oxygen species (ROS) as well as microglial activation, we hypothesized that cocaine-mediated microglial activation involves both ROS and NLRP3 signaling pathways. We examined activation of the NLRP3 pathway in microglia exposed to cocaine, followed by validation in mice administered either cocaine or saline for 7 days, with or without pretreatment with the NLRP3 inhibitor, MCC950, and in postmortem cortical brain tissues of chronic cocaine-dependent humans. We found that microglia exposed to cocaine exhibited significant induction of NLRP3 and mature IL-1β expression. Intriguingly, blockade of ROS (Tempol) attenuated cocaine-mediated priming of NLRP3 and microglial activation (CD11b). Blockade of NLRP3 by both pharmacological (MCC950) as well as gene silencing (siNLRP3) approaches underpinned the critical role of NLRP3 in cocaine-mediated activation of inflammasome and microglial activation. Pretreatment of mice with MCC950 followed by cocaine administration for 7 days mitigated cocaine-mediated upregulation of mature IL-1β and CD11b, in both the striatum and the cortical regions. Furthermore, cortical brain tissues of chronic cocaine-dependent humans also exhibited upregulated expression of the NLRP3 pathway mediators compared with non-cocaine dependent controls. Collectively, these findings suggest that cocaine activates microglia involving the NLRP3 inflammasome pathway, thereby contributing to neuroinflammation. NLRP3 can thus be considered as a potential therapeutic target for alleviating cocaine-mediated neuroinflammation.

Keywords Cocaine · NLRP3 · IL-1β · Reactive oxygen species · Tempol · MCC950

Abbreviations
ROS	Reactive oxygen species
NLRP3	NLR family pyrin domain containing 3
ASC	Apoptosis-associated speck-like protein containing a CARD
CUD	Cocaine use disorder
LPS	Lipopolysaccharide

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Background

Cocaine use disorder (CUD) is estimated to affect up to 22.5 million people worldwide resulting in marked changes in behavior and lifestyle emanating from its psychoactive and addictive effects [1]. In the USA, cocaine use is increasing among young adults suggesting that cocaine abuse is an emerging issue among young adults [2]. Accumulating evidence suggests a close link between drug abuse and neuroinflammation [3, 4]. In fact, CUD is associated with enhanced immune activation, inflammation, and neuronal injury which, in turn, further contributes to behavioral alterations [5–9]. Several mechanism(s) such as cocaine-induced production of reactive oxygen species (ROS) and inflammasome activation have been suggested to underlie cocaine-associated behavioral deficits [10, 11]. Cocaine is a well-known inducer of

ROS [6], which has been implicated as an upstream mediator in the activation of NLR family pyrin domain containing 3 (NLRP3) inflammasome [12, 13].

Several studies have demonstrated the contribution of ROS in cocaine-mediated physiological alterations and addictive behavior [6, 14–18]. Increased ROS production is shown to be associated with oxidized metabolites of cocaine which, subsequently activate the redox cycling pathways and subsequent electron transfer [10, 19]. Increased ROS has also been well-documented to elicit inflammatory responses within the CNS via activation of glial cells such as the microglia—the primary immunocompetent cells of the brain [15, 20–22]. Under normal homeostasis, microglia play essential roles involving surveillance of the brain environment, phagocytosis, synaptic pruning, and neuronal plasticity [23–25]. Several psychostimulant drugs, such as cocaine and methamphetamine, have been shown to activate microglia, resulting in the production of pro-inflammatory cytokines, ultimately contributing to a neuroinflammatory milieu that underlies neurodegeneration and behavioral changes [26–29]. The interrelationship of cocaine-induced ROS and inflammasome activation, however, remains poorly understood. In this study, we rationalized that inhibition of cocaine-mediated ROS generation could result in abrogation of cellular activation by dampening activation of the NLRP3 inflammasome, which has been linked to ROS [12, 13].

Activation of the NLRP3 inflammasome is a two-step process comprising of signals 1 and 2; with the first signal priming the pathway by enhancing levels of NLRP3, followed by the second signal that triggers caspase-1 dependent cleavage, maturation and release of the pro-inflammatory cytokines IL-1β and IL-18 [30–34]. Although a previous report has shown an increase in the expression of NLRP3 mRNA in macrophages exposed to cocaine and HIV-1 [35], the causal association between NLRP3 activation and ROS in microglia and its contribution to cocaine-induced behavioral changes, however, remains poorly understood. Herein, we investigated the molecular mechanism(s) underlying cocaine-mediated NLRP3 inflammasome activation in microglia and the in vivo effects of NLRP3 blockade in reducing microglial activation and inflammation, in mice administered cocaine.

Using both primary microglia and cocaine administration of mice for 7 days, our findings suggested that cocaine activates microglia via the NLRP3 inflammasome. Moreover, our findings also identified ROS-dependent priming of NLRP3 as a potential mechanism underlying cocaine-induced activation of microglia and neuroinflammation. Intriguingly, pharmacological inhibition of NLRP3 was shown to dampen cocaine-mediated microglial activation in mice. Overall, these findings thus demonstrate a critical association between ROS, NLRP3 inflammasome, and microglial activation as contributors of neuroinflammation.

Methods

Reagents

Antibodies and reagents used in this work were purchased from the indicated sources: NLRP3 (AdipoGen; AG-20B-0014); ASC (Novus; NB1-78978 for Western blots and Adipogen; AG-25B-0006 for IHC), caspase-1 p20 (Santa Cruz; sc-398715), Iba-1 (Novus NB1001028 or Wako; 19-19741), CD11b (Novus; NB11089474), goat anti-mouse-HRP (Santa Cruz Biotechnology; sc-2005), and goat anti-rabbit-HRP (Santa Cruz Biotechnology; sc-2004); IL-1 beta (Abcam; ab9722); actin (Sigma-Aldrich; A1978). Cryopyrin/NLRP3 small interfering RNA (siRNA) (sc-45470) was from Santa Cruz Biotechnology. MCC950/CP-456773 (Sigma; pz0280) was from Sigma-Aldrich. Cocaine hydrochloride (C5776) and LPS (L2880) was from Sigma-Aldrich. Sterile water was used to dissolve cocaine and LPS. ATP (tlrl-atpl) was from Invivogen. FAM-FLICA caspase assay kit (#97) was from ImmunoChemistry

Animals

All animal procedures were performed according to the protocols approved by the Institutional Animal Care and Use Committee of the University of Nebraska Medical Centre and the National Institutes of Health. The C57BL/6N mice were purchased from Charles River Laboratories (Wilmington, MA, USA) and housed under standard vivarium conditions. Food and water were available ad libitum [34]. C57BL/6 wild-type mice were divided into groups; receiving cocaine or saline injections for seven consecutive days in the presence of absence of MCC950 pretreatment. Mice were injected with NLRP3 inhibitor MCC950 (50 mg/kg or vehicle, i.p.) daily followed by cocaine administration (20 mg/kg, i.p.) an hour later. Mice were sacrificed 1 h following the last injection. Brain tissues were dissected, and homogenates of the cortex and the striatum were used to assess the protein levels of NLRP3 and microglial activation markers.

Human Tissues

Frozen frontal cortex tissues from cocaine abusers or non-cocaine abusing controls or were obtained from the Douglas-Bell Canada Brain Bank (DBCBB; Douglas Mental Health University Institute, Montreal, Quebec, Canada). The DBCBB samples were collected postmortem following consent according to tissue banking practices regulated under *IUSMD-04-21* Banque de cerveaux suicides du Québec. Patient characteristics are shown in Table S1. Protein was isolated and the expression of NLRP3 pathway was analyzed by Western blotting.

Isolation of Primary Mice Microglial Cells

Primary microglial cells were isolated from C57BL/6N newborn mice pup brains as previously described [22, 34, 36]. The purity of isolated microglia was confirmed by immunohistochemical staining with antibodies specific for Iba-1 and was routinely found to be > 95% pure [34, 36].

BV-2 Cells

Mouse BV-2 microglial cell line was generously provided by Dr. Sanjay Maggirwar (George Washington University, Department of Microbiology, Immunology, and Tropical Medicine, USA). These cells were grown and routinely maintained at 37 °C, and 5% CO_2 in DMEM supplemented with 10% heat-inactivated Fetal Calf Serum (# S11150H, Atlanta Biologicals, GA, USA), 100 IU/ml penicillin, and 100 μg/ml streptomycin as previously described [36].

