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Methamphetamine exacerbates neuroinflammatory response to lipopolysaccharide by activating dopamine D1-like receptors

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ABSTRACT

Methamphetamine (METH) is a highly addictive and widely abused drug worldwide. Although much research is on the drug's direct effects, METH may also alter host immunity. The mechanism by which METH influences immunity remains elusive. Here, C57BL6/J mice were intraperitoneally injected with 5 mg/kg METH four times at two-hour intervals. The microglial inhibitor minocycline or dopamine D1-like receptor antagonist SCH-23390 was also applied prior to METH injection. Twenty-four hours following the first METH injection, mice were challenged by lipopolysaccharide (LPS) at a dose of 330 μg/kg, and the hippocampus (Hip), caudate putamen (CPU), nucleus accumbens (NAc) and prefrontal cortex (PFC) were collected 4 h after LPS administration. IL-6 and TNF-α levels were detected by ELISA. The activation of D1-like receptors and microglial marker Iba1 were examined by immunohistochemical staining and Western blot. Finally, we examined the phosphorylation of ERK1/2 and CREB. We found that METH exposure increased LPS-induced IL-6 and TNF-α production in the Hip, CPU and NAc regions. METH also augmented microglia activation and D1/5DR expression in response to LPS. Moreover, administering SCH-23390 significantly reduced IL-6 and TNF-α production and Iba1 expression following LPS challenge. Similar inhibitory effects were also observed by minocycline administration. Moreover, phosphorylation of ERK1/2 and CREB was increased after METH and LPS exposure but decreased by SCH-23390. These data illustrate that METH exacerbates neuroinflammation response in LPS-stimulated mouse brains through dopamine D1-like receptors, microglia, and relevant signaling proteins, which may have therapeutic implications.

1. Introduction

Methamphetamine (METH) is a highly addictive and widely consumptive drug in the world [1]. METH can be rapidly delivered to the brain to induce immediate and intense euphoria [2]. However, METH also results in adverse neurodegenerative effects and neuropsychiatric complications, including long-lasting brain dysfunction and abnormal behaviors [3]. Mechanisms underlying these adverse impacts have garnered intense interest in the past several decades. Besides the direct impact on neurons, METH-induced immune regulation also attracts increasing attention in recent years as dysfunction of the immune system may result in pathological consequence in the central nervous system (CNS) [4–6]. For example, higher levels of pro-inflammatory cytokines including tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) are found in the brain of Parkinson's patients, which may be a potential mediator or even initiator of the disease [7]. In traumatic brain injury, neuroinflammation is proposed as an important aspect of secondary injury in animal and human studies [8]. Given the serious neurotoxicity, METH is an important focal point for mechanistic understanding inflammation in CNS.

In the periphery, METH clutters the production of cytokines and the population of immune cells with or without subsequent peripheral immune stimulation [9,10]. But in the CNS, most research focuses on the consequences of METH itself while limited studies have been conducted to determine its relevance to immune stimulation. With a contaminated needle for drug abuse, METH takers are likely to expose to

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pathogens and highly susceptible to infectious diseases [11]. Thus, it is important to elucidate the impact of METH in immune function with an animal model. In the brain, microglia are the predominant immune cells of the brain and play a substantial role in immune surveillance and inflammatory reactions [12]. METH can activate microglia, which has been observed in animal subjects and human users [13]. Additionally, microglia are the primary source of pro-inflammatory mediators, such as IL-6 and TNF-α, which may cause neuronal damage [12]. Therefore, the influence of METH on microglia and the underlying mechanism need to be better characterized.

METH functions as a regulator of dopamine (DA) *in vivo*, including inducing a rapid decrease in DA transporter (DAT), redistributing cytosolic DA and up-regulating its extracellular levels [14]. Currently, DA receptors (DARs) are recognized and subdivided into two families: D1-like DARs (D1DR and D5DR) and D2-like DARs (D2DR, D3DR and D4DR) [15]. These receptors, especially D1-like DARs, are reported to be involved in METH-related CNS dysfunction [15–17]. In addition, several lines of evidence indicate their potential roles in immune regulation, such as the proliferation and migration of immune cells and synthesis of cytokines, by altering the activation of extracellular signal-regulated kinase 1/2 (ERK1/2) and cAMP-response element binding protein (CREB) [18–21]. Importantly, D1-like DARs are highly expressed on the surface of microglia [22]. Herein, we hypothesized that both microglia and D1-like DARs may be involved in METH-mediated neuroinflammation.

We investigated the roles of METH in immune response to peripheral lipopolysaccharide (LPS) challenge in various brain regions of C57BL/6 mice, hippocampus (Hip), caudate putamen (CPU), nucleus accumbens (NAc) and prefrontal cortex (PFC). These regions have been chosen because they are strongly associated with behavioral disorders and neurotoxicity induced by METH [23–25]. We detected the activation of microglia, expression of D1-like DARs and phosphorylation levels of ERK1/2 and CREB. We found that exposure to METH increases the LPS-induced inflammatory cytokines and activates D1-like DARs and microglia in mouse brains.

2. Materials and methods

2.1. Animals

Male C57BL/6 mice were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. Eight-week-old mice (weighing approximately 22 ± 2 g) were used. Three or four animals per cage were bred under specific pathogen-free conditions in Xi'an Jiaotong University's animal facility, in which the environment was 23 ± 1 °C, 50 ± 5% humidity with a 12:12 h light/dark cycle (lights on at 07:00). Autoclaved food and water were available *ad libitum*. All mice were divided into experimental and control groups using a table of random numbers. The investigators were blinded to all the groups, until the end of the experiment. All experimental procedures were pre-registered by the Institutional Animal Care and Use Committee of Xi'an Jiaotong University and conducted according to the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (United States). Every effort was made to minimize the number of animals and avoid unnecessary harm to the animals.