Western Blotting

Mouse brain tissue homogenates or microglia exposed to cocaine were lysed with RIPA buffer supplemented with a protease inhibitor cocktail (#78430, ThermoFisher Scientific, MA) followed by ultrasonication for 15 s at 80% amplitude. Western blotting was performed as previously described [22, 34, 36, 37]. Densitometric analyses was performed using NIH ImageJ software (ImageJ v1.44, NIH) as previously described [37]. Protein amounts for bands of interest were normalized to β-actin.

Small Interfering RNA Transfection

siRNA transfections were performed using Lipofectamine 2000 (11668027, Life Technologies, CA) according to the manufacturer's instructions. Briefly, cells were transfected with targeted siRNA or scrambled siRNA (20 pM) mixed with 6 μl of Lipofectamine 2000 diluted in 150 μl Opti-MEM Reduced Serum Medium (#31985062, Life Technologies, CA). The resulting siRNA-lipid complexes were added onto cells, incubated for 6 h, and the medium was refreshed and maintained up to 24 h. Cells were subsequently treated with cocaine (10 μM) or left untreated and harvested after an additional 24 h as previously described [22, 34, 36]. Knockdown efficiencies were determined by Western blotting.

FAM-FLICA Caspase-1 Assay

Microglia were seeded into 24-well plates overnight, followed by exposure to medium with cocaine, LPS, or left untreated for 24 h. Cells were treated with ATP (1 mM) for 1 h followed by FAM-FLICA assay (#97) according to the manufacturer's instructions.

IL-1β Cytokine Assay

Microglia were seeded onto 96-well plates (8000 cells/well) overnight, and subsequently exposed to medium with cocaine or left untreated. Supernatant fluids were collected at 24 h post-cocaine treatment, followed by quantification of IL-1β by ELISA (MLB00C, R&D, Minneapolis, USA) according to the manufacturer's instructions.

Immunohistochemistry

Double immunofluorescence staining for NLRP3 and Iba1 or ASC was performed in the whole brain sections from mice injected with either cocaine (20 mg/kg body weight, i.p.) or saline for 7 consecutive days or on microglia cultured as previously described [22]. Briefly, for brain slides, formalin-fixed, paraffin-embedded brain slides were baked overnight at 55 °C. The sections were deparaffinized using xylene and rehydrated by incubating the slide in graded series (100, 95, and 70%; each 5 min) of alcohol. Next, the slides were subjected to antigen retrieval by boiling them in Tris/EDTA buffer (pH 9) for about 20 min. Microglia cells on coverslips were fixed with 4% formaldehyde in PBS for 10 min at room temperature, washed three times with PBS, permeabilized with 0.3% Triton X-100 in PBS with 10% goat serum for 1 h as previously described [22]. The tissues were then blocked with 10% goat serum (#S-1000-20, Vector Laboratories, CA, USA) in PBS for 2 h followed by overnight co-incubation with primary antibodies (NLRP3, ASC or Iba-1) at 4 °C. Next day, the slides were washed with PBS for three times, followed by incubation with corresponding secondary Alexa Fluor 488 goat anti-mouse IgG (#A-11008, Invitrogen, CA) or Alexa Fluor 594 goat anti-rabbit (#A-11032, Invitrogen, CA) for 2 h. Finally, the slides were washed with PBS for three times and mounting with ProLong Gold Antifade Reagent with DAPI. Fluorescent images were taken on a Zeiss Observer using a Z1 inverted microscope (Carl Zeiss, Thornwood, NY, USA). The acquired images were analyzed for the fluorescence intensity and co-localization using the Axio Vs 40 Version 4.8.0.0 software (Carl Zeiss MicroImaging GmbH). To calculate microglial process length, images were converted to a binary format and skeletonized for analysis using ImageJ software. All experiments were repeated at least three times.

Statistical Analysis

Graphs and statistical analyses were performed using GraphPad software V5.0 (GraphPad Prism Software). Student's *t* test was used to compare results between test and controls. One-way ANOVA was used for multiple comparisons. *P* values less than 0.05 were considered statistically significant.

Results

Cocaine Exposure Upregulated the Expression of NLRP3 in Microglia

We first sought to examine whether exposure of microglia to cocaine could activate these cells involving the NLRP3 inflammasome. Mouse primary microglia (mPM) or BV-2 microglial cells were exposed to various concentrations of cocaine (0, 1, 10, 50, 100 μM) for 24 h and analyzed for the expression of NLRP3. As shown in Fig. 1a and b, cocaine dose-dependently increased the expression of NLRP3, with significant increases at 10–100 μM in both mPM and BV-2 cells. As anticipated, exposure of cells to bacterial lipopolysaccharide (LPS) (50 ng/ml), also caused a significant increase in NLRP3 expression compared with control cells (Fig. 1a and b). Based on these findings, we chose the lowest dose of cocaine (10 μM) that upregulated expression of NLRP3 in BV-2 cells, for all the ensuing experiments.

The next step then was to assess the time-course of cocaine-mediated upregulation of NLRP3. As shown in Fig. 1c, we found significant upregulation of NLRP3 protein at 6 h post-cocaine exposure (10 μM) that was sustained up to 48 h ($p < 0.05$) in BV-2 cells. These findings were also confirmed in mPMs exposed to cocaine which revealed a sustained increase in the expression of NLRP3 up to 48 h (Fig. 1d). To further validate cocaine-induced upregulation of NLRP3, immunofluorescence imaging of mPMs exposed to cocaine was performed. As shown in Fig. 1e, cocaine increased the expression of both NLRP3 and Iba-1 compared with control cells. Quantification of NLRP3 fluorescence intensity is shown in Fig. 1f. Collectively, these data demonstrated that cocaine upregulated the expression of microglial NLRP3.

Effect of Cocaine on NLRP3 Inflammasome Formation and Activation of Caspase-1 and IL-1β in Microglial Cells

Next, we examined whether cocaine exposure could increase generation of the NLRP3 inflammasome complex by immunostaining microglia for both NLRP3 and the adaptor molecule, apoptosis-associated speck-like protein containing a CARD (ASC) by immunofluorescence. As shown in Fig. 2a, ASC speck formation was observed in the cocaine-exposed or LPS-positive control cells compared with untreated control cells. We next sought to assess whether exposure of microglia to cocaine could activate pro-caspase-1 which undergoes proteolytic maturation leading to detection of active caspase-1 using the FAM-FLICA assay. The FLICA probe is a non-cytotoxic fluorescent-labeled inhibitor of caspase-1 that covalently bind to active caspase-1 enzyme. Mouse primary

microglia were seeded in 24-well plates and exposed to cocaine for 24 h followed by subjecting cells to the FAM-FLICA assay according to manufacturer's instructions. As shown in Fig. 2b, exposure of microglia to cocaine (10 μM) or LPS (50 ng/ml) resulted in increased FLICA fluorescence signal. FLICA intensity was higher in cells exposed to cocaine or LPS in the presence of ATP. The effect of LPS in increasing FLICA intensity was at least twice as much as that of cocaine suggesting that cocaine-induced caspase-1 activation is milder than that induced by LPS at the concentrations assayed. Quantification of FLICA fluorescence intensity is shown in Fig. 2c. To validate caspase-1 cleavage, we analyzed expression of both total and cleaved caspase-1 p20 by Western blot. As shown in Fig. 2d and quantified in Fig. 2e, exposure of cells to cocaine increased the expression of cleaved caspase-1.

To further validate cocaine-mediated activation of NLRP3 inflammasome, we determined the expression levels of IL-1β in microglia exposed to cocaine over a 48-h time-course. As shown in Fig. 3a and b, mPMs exposed to cocaine demonstrated a time-dependent increase of the mature IL-1β, (p17) at 6–48 h. We also sought to examine cocaine-mediated release of IL-1β into the supernatant fluids. As shown in Fig. 3c, there was a significantly ($p < 0.05$) increased release of IL-1β 24 h in mPMs exposed to cocaine compared with cells not exposed to cocaine.

Genetic Silencing of NLRP3 Attenuated Cocaine-Mediated Activation of NLRP3 Inflammasome and Cellular Activation in Microglia

Next, we used a gene silencing approach to validate the role of NLRP3 in cocaine-induced activation of the NLRP3 inflammasome. Herein, mPMs were transfected with either scrambled or NLRP3 siRNA (24 h) followed by exposure of transfected cells to cocaine for an additional 24 h. As shown in Fig. 4a, in cells transfected with NLPR3 siRNA, cocaine failed to upregulate the expression of NLRP3 compared with cells transfected with the scrambled siRNA. Similarly, there was abrogation of cocaine-mediated maturation of IL-1β as evidenced by reduced expression of mature IL-1β in cells silenced for NLRP3 (Fig. 4b). In addition, cells silenced for NLRP3 failed to demonstrate cocaine-mediated activation of cellular activation markers Iba-1 and CD11b (Fig. 4c and d). These findings thus underscore the role of NLRP3 in cocaine-mediated activation of both microglial inflammasome as well as cellular activation.

Cocaine Upregulated NLRP3 Inflammasome Pathway via the Sigma Receptor in Microglia

Having demonstrated that cocaine activated microglia via the NLRP3 pathway, we next explored the role of cognate cocaine receptor—sigma-1 receptor (σ-1R), in this process.

Fig. 1 Cocaine upregulates the expression of NLRP3 in microglia cells in a dose- and time-dependent manner. BV-2 cells and mouse primary microglia (mPMs) were seeded into 6-well plates and exposed to the indicated doses of cocaine for 24 h. Cocaine (10 μM) concentration increased the expression of NLRP3 in both cell types (**a**, **b**). BV-2 cells and mPMs were seeded into 6-well plates and exposed to cocaine (10 μM) for the indicated time periods. Exposure of these cells to cocaine increase the expression of NLRP3 time dependently in both cell types (**c**, **d**). mPMs were seeded into 24-well plates and exposed to cocaine for 24 h. The expression of NLRP3 and Iba-1 was analyzed at 24 h post-exposure (**e**). Quantification of NLRP3 fluorescence intensity (**f**). All experiments were done at least three independent times, and representative figures are shown. LPS and actin served as positive or loading controls respectively. Quantification of western blots is shown under each blot. Scale bar = 10 μm. Data are shown as mean ± SEM and *$p < 0.05$ vs. control
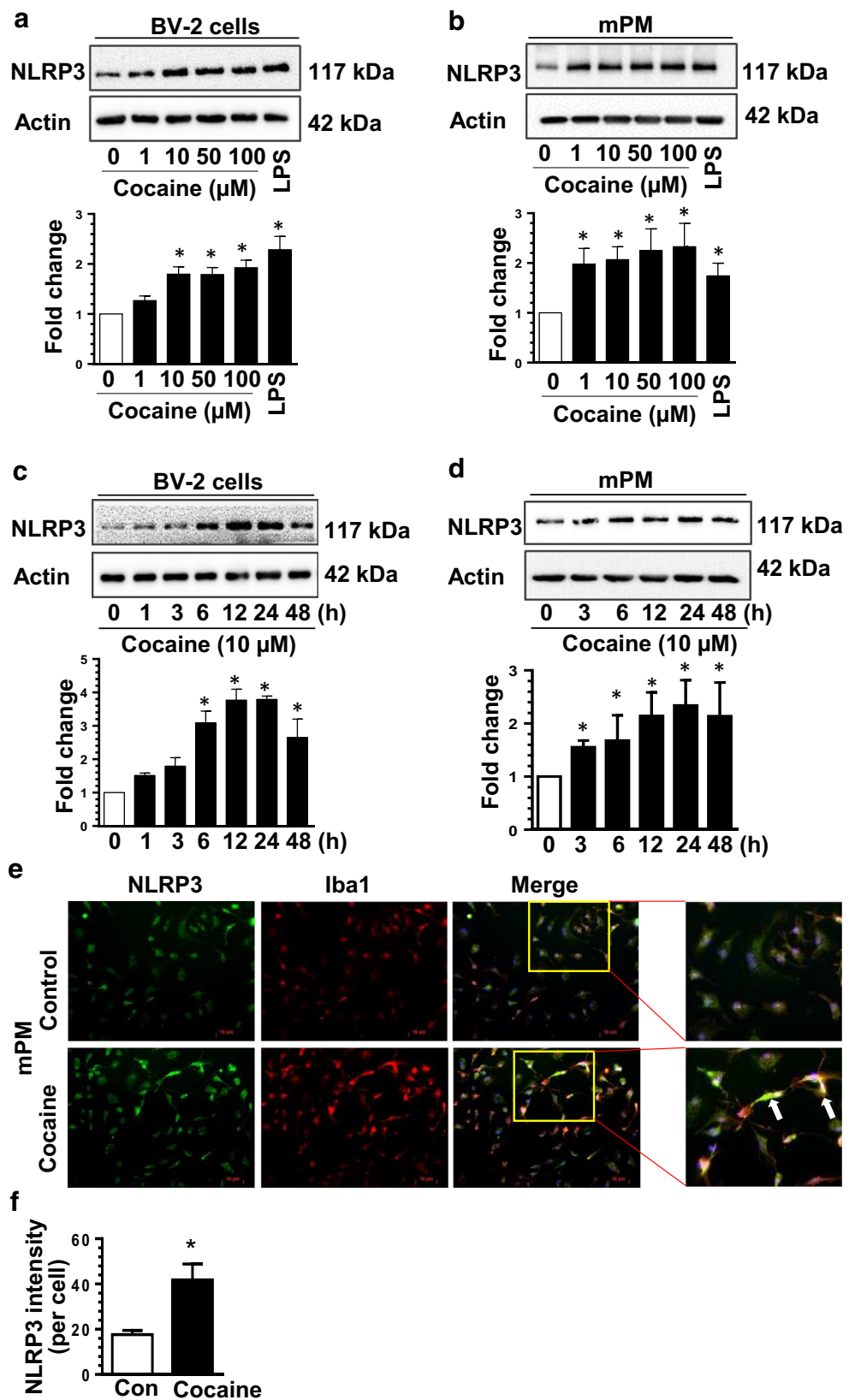

The σ-1R is shown to be expressed in most cells of the CNS and has been shown to be activated following binding of cocaine [38–40]. Its role in the priming of NLRP,3 however, remains to be investigated. To address this, we assessed the

expression of σ-1R in mPMs exposed to cocaine. As shown in Fig. 5a, the expression of σ-1R remained constant to the last time point analyzed (48 h). Next, mPMs were pretreated with the σ-1R inhibitor—BD1047 (10 μM), followed by exposure of cells to cocaine for 24 h and analyzed for the expression of NLRP3 and the activation marker CD11b, by Western blotting. As presented in Fig. 5b, cocaine exposure alone increased the expression of NLRP3 compared with untreated control cells. Pretreatment of mPMs with BD1047, on the other hand, resulted in significant abrogation of cocaine-mediated upregulation of NLRP3 compared with cells exposed to cocaine alone. In keeping with these findings, there was also inhibition of CD11b expression (Fig. 5c) in cells exposed to the pharmacological inhibitor of σ-1R, followed by exposure to cocaine. Overall, these results underscore the involvement of σ-1R in cocaine-mediated upregulation of NLRP3 inflammasome and subsequent activation of microglia.