2.2. Drugs

METH (methamphetamine hydrochloride) was provided by the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, People's Republic of China). *Escherichia coli* LPS (serotype O55:B55) and D1-like receptors inhibitor SCH-23390 were purchased from Sigma-Aldrich (St. Louis, U.S.A.). Minocycline was bought from Selleck Chemical (Houston, U.S.A.). METH, LPS and SCH-23390 were dissolved in sterile 0.9% physiological saline before experiments.

2.3. Animal treatments

METH was injected i.p. at a dose of 5 mg METH·HCl/kg body weight four times at 2 h intervals. This dosing schedule was based on the reports by Thomas et al. and expected to regulate the immune response in brains [26,27]. Twenty-four hours after the first METH injection, mice received i.p. injections of LPS (330 μg/kg). The injection volume for METH and LPS was controlled at 0.01 ml/g. Minocycline (10 mg/kg or 40 mg/kg) and SCH-23390 (0.05 mg/kg or 0.5 mg/kg) were injected 30 min before METH when needed. Control mice received an equivalent volume of saline. Four hours after LPS injection, mice were anesthetized and sacrificed by decapitation.

2.4. Tissue preparation

Immediately after the mice were killed, Hip, PFC, CPU, and NAc were dissected bilaterally on dry ice. The samples were homogenized in an ice-cold RIPA buffer (Solarbio, China) with protease and phosphatase inhibitors cocktail (Roche, Switzerland). The mixtures were incubated on ice for 30 min and centrifuged at 12,000*g* for 15 min at 4 °C. Supernatants were collected and protein concentrations were determined by a BCA protein assay kit (Beyotime, China). Then, they were stored at −80 °C until further use.

2.5. Immunohistochemistry

After being deeply anesthetized, the mice were perfused transcardially with sterile 0.9% physiological saline and 4% paraformaldehyde. The whole brain was removed and sliced into three parts. They were post-fixed in 4% paraformaldehyde for 1 day and then dehydrated by 30% sucrose. All brain tissues were embedded in OCT and sectioned coronally into 12 μm-thick sections using a freezing microtome. One section per 120 μm was picked for the following experiment, and 4 sections were used per animal. All of the sections were adhered to a glass slide, treated by 3% hydrogen peroxide for 10 min and blocked by normal goat serum for 30 min. The sections were then incubated with rabbit anti-mouse D1-like DARs antibody (1:100, Santa Cruz, U.S.A.) overnight at 4 °C. After washing by PBS, the sections were then incubated with horseradish peroxidase (HRP)-labeled goat anti-rabbit immunoglobulin G (IgG, Abgent, U.S.A.) for 30 min and subsequently stained by DAB (Boster Biological Technology, China). As a control, the primary antibody was omitted, or isotypic antibody (normal rabbit IgG) was used to confirm the specificity of immunohistochemistry. Histological sections were photographed with an Olympus BX-51 microscope and DP71 camera (Olympus, Japan). Immunoreactivity was quantified using Image J software by double blind assessment. The mean optical density (MOD) and area ratio of DRs positive cells (area of positive signal/area of region of interest) were measured.

2.6. Western blot

The samples were adjusted to the same protein concentrations and treated with a 5 × SDS-PAGE loading buffer including β-mercaptoethanol (5%, w/v). Then, they were denatured at 95 °C for 5 min. The samples were separated on 10% SDS-PAGE gels and electrophoretically transferred onto polyvinylidene fluoride membranes (PVDF, 0.22 μm, Millipore, U.S.A.) with a semidry blotting system (BIO-RAD, U.S.A.). The loading quantity of protein sample for ERK1/2 and CREB were 10 mg and 20 mg, respectively. After blocking with 5% non-fat milk for 2 h, the membranes were incubated with primary antibodies overnight at 4 °C. p-ERK1/2 and ERK1/2 (Cell Signaling Technology, U.S.A.) were used at 1:2000 dilutions, and p-CREB and CREB (Cell Signaling Technology, U.S.A.) were used at 1:1000 dilutions. After washing, the membranes were incubated with HRP-conjugated secondary antibody (1:1000) for 1 h. Enhanced chemiluminescent substrate (ECL, Thermo Fisher Scientific, U.S.A.) was used to visualize the bands in the

developing machine (Fusion Fx5, China). The pictures were evaluated by the gel image analysis software ImageJ 2.1.4.7.

2.7. Cytokine analysis

After the protein concentrations were determined by the BCA protein assay kit, cytokines were measured in duplicate by enzyme-linked immunosorbent assay (ELISA, eBioscience, U.S.A.) according to the manufacturer's recommendations. The detection limit for IL-6 and TNF-α was 6.5 pg/ml and 3.7 pg/ml, respectively.

2.8. Statistical analysis

All data were presented as the means ± SEM. Before statistical analysis, normality and homogeneity of equal variance were confirmed. One-way analysis of variance (ANOVA) or two-way ANOVA was used. When ANOVAs revealed a significant effect of main factors or main factor interactions, differences in treatment group means were tested using Fisher's least-significant differences (LSD). Sample size was determined by our preliminary results and power analysis. No outlier or missing data were found. Statistical analyses were performed with IBM SPSS Statistics 20.0. $p < 0.05$ was considered statistically significant.

3. Results

3.1. METH administration aggravates LPS-induced neuroinflammation

We investigated whether METH administration would alter neuroinflammation response to a peripheral immune stimulus. As an effective stimulant, LPS was used to induce an inflammatory response in METH-treated mice [28]. We tested the changes in inflammatory cytokines in the four brain regions after METH administration and LPS challenge. LPS caused an increase in IL-6 production in the brain (Fig. 1a–d). A similar effect of LPS on TNF-α production was also observed (Fig. 1e–h). Importantly, METH-pretreatment strongly increased LPS-induced IL-6 in all the regions detected, while METH alone exhibited no effects (Fig. 1a–d). Similarly increased levels of TNF-α were also found in Hip, CPU and NAc while not in PFC (Fig. 1e–h). Taken together, these data suggest that METH exposure alone has not influenced the inflammation response but altered the immune response to LPS in mouse brains.

3.2. Microglia mediate METH-altered neuroinflammation

Microglia are the primary immune cells in the brain and promptly migrate to the site where it is challenged by external and internal factors [12]. We therefore investigated whether METH administration altered the microglial response to LPS. Iba1 is highly expressed in microglia, and is widely used as a marker for microglial activation [29]. Western blot showed that LPS influenced the activation of microglia in PFC but not Hip, CPU or NAc (Fig. 2a–d). METH not only increased Iba1 expression in Hip, NAc and PFC, but also further potentiated the stimulation following LPS challenge (Fig. 2a–d). These data suggest that METH exposure activates microglia to exert its regulatory role in LPS-induced neuroinflammation. To verify whether the altered neuroinflammation was due to microglial activation in the mice, we applied minocycline, an effective inhibitor of microglia to mice. Minocycline promotes M2 microglia polarization to exert anti-inflammatory activity in the CNS [30]. As shown, both low and high doses of minocycline showed no effect on IL-6 or TNF-α production in the four brain regions in resting mice (Fig. 2e–l). However, when injected before METH and LPS, a 40 mg/kg dose of minocycline significantly abolished the increase in IL-6 and TNF-α in four brain regions while a 10 mg/kg dose of minocycline inhibited the cytokines in NAc and PFC (Fig. 2e–l). These data indicate the potential function of microglia in METH-associated neuroinflammation.

3.3. METH up-regulates D1DR and D5DR expression

It is widely accepted that METH mainly acts on the synaptic cleft and plays a role in activation of DARs [14,31]. We previously found that METH altered IL-6 and TNF-α production *in vitro* by up-regulating the cAMP concentration [32]. Thus, we posit that D1-like DARs, which induce the accumulation of cAMP, might be regulated by METH exposure. After analyzed mean optical density (MOD) value, we found that both LPS and METH caused high expression of D1DR in CPU and

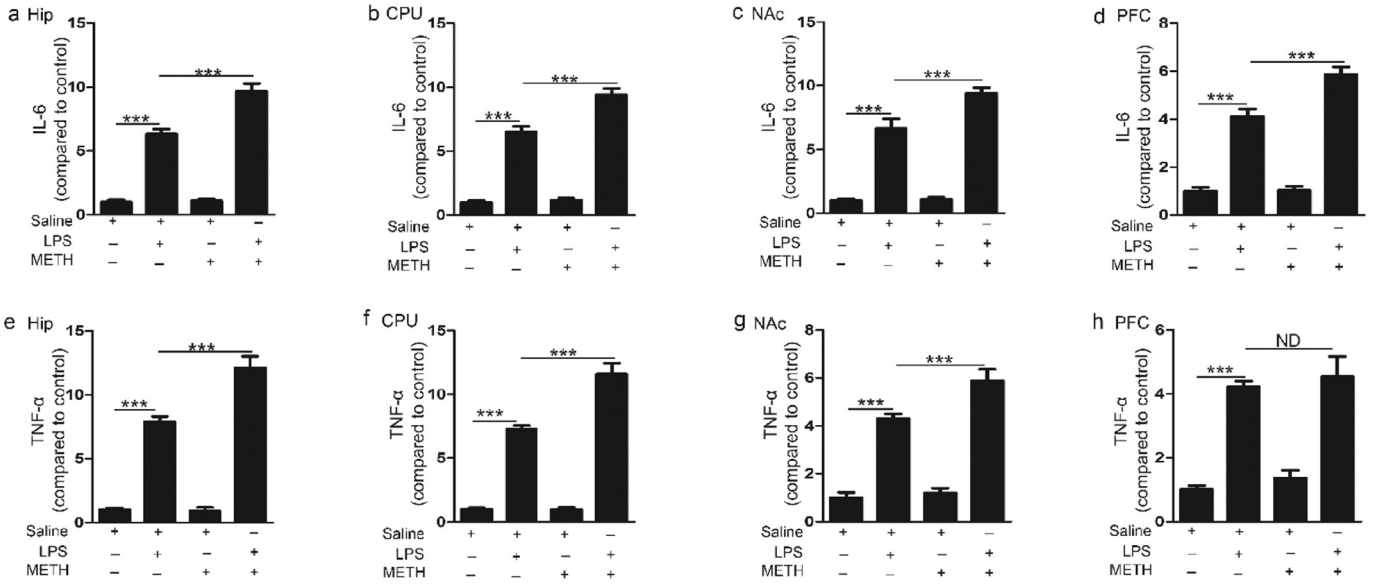

Fig. 1. Methamphetamine administration enhances LPS-induced neuroinflammation.
(a–d) IL-6 concentrations in Hip, CPU, NAc, and PFC. (e–h) TNF-α concentrations in Hip, CPU, NAc, and PFC. Mice were subjected to four injections of 5 mg/kg METH at 2 h intervals and then challenged by 330 μg/kg LPS 24 h after the first METH injection. Brain regions were collected 4 h after LPS administration. Data are represented as the means ± SEM, n = 6 mice/group. Statistical analysis was performed using two-way ANOVA and Fisher's LSD test. ***$p < 0.001$, ND means no significant difference.