Cocaine-Induced ROS Is Involved in the Upregulation of NLRP3 Inflammasome Pathway and Activation of Microglia

Previous reports have demonstrated cocaine-mediated induction of ROS in microglia [22]. Herein, we sought to explore whether inhibiting ROS could lead to inhibition of both NLRP3 priming and microglial activation. To test this hypothesis, mPMs were pre-treated with tempol—a brain penetrant ROS inhibitor (1 h), followed by exposure of cells to cocaine (10 μM, 24 h) and assessed for the expression of NLRP3 and CD11b. As shown in Fig. 5d, and as expected, exposure of mPMs to cocaine resulted in an increase in the expression of NLRP3 compared with the control cells. Interestingly, pretreatment of mPMs with tempol attenuated cocaine-mediated upregulation of NLRP3 compared with cells exposed to cocaine alone. As expected, pretreatment of mPMs with tempol alone did not upregulate the expression of NLRP3. In keeping
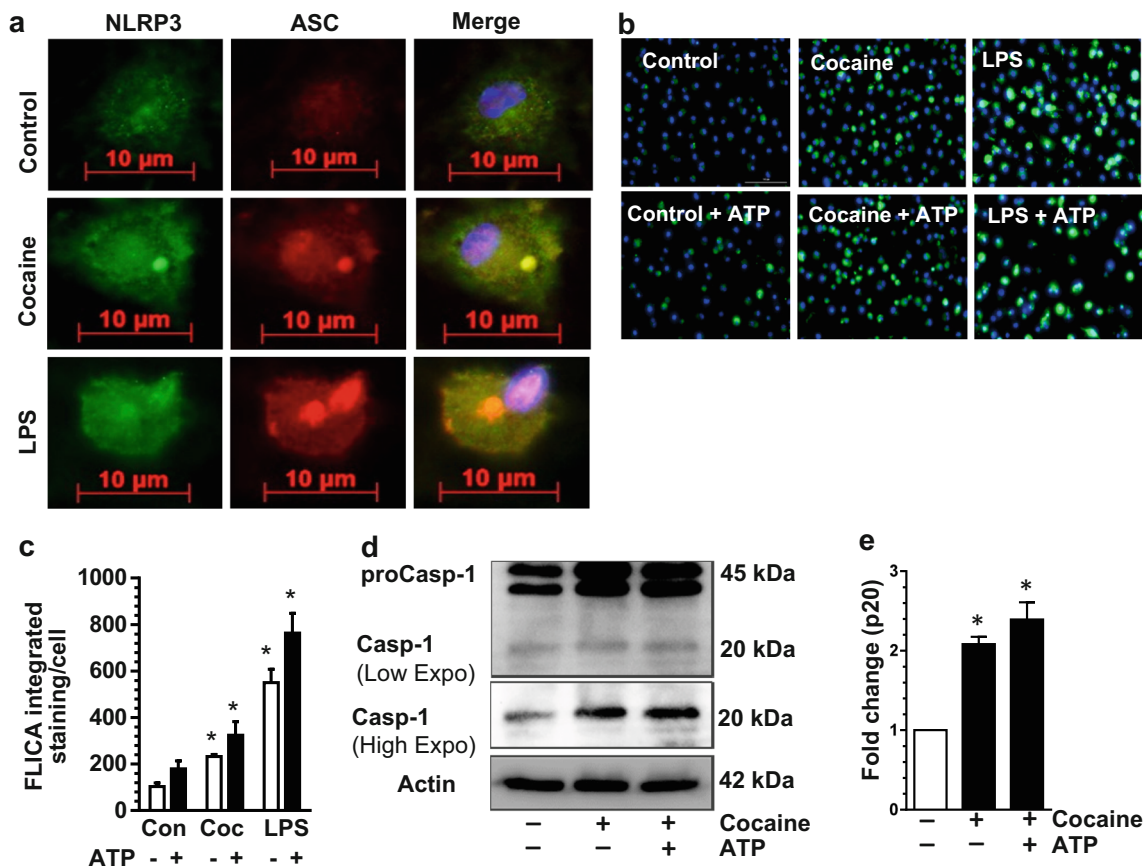
Fig. 2 Cocaine induced NLRP3 inflammasome formation and activation caspase-1 in microglia. Mouse primary microglia (mPMs) were seeded into 24-well plates and exposed to cocaine for 24 h. The expression and presence of ASC specks was analyzed at 24 h post-exposure (**a**). Cells were seeded in 24-well black plates with glass bottom and exposed to cocaine (10 μM) or LPS (50 ng/ml) for 24 h followed by addition of ATP (2.5 mM) or left untreated for 1 h. The FLICA probe was added for 1 h, followed by washing and fluorescence imaging (**b**). Densitometric quantification of FLICA fluorescence (**c**). Expression of procaspase-1 and cleaved caspase-1, p20. Cleaved caspase-1 p20 is shown at both low- and high exposure (**d**). The high exposure images of caspase-1 p20 were quantified (**e**). All experiments were done at least three independent times. Data are shown as mean ± SEM and *$p < 0.05$ vs. control
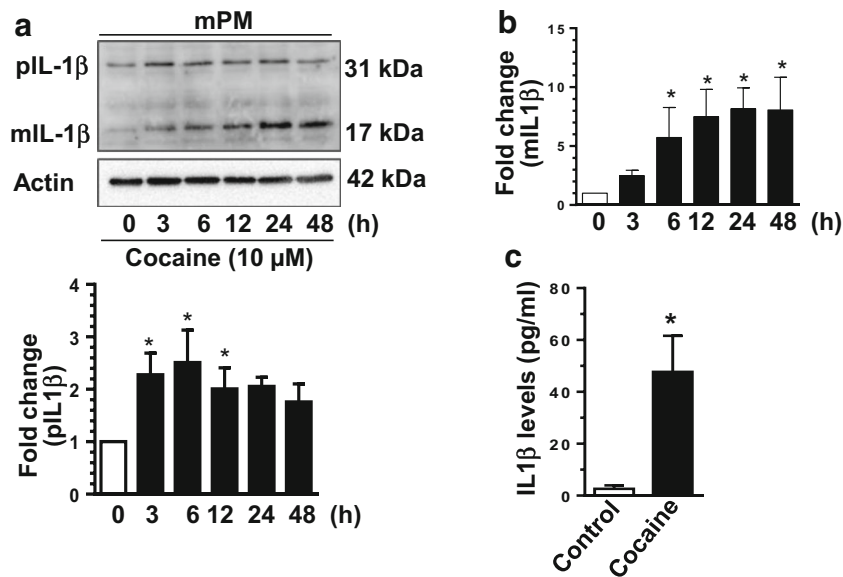
Fig. 3 Cocaine mediated the maturation of IL-1β in microglia. Mouse primary microglia (mPMs) were seeded into 6-well plates and exposed to cocaine (10 μM) for the indicated time periods. Exposure to cocaine induced the expression of mature IL-1β (mIL-1β) time-dependently (**a**, **b**). Cocaine exposure resulted in increased release of IL-1β into supernatants from mPMs as quantified by ELISA (**c**). ELISA experiments were done with 6 replicates per each condition. All experiments were done at least three independent times. Actin served as a loading control. Quantification of pro IL-1β (pIL-1β) and mIL-1β is shown in (**a**) and (**b**) respectively. Data are shown as mean ± SEM and *p < 0.05 vs. control