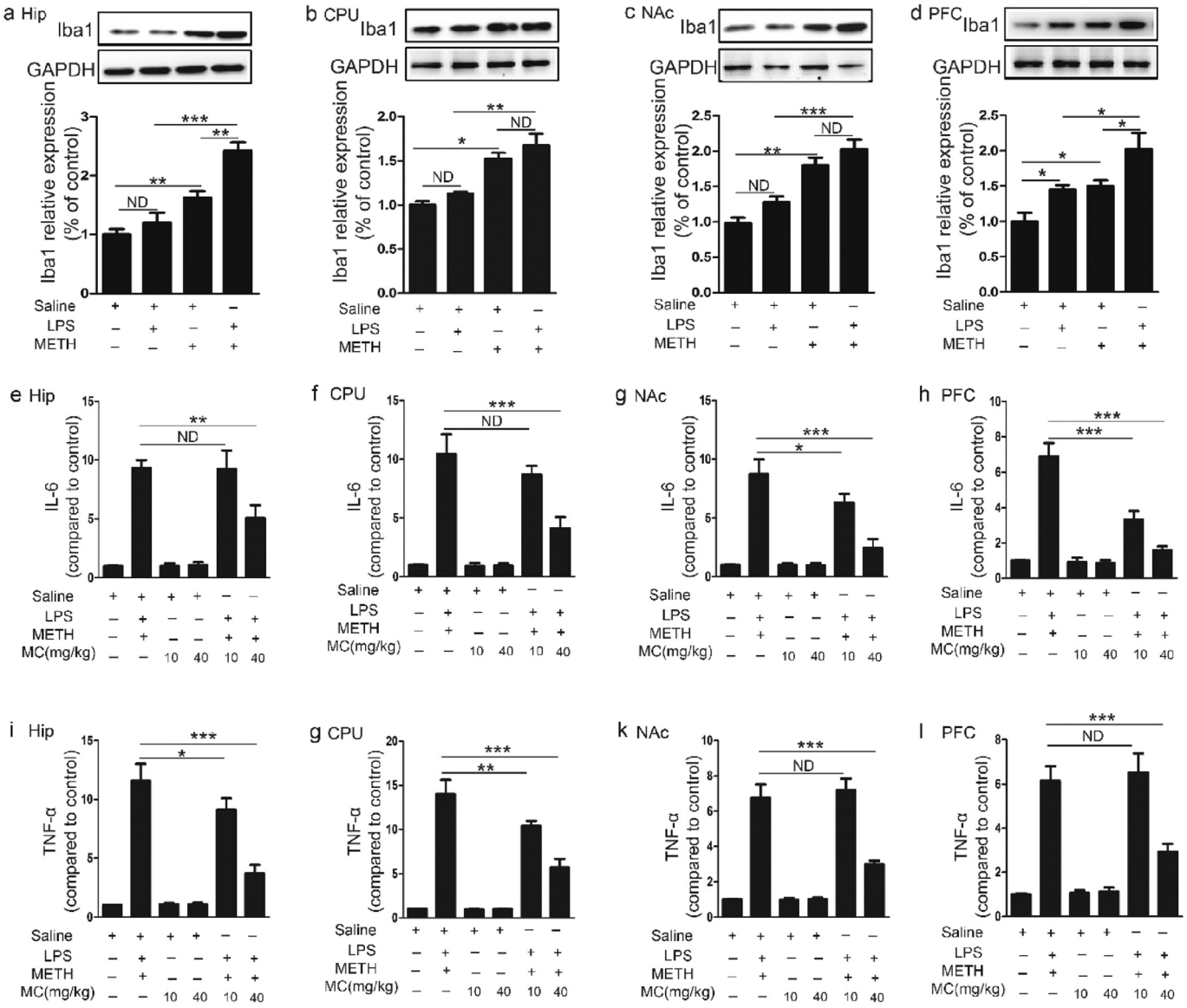

Fig. 2. Microglia mediate METH-altered neuroinflammation.
(a–d) Western blot of Iba1 in four brain regions. Mice were subjected to four injections of 5 mg/kg METH at 2 h intervals and then challenged by 330 μg/kg LPS 24 h after the first METH injection. Brain regions were collected 4 h after LPS administration. n = 4 mice/group. (e–l) IL-6 and TNF-α concentrations in four brain regions. Minocycline (MC, 10 mg/kg or 40 mg/kg) was given to mice 30 min before every injection of METH. n = 6 mice/group. Data are represented as the means ± SEM. Statistical analysis was performed using one-way (e–l) or two-way (a–d) ANOVA and Fisher's LSD test. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ND means no significant difference.

PFC (Fig. 3a–e). Importantly, METH exposure significantly exacerbated the LPS-induced increase in D1DR in four brain regions when compared to the LPS group (Fig. 3a–e). Same results were also obtained by analysis of D1DR-positive cell ratio (Fig. 3f–i). Meanwhile, D5DR expression was also increased by LPS in CPU and PFC and by METH in Hip, NAc and PFC (Fig. 4a–i). Remarkably, when compared to LPS group, pre-treatment with METH augmented the expression of D5DR in all the brain regions (Fig. 4a–i). Taken together, above data reveal the changes of D1-like DARs in mouse brains after administration of LPS and METH. These findings suggest that METH aggravates the expression of D1-like DARs, which may be responsible for the METH-mediated alteration of the LPS-induced neuroinflammation.

3.4. D1-like DARs are involved in METH-augmented neuroinflammation by regulating microglial activation

To further elucidate the involvement of D1-like DARs, their common antagonist SCH-23390 was used to block DARs in the following experiments. In sham controls, both low and high doses of SCH-23390 (0.05 mg/kg and 0.5 mg/kg) showed no effects on the production of IL-6 or TNF-α (Fig. 5a–h). 0.5 mg/kg SCH-23390 abolished the up-regulation of IL-6 and TNF-α production in four regions compared to the control group, whereas 0.05 mg/kg only inhibited levels of IL-6 in PFC and TNF-α in CPU by METH and LPS exposure (Fig. 5a–h). These findings imply the involvement of D1-like receptors in the METH-augmented neuroinflammation in response to LPS. In view of the accumulation of Iba1 marker, we speculated whether D1-like DARs modulated neuroinflammation by activating microglia. To determine this point, 0.5 mg/kg SCH-23390 was used to inhibit D1-like DARs and four brain regions were collected to evaluate the expression levels of Iba1. We found that SCH-23390 alone had no effects on Iba1 expression in all of the brain regions without stimulation (Fig. 5i–l). However, when given before the combination of METH and LPS, SCH-23390 significantly inhibited Iba1 production in Hip, CPU, NAc and PFC