Fig. 4 Cocaine-mediated NLPR3 inflammasome and cellular activation was attenuated by *NLRP3* siRNA. Mouse primary microglia were seeded into 6-well plates and treated with either siNLRP3 or control siRNA. Cells were subsequently exposed to cocaine (10 μM) or left untreated for 24 h and expression of NLRP3, IL-1β, Iba-1, and CD11b analyzed by Western blotting. NLRP3 expression was reduced by siNLRP3 knockdown (**a**). Exposure of cells treated with siNLRP3 to cocaine did not increase NLRP3 levels compared with control cells (**a**); or mature IL-1b, Iba-1, and CD11b (**b–d** respectively). All experiments were done at least three independent times, and representative figures are shown. Actin served as a loading control and quantification of western blots are shown under each blot. Data are shown as mean ± SEM and *p < 0.05 vs. control or # vs. cocaine/control siRNA group
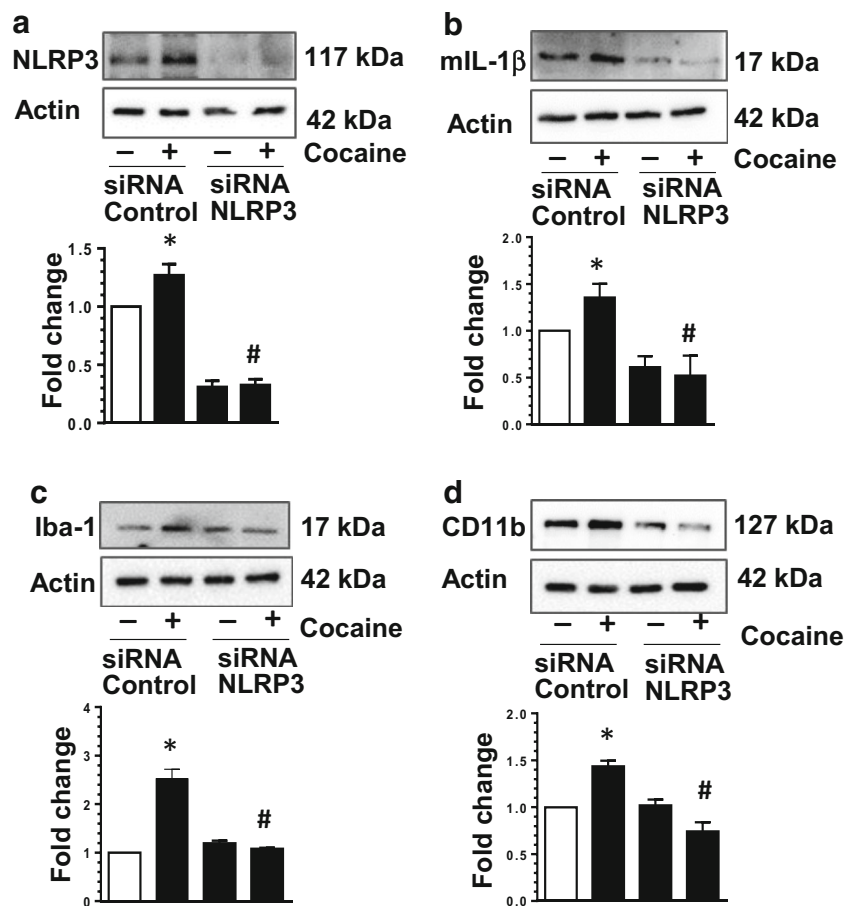
Fig. 5 Cocaine-induced ROS and the sigma receptor are involved in the upregulation of NLRP3 and cellular activation in microglia. Mouse primary microglia (mPMs) exposed to cocaine express the sigma-1 receptor (**a**). mPMs were pretreated with BD1047 (10 μM) for 1 h followed by exposure to cocaine for 24 h. Exposure of cells to cocaine resulted in increased expression of NLRP3. Pre-treatment of cells with BD1047 attenuated cocaine-mediated upregulation of NLRP3 expression (**b**). Similarly, BD1047 attenuated cocaine-mediated upregulation of CD11b (**c**). mPMs were pretreated with tempol (50 μM) for 1 h followed by exposure to cocaine for 24 h. Exposure of cells to cocaine resulted in increased expression of NLRP3. Pre-treatment of cells with tempol attenuated cocaine-mediated upregulation of NLRP3 expression (**d**). Similarly, tempol attenuated cocaine-mediated upregulation of CD11b (**e**). All experiments were done at least three independent times, and representative figures are shown. Actin served as a loading control and quantification of Western blots are shown under each blot. Data are shown as mean ± SEM and *$p < 0.05$ vs. control or # vs. cocaine group
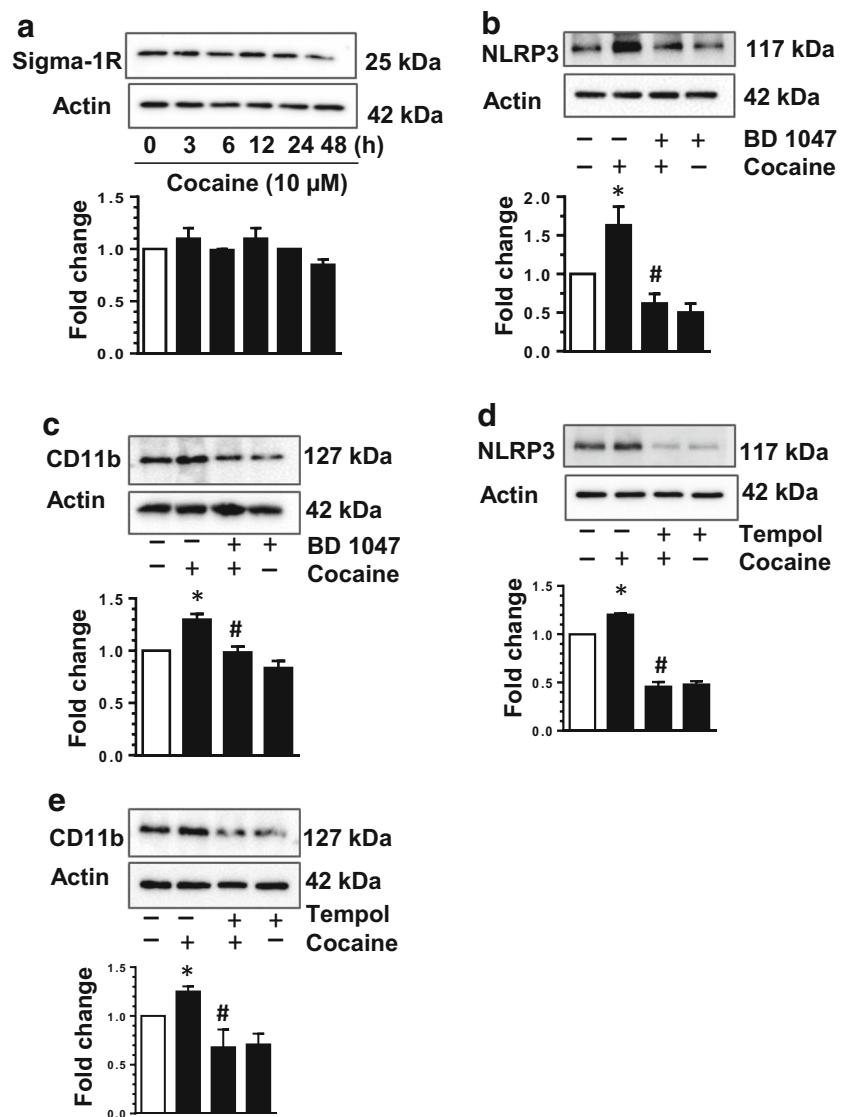

with the findings on NLRP3 expression, tempol pretreatment also abrogated cocaine-mediated upregulation of the microglial activation marker, CD11b (Fig. 5e), thereby underscoring the role of ROS in cocaine-mediated activation of microglia. Together, these data suggest that ROS lies upstream of cocaine-mediated induction of the NLRP3 inflammasome and cellular activation, thereby underscoring the potential role of ROS inhibitor(s) in ameliorating cocaine-mediated activation of the NLRP3 inflammasome.

In Vivo Administration of Cocaine Upregulated the Expression of NLRP3 Inflammasome Mediators and Microglial Activation

Having demonstrated the effects of cocaine on the NLRP3 inflammasome and microglial activation in vitro, we next sought to validate these findings in vivo in mice administered cocaine for 7 days. Mice were randomly assigned into two

groups ($n = 4$/group) and administered either saline or cocaine (20 mg/kg, i.p.) once a day for 7 consecutive days. One hour following the last cocaine injection, mice were sacrificed, brains removed, and striatal tissue homogenates assessed for expression of NLRP3 as well as markers of microglial activation. Our findings demonstrated that cocaine administration resulted in significant upregulation of NLRP3 in the striatal brain tissues compared with the striatum from saline-injected controls (Fig. 6a). Additionally, to further validate these findings, we also assessed the expression of mediators such as caspase-1, ASC, and IL-1β in the striatum of cocaine-administered mice. As shown in Fig. 6b–d, expression of pro- and cleaved forms of caspase-1, ASC, and mature IL-1β was significantly upregulated in cocaine-administered mice compared with the saline-injected controls. To further ascertain the role of microglia in this process, we also monitored the expression of microglial activation markers CD11b and Iba-1 in the brains of these mice. As expected, and similar
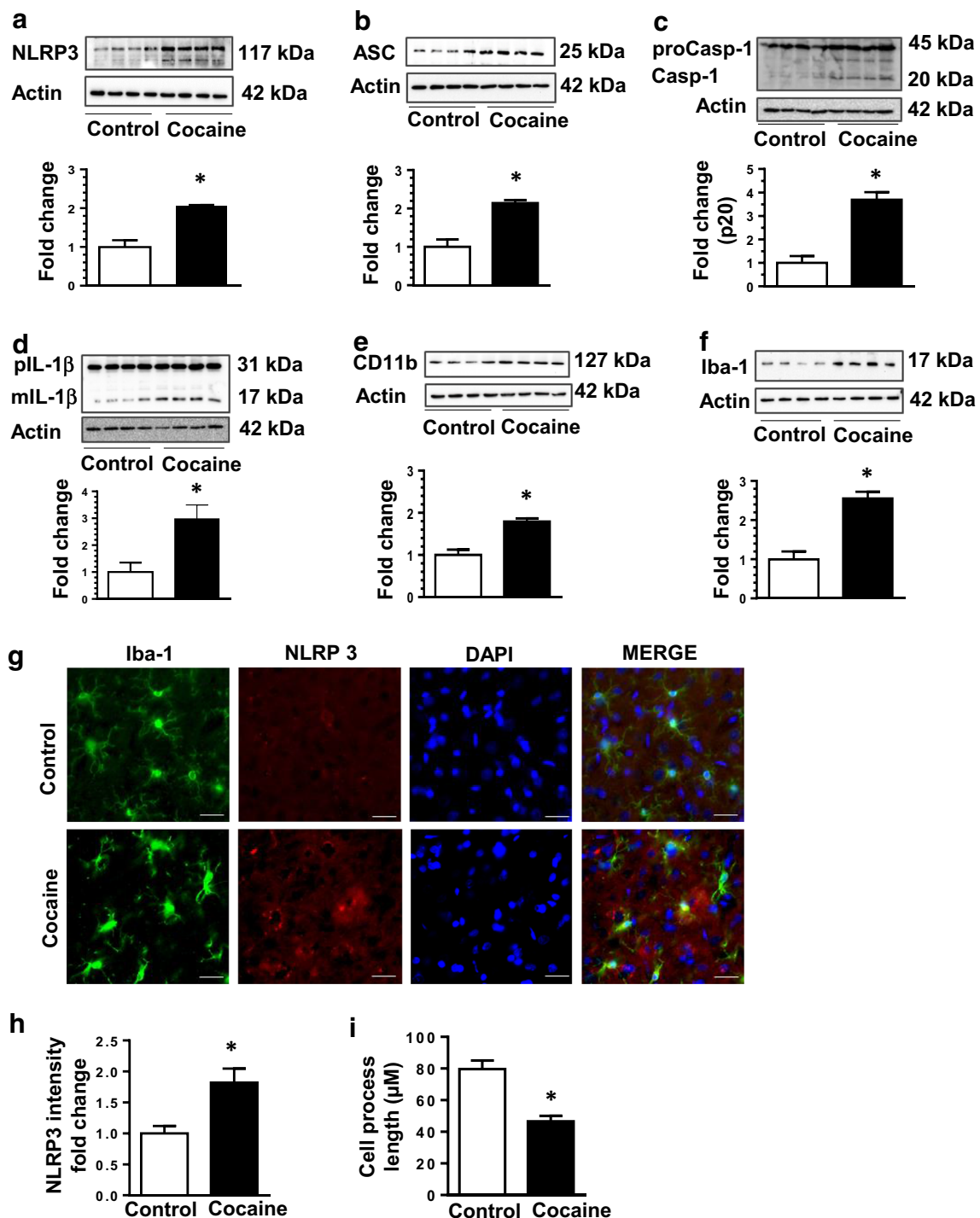
Fig. 6 Cocaine upregulated the expression of NLRP3 pathway and microglial activation in the striatum. Wild-type mice (C57BL/6) were administered cocaine (i.p. 20 mg/kg) or saline for 7 consecutive days followed by euthanasia within 1 h of the last injection and removal of brains for analysis of NLRP3 pathway mediators. The expression of NLRP3, ASC, caspase-1, and IL-1β were significantly upregulated in the striatum (**a**–**d**). The expression of microglial markers CD11b and Iba-1 was upregulated in cocaine treated mice (**e**, **f**). Immunofluorescence analysis similarly showed increased NLRP3 and Iba-1 staining in presence of cocaine (**g**) and the fold change in NLRP3 fluorescence intensity analyzed (**h**). Cocaine administration in these mice decreased microglial cell process length (i). $N = 4$/group. Quantification of Western blots is shown under each blot. Data are shown as mean ± SEM and *$p < 0.05$

to our in vitro studies, cocaine administration upregulated the expression of CD11b and Iba-1 proteins (Fig. 6 e and f) compared with the saline administered controls.

To further validate cocaine-mediated activation of the microglial NLRP3 inflammasome, we also performed immunofluorescence staining for NLRP3 and Iba-1 or ASC in

striatal brain sections obtained from mice administered either cocaine or saline for 7 consecutive days. Our results demonstrated increased expression of NLRP3 in Iba-1-positive microglial cells in the striatum of cocaine administered mice versus control animals (Fig. 6g). As shown in Fig. 6h, the fluorescence intensity of NLRP3 in cocaine-administered mice was upregulated (1.82-fold, $p < 0.05$) compared with control mice. Furthermore, microglial process length was significantly reduced in cocaine-administered mice compared to control mice (Fig. 6i). As shown in supplementary Fig. 1, formation of ASC specks was increased in Iba-1-positive cells in cocaine-administered mice compared with control animals. Increased expression of ASC is also seen in non-Iba-1-positive cells. Together, these data suggest that cocaine exposure modulates the NLRP3 inflammasome in vivo in the brains of mice.

Pharmacological Inhibition of NLRP3 Attenuated Cocaine-Induced Upregulation of Inflammasome Markers and Microglial Activation

Next, we investigated in vivo the effects of pharmacological inhibition of NLRP3 by the small molecule inhibitor, MCC950. C57BL/C mice were randomly divided into four groups; receiving cocaine or saline injections for seven consecutive days in the presence or absence of MCC950 pretreatment. Mice were injected with NLRP3 inhibitor MCC950 (50 mg/kg or vehicle, i.p.) followed by cocaine administration (20 mg/kg, i.p.) an hour later, daily. On the seventh day, mice were euthanized and brains removed for expression of proteins by Western blotting. As shown in Fig. 7a and b and as expected, cocaine increased the expression of mature IL-1β in both the striatum and cortex compared with control animals. Importantly, pre-treatment with MCC950 suppressed the expression of mature IL-1β in both the brain regions (Fig. 7a and b), thereby suggesting MCC950 mediated inhibition of cocaine-induced NLRP3 inflammasome activation. We also monitored the expression of the microglial activation marker CD11b in the striatum and cortex regions of these mice and found that MCC950 also suppressed cocaine-induced expression of CD11b (Fig. 7c, d).

Chronic Cocaine Dependence Upregulated the Expression of NLRP3 Pathway Mediators and Microglial Activation in Human Brains of Cocaine Addicts

We next sought to validate the effects of cocaine on the NLRP3 inflammasome in archival human brain sections with or without cocaine abuse. To address this, we assessed the expression of NLRP3 pathway mediators in frontal cortices of postmortem brain tissues from cocaine abusers and non-cocaine using controls. We found increased expression of

NLRP3, ASC, caspase-1, and IL-1β in the brains of cocaine abusers compared with cocaine naïve controls (Fig. 8a–d). As shown in Fig. 8 e and f, expression of the microglial markers Iba-1 and CD11b was also increased in the brains of cocaine addicts compared with cocaine naive controls. Together, these results suggest the involvement of NLRP3 inflammasome and microglial activation in cocaine-induced inflammation in humans with chronic cocaine use.

Discussion

CUD remains a significant public health concern globally that affects up to 22 million people worldwide and is closely associated with severe dysregulation of the immune system in both periphery and the CNS [1, 2, 7–9]. Previous studies have demonstrated the involvement of reactive oxygen species, activation of NF-kB, and induction of proinflammatory cytokines such as IL-1β and TNFα, in functional neurocognitive decline associated with cocaine [6–8, 11, 15, 41, 42]. Interestingly, while activation of the NLRP3 inflammasome has been implicated in cocaine-induced activation of peripheral macrophages [35], the causal association between NLRP3 activation, ROS, and cocaine-induced behavioral changes remains poorly understood. The current study provides insights into the mechanism(s) of cocaine-induced priming and activation of the NLRP3 inflammasome. Our findings suggest that cocaine administration activates the NLRP3 inflammasome resulting in increased levels of proinflammatory IL-1β.