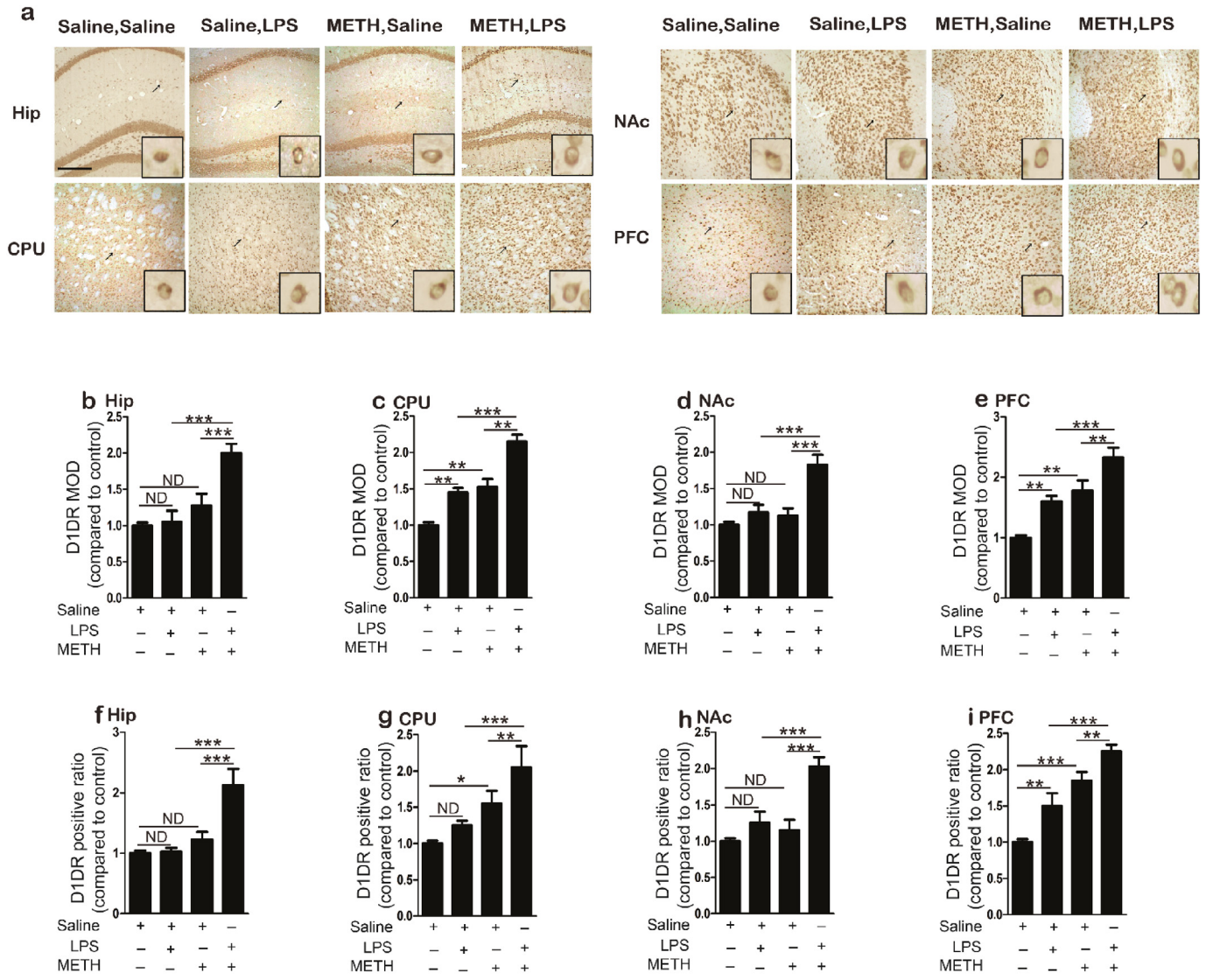

Fig. 3. METH promotes D1DR expression in LPS conditions.
(a) Immunohistochemical staining of D1DR in Hip, CPU, NAc, and PFC after the METH treatment. Mice were subjected to four injections of 5 mg/kg METH at 2 h intervals and then challenged by 330 μg/kg LPS 24 h after the first METH injection. (b–e) MOD value of D1DR in four brain regions. (f–i) D1DR-positive cells ratio in four brain regions. Scale bar = 200 μm. Data are represented as the means ± SEM, n = 4 mice/group. Statistical analysis was performed using two-way ANOVA and Fisher's LSD test. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, ND means no significant difference.

(Fig. 5i–l). These data suggest that D1-like DARs take part in METH/LPS-associated microglial activation and further alter the inflammation levels in mouse brain.

3.5. ERK1/2 and CREB are involved in the D1-like DARs-mediated neuroinflammation following METH/LPS exposure

The above results defined the role of D1-like DARs in METH-mediated neuroinflammation while the underlying signaling molecules remain to be defined. We previously found that both ERK1/2 and CREB signaling pathways were involved in METH-induced spatial memory changes [28]. Specifically, ERK1/2, a member of the MAPKs family, plays a role in METH-mediated behavioral alterations [28,33,34]. CREB as one of the most prominent transcription factors, is also critical in METH-initiated psychological disorders [35,36]. To elucidate the mechanistic detail in METH/LPS-mediated immune response to LPS in mouse brains, we detected the phosphorylation levels of ERK1/2 and CREB. The combination of METH and LPS obviously increased the ratio of p-ERK1/2 to ERK1/2 and p-CREB to CREB in Hip, CPU, NAc, and PFC (Fig. 6a–h). Importantly, the phosphorylation of ERK1/2 was significantly abolished by SCH-23390 in all of the brain regions (Fig. 6a–d). The phosphorylation of CREB was also inhibited in Hip, CPU, and PFC (Fig. 6e–h). These data suggest that both ERK1/2 and CREB are involved in the D1-like DARs-mediated neuroinflammation.

4. Discussion

Consumption of METH causes neuropsychiatric disorders and is also recently implicated in increasing vulnerability to other diseases, such as heart diseases, HIV and hepatitis C [37,38]. In fact, METH users appear to have increased susceptibility to bacterial and viral infection, which can result in cognitive decline, dementia and immunodeficiency [39]. Thus, it is important to elucidate how METH regulates immune responses. In a mouse model, we used an intraperitoneal injection of METH four times at a concentration of 5 mg/kg followed by a peripheral LPS challenge. We found that the expression of Iba1 was increased by injecting METH, but the secretion of IL-6 and TNF-α in all the four brain regions (Hip, PFC, CPU, and NAc) showed no changes. Buchanan's