NLRP3 is highly expressed in microglia and is activated by various external and internal stimuli including urea crystals, asbestos, and viral and bacterial proteins [30–32, 43]. Activation of the NLRP3 inflammasome leads to increased levels of IL-1β, which, in turn, modulates neuronal excitability of the neighboring cells [44–46]. NLRP3 inflammasome has been demonstrated to play critical roles in microglial overactivation, which is an underlying mechanism critical for promoting the pathogenesis of multiple neurodegenerative diseases including Parkinson's diseases (PD), Alzheimer's disease, and multiple sclerosis [47–52]. Additionally, chronic unpredictable mild stress has also been reported to accelerate LPS-induced NLRP3 inflammasome activation in the PD model of rats, resulting in the death of dopaminergic neurons in this rat model, thereby signifying the importance of microglial activation in NLRP3 signaling [53]. Cocaine is well-known to activate glial cells in both in vitro and in vivo model systems [22, 36, 54]. Whether NLRP3 is also involved in cocaine-mediated activation of microglia and subsequent behavioral changes has not been explored. In this study, we demonstrated that exposure of mPMs to cocaine resulted in the upregulation of NLRP3, followed by recruitment of ASC and release of IL-1β from microglial cells. Our findings suggested that a physiologically relevant dose of cocaine (10 μM)

Fig. 7 Pharmacological inhibition of NLRP3 attenuated cocaine-induced upregulation of inflammasome markers and microglial activation. C57BL/6 wild-type mice were divided into groups; receiving cocaine or saline injections for seven consecutive days in the presence or absence of MCC950 pretreatment. Mice were injected with NLRP3 inhibitor MCC950 (50 mg/kg or vehicle, i.p.) daily followed by cocaine administration (20 mg/kg, i.p.) an hour later. Mice were euthanasia within 1 h of the last injection followed by removal of brains for analysis of IL-1β and microglial activation. Cocaine increased the expression of mature IL-1β in both the striatum and cortex compared with control animals. Pre-treatment with MCC950 suppressed the expression of mature IL-1β in both the brain regions (**a**, **b**). The expression of microglial activation markers Iba-1 and CD11b in the striatum and cortex regions was attenuated by MCC950 (**c**, **d**). *N* = 3/group. Quantification of western blots is shown under each blot. Data are shown as mean ± SEM and *p < 0.05
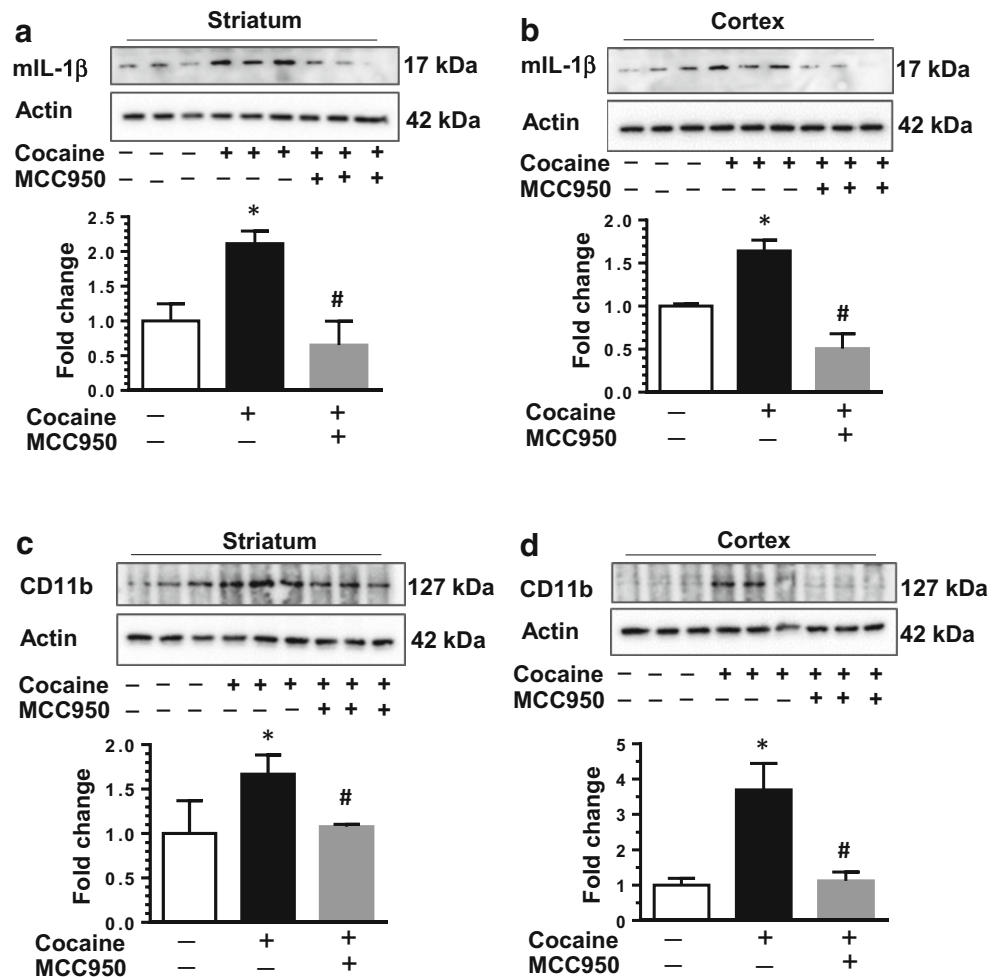

resulted in activation of the NLRP3 inflammasome as demonstrated by increased expression of mature IL-1β in microglia. It has been shown that the plasma levels of cocaine in humans who administered cocaine intranasally range between 0.4 and 1.6 μM [55], while the plasma cocaine levels in tolerant abusers was found to be 13 μM [56]. Further, the levels of cocaine in postmortem brains of chronic cocaine users with acute intoxications has been found to be greater than 100 μM [57]. Cocaine concentrations used in this study are in keeping with the physiological levels observed in humans abusing cocaine. Our in vivo validation study also demonstrated that the expression of multiple NLRP3 pathway mediators (NLRP3, ASC, caspase-1, and IL-1β) as well as microglial activation (Iba-1 and CD11b) was upregulated in the striatum of mice administered cocaine repeatedly (20 mg/kg i.p., daily for 7 days) as well as in humans with reported chronic cocaine abuse compared to the levels in the control brains, thereby underscoring the involvement of NLRP3 in cocaine-induced microglial activation and neuroinflammation. Furthermore, our results demonstrating increased expression of Iba-1 in microglia exposed to cocaine are consistent with previous

studies that have also shown upregulation of Iba-1 following cocaine exposure [58–60].

To examine the molecular changes involved in cocaine-mediated activation of microglia, we employed pharmacological approaches. Our findings demonstrated that pharmacological inhibition of ROS using tempol reduced cocaine-mediated priming of NLRP3 inflammasome. These data thus implicate the role of ROS in cocaine-induced priming of NLRP3, a finding that is consistent with previous studies showing ROS-mediated priming of the NLRP3 inflammasome [12, 13, 15]. Tempol has been previously shown to reduce oxidative damage and the development of behavioral sensitization in rodents [17]. Recently, in an in vitro model of diabetic nephropathy, exposure of HK-2 cells (an immortalized proximal tubule epithelial cell line from normal adult human kidney) to high glucose demonstrated increased expression of ROS-mediated NLRP3 activation, which was notably inhibited in cells pretreated with Tempol [61]. Our findings also suggest that ROS elevation lies upstream of NLRP3 activation.