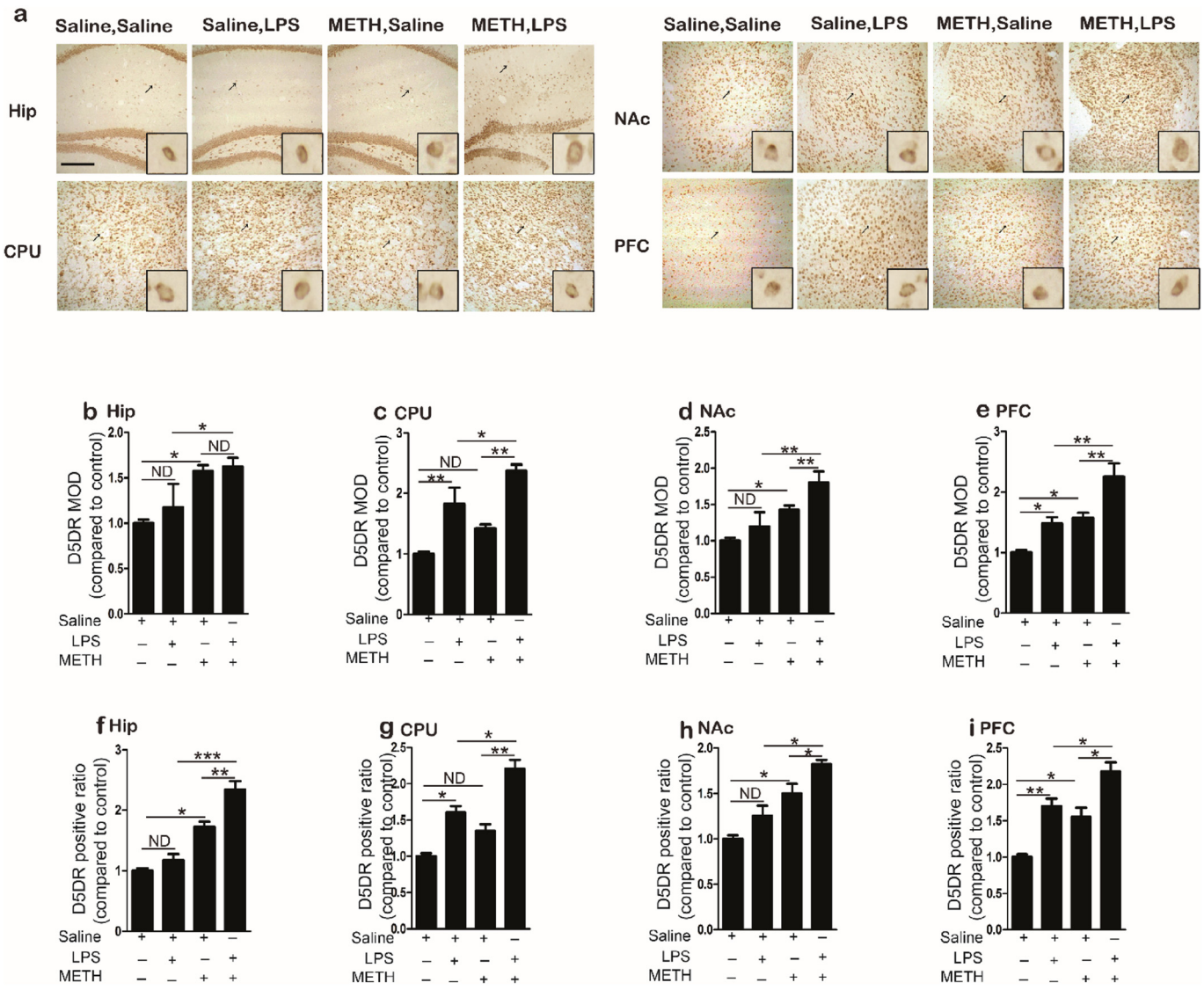

Fig. 4. METH promotes D5DR expression in LPS condition.
(a) Immunohistochemical staining of D5DR in Hip, CPU, NAc, and PFC after the METH treatment. Mice were subjected to four injections of 5 mg/kg METH at 2 h intervals and then challenged by 330 μg/kg LPS 24 h after the first METH injection. (b–e) MOD value of D5DR in four brain regions. (f–i) D5DR-positive cells ratio in four brain regions. Scale bar = 200 μm. Data are represented as the means ± SEM, n = 4 mice/group. Statistical analysis was performed using two-way ANOVA and Fisher's LSD test. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, ND means no significant difference.

experiments show that METH alone does not augment the production of IL-1β and IL-6 mRNA in the brain, but TNF-α is elevated in the hippocampus and striatum [26]. The reason for the difference may be that the production of protein and mRNA is different because mRNA needs time to translate into protein, of which the progress may be also affected by other factors [40–42]. The elevated expression of Iba1 might hint that METH has the ability to change the state of microglia from the resting state to an activated state while no alteration in the secretion of inflammatory factors. After LPS challenge, the amount of IL-6 and TNF-α increased significantly in the group with the METH pretreatment. Moreover, the altered activation state in microglia, the chief reason for this phenotype in the brain, may be due to increased BBB permeability with alterations on the expression of endothelial intercellular proteins and vascular adhesion molecules [43]. This reveals that METH abuse causes neuroinflammatory response after peripheral immune stimulation.

Highly-activated microglial cells are not merely a subsequent event but a specific pharmacological marker for METH-induced neurotoxicity [44–46]. Moreover, as the main immune cells of the brain, microglial response to METH precedes both terminal neuronal degeneration and astrocyte activation [44]. In the present study, METH alone could trigger the activation of microglia and also significantly enhanced LPS-induced microglial activation. These results indicate that METH can trigger the activation of intracerebral microglia and up-regulate the ability and intensity of microglia that respond to peripheral immune stimuli. Moreover, the activation of microglia will sustain for a long time as it has been found to exist for years in the brains of drug abusers despite cessation [13]. We also reported that the strong activation of microglia and increased production of IL-6 and TNF-α by METH pretreatment were impeded by minocycline. Minocycline is known as the second-generation tetracycline antibiotic drug with powerful anti-inflammatory and neuroprotective effects [47]. The beneficial effects of minocycline are mediated by modulating oxidative and nitrosative stress [48]. Moreover, minocycline is also proved to have the ability to modify a variety of morphine and METH effects [49,50]. These findings illustrate that minocycline might be considered a promising therapeutic agent in reducing or even preventing the neurotoxicity in METH-dependent individuals.