Our in vivo findings demonstrated that expression of mature IL-1β was upregulated in the striatum of cocaine-

Fig. 8 Chronic cocaine dependence upregulated the expression of NLRP3 pathway mediators and microglial activation in human brains of cocaine addicts. Postmortem cortex brain tissues from chronic cocaine-dependent addicts and non-cocaine using controls were analyzed for the expression of NLRP3 pathway mediators. The expression of NLRP3, ASC, caspase-1, and IL-1β was increased in the brains of cocaine addicts compared with no-cocaine using controls (**a**–**d**). As shown in **e** and **f**, expression of the microglial markers Iba-1 and CD11b was also increased in the brains of cocaine abusers compared with no-cocaine using controls. $N = 4$/group. Quantification of Western blots is shown under each blot. Data are shown as mean ± SEM and *$p < 0.05$
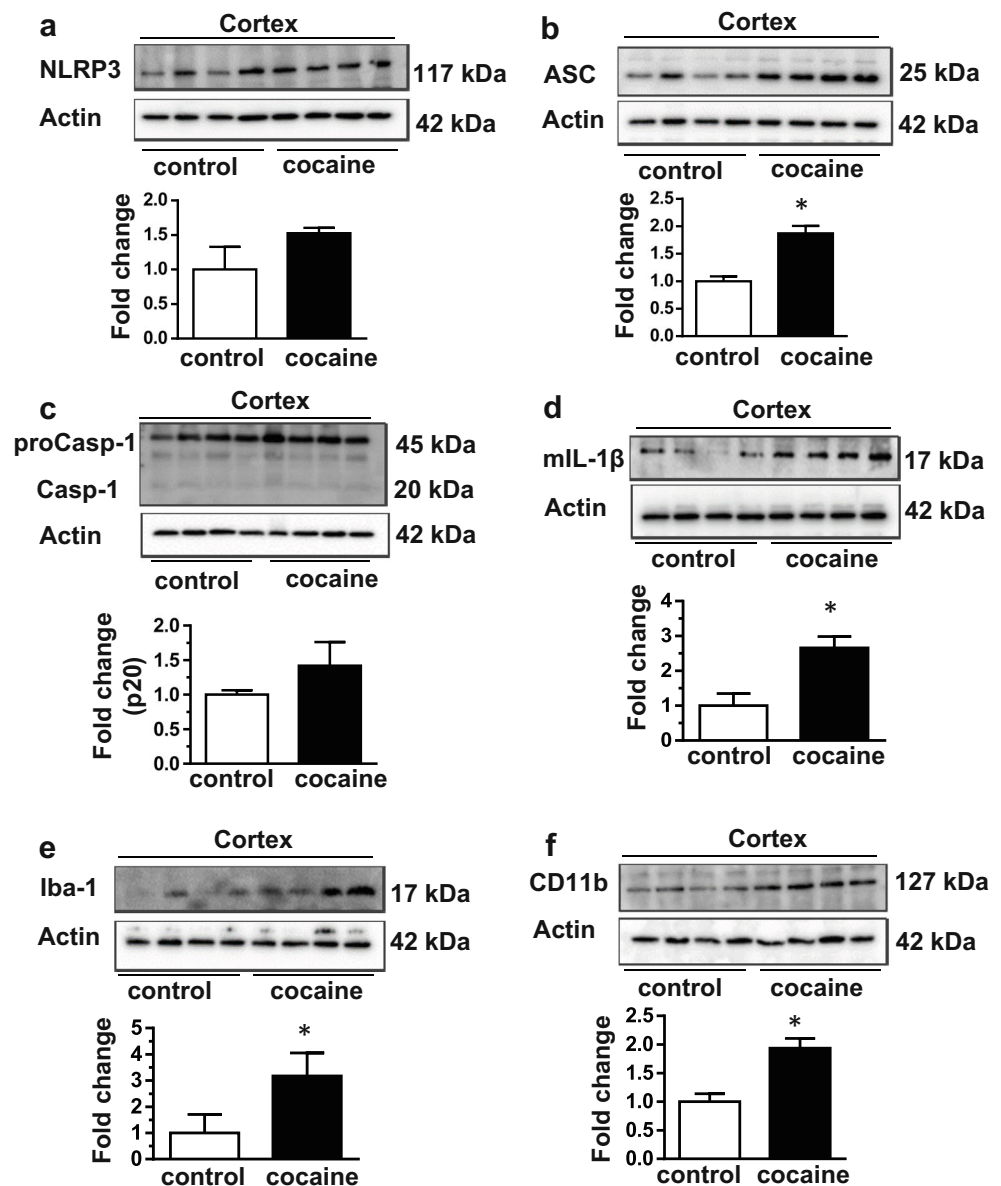
administered mice compared with control mice, thus suggesting cocaine-induced cleavage of pro-IL-1β. In keeping with these findings, it has also been reported that circulating levels of IL-1β are increased in humans with crack-cocaine disorders and could be associated with an activation of the reward, immune, and inflammatory systems [8]. Activation of the NLRP3 inflammasome by mechanism(s) involving ROS, lysosome rapture, or potassium efflux has been well documented [12, 30–32, 62, 63]. The effects of cocaine on the cleavage and release of mature IL-1β, however, has not yet been clearly elucidated. In this study, we provide data that cocaine-exposed microglia activate NLRP3 inflammasome signaling with the release of IL-1β involving the upstream activation of ROS. Studies are underway to ascertain the detailed molecular mechanism(s) underlying cocaine-mediated activation of the NLRP3 inflammasome.

The critical role of NLRP3 in cocaine-mediated microglial activation was further explored by both pharmacological and gene silencing (siRNA) approaches. Our results demonstrated that both MCC950 (NLRP3 inhibitor) as well as the NLRP3 siRNA attenuated cocaine-mediated activation of the NLRP3 inflammasome, as evidenced by the reduced expression of mature IL-1β. There are numerous studies reporting the use of MCC950 as a potential pharmacological inhibitor of NLRP3 to alleviate the NLRP3-mediated immune activation in various disease settings [52, 64–78]. Our findings also demonstrated that both pharmacological inhibition and gene silencing of NLRP3 resulted in significant inhibition of cocaine-mediated expression of IL-1β and activation of microglia, as evidenced by decreased expression of both Iba-1 and CD11b. Our results thus suggest that targeting the NLRP3
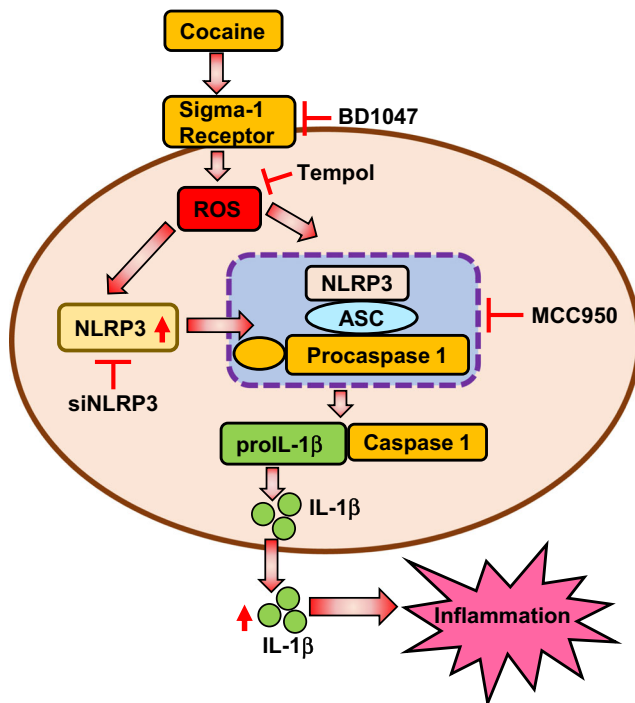
Fig. 9 Schematic description of cocaine-mediated activation of the NLRP3 inflammasome in microglia. Cocaine upregulates the expression of NLRP3 and increases generation of ROS. The NLRP3 inflammasome is subsequently activated resulting in the maturation of IL-β that contributes to inflammation. Blockade of ROS (Tempol) attenuates cocaine-mediated induction of NLRP3. Blockade of NLRP3 by either pharmacological (MCC950) or gene silencing (siNLRP3) approaches reduced cocaine-mediated activation of the inflammasome

inflammasome could be developed as an adjunctive therapeutic approach to ameliorate the deleterious effects of cocaine.

Conclusion

In summary, herein we report that the NLPR3 inflammasome signaling involving generation of reactive oxygen species plays a role in cocaine-mediated activation of microglia. Blockade of NLRP3 reduced cocaine-mediated activation of NLRP3 inflammasome and ensuing microglial activation and inflammation is depicted in Fig. 9. Taken together, inhibiting the NLRP3 inflammasome could thus be developed as a potential therapeutic strategy for dampening cocaine-mediated neuroinflammation.

Data Availability All data generated or analyzed during this study are included in this published article and its supplementary information files.

Compliance with Ethical Standards

Competing Interests The authors declare that they have no competing interests.

Ethics Approval and Consent to Participate All animal procedures were performed according to the protocols approved by the Institutional Animal Care and Use Committee of the University of Nebraska Medical Centre and the National Institutes of Health.

Consent for publication Not applicable.

Code Availability Not applicable.

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