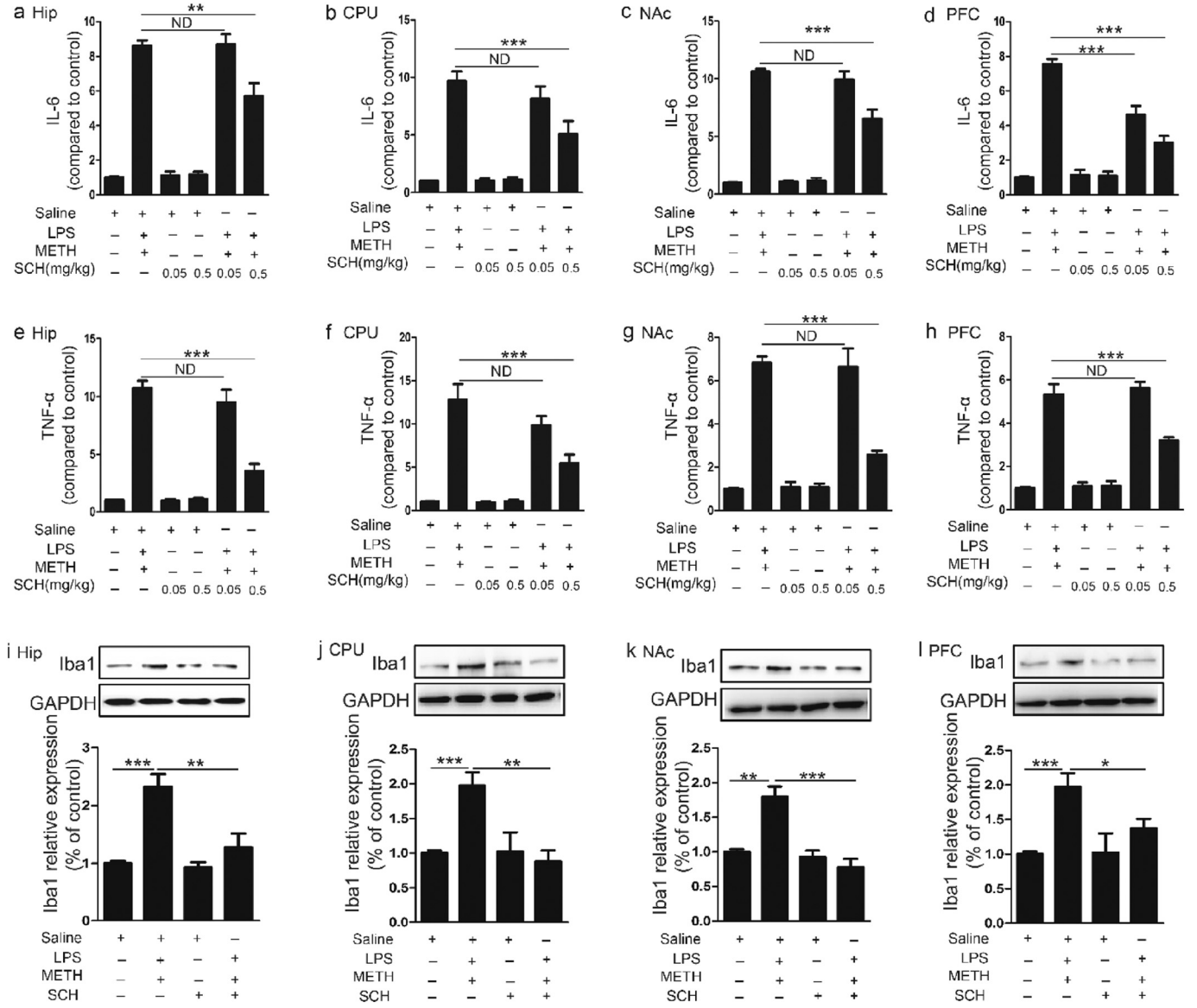

Fig. 5. D1-like DARs are involved in METH-augmented neuroinflammation by regulating microglial activation.
(a–d) IL-6 concentrations in four brain regions. (e–h) TNF-α concentrations in four brain regions. Mice were subjected to four injections of 5 mg/kg METH at 2 h intervals and then challenged by 330 μg/kg LPS at 24 h after the first METH injection. SCH-23390 (SCH, 0.05 mg/kg or 0.5 mg/kg) was given to mice 30 min before every injection of METH. n = 6 mice/group (e–h) Western blot of Iba1 in four brain regions. SCH (0.5 mg/kg) was given to mice 30 min before the injection of METH. Data are represented as the means ± SEM, n = 4 mice/group. Statistical analysis was performed using one-way (a–h) or two-way (i–l) ANOVA and Fisher's LSD test. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, ND means no significant difference.

METH damages dopamine nerve endings by a process that has been linked to microglial activation, but the signaling pathways that mediate this response have not yet been delineated [51]. It is well known that METH acts mainly through the activation of dopamine receptors [52]. Indeed, five dopamine receptors, especially D1-like receptors, have been shown to express on the surface of microglia and regulate the cellular functions, such as inflammatory factor secretion and microglia migration *in vitro* [22]. Here we reported that METH dramatically increased the expression of D1DR and D5DR in brain PFC, Hip, NAc and CPU regions after LPS stimulation. Further, the activation of microglia and the production of IL-6 and TNF-α were significantly abolished when mice were pretreated with the D1-like DARs antagonist SCH-23390. This suggests that METH activates microglia through D1-like DARs and then regulates their immune response to LPS. In human naïve CD4+ T cells, dopamine increases IL-6-dependent IL-17 production *via* D1-like receptors, and in rheumatoid arthritis mice, SCH-23390 strongly suppresses the accumulation of IL-6+ and IL-17+ T cells [53]. *In vivo*, D1-like receptors also modulate the activation state of NLRP3 inflammatory bodies in spinal cord injured rats [54]. These reports coin the immune regulatory role of D1-like receptors in CNS. However, D1-like receptors are also expressed on astrocytes and neuronal cells [18,19]. However, whether the D1-like DARs of microglia and/or other cells regulate the activation of microglia remains to be determined.

Studies in numerous brain regions and cell types have established that activation of D1-like receptors is linked to increased phosphorylation of a number of targets, including ERK1/2 and CREB, which can occur either directly or indirectly *via* the D1-like receptors [55–58]. Here, we found that the increased phosphorylation of ERK1/2 in METH and LPS condition was inhibited by the D1-like DARs antagonist SCH-23390. In Hip and PFC, the phosphorylation level of CREB was also considerably elevated by METH and LPS, which could be reversed by and by SCH-23390. Similar trends were also observed in CPU and NAc. It has been reported that METH can enhance memory by activating ERK1/2 and D1-like receptors are involved in this process [59,60].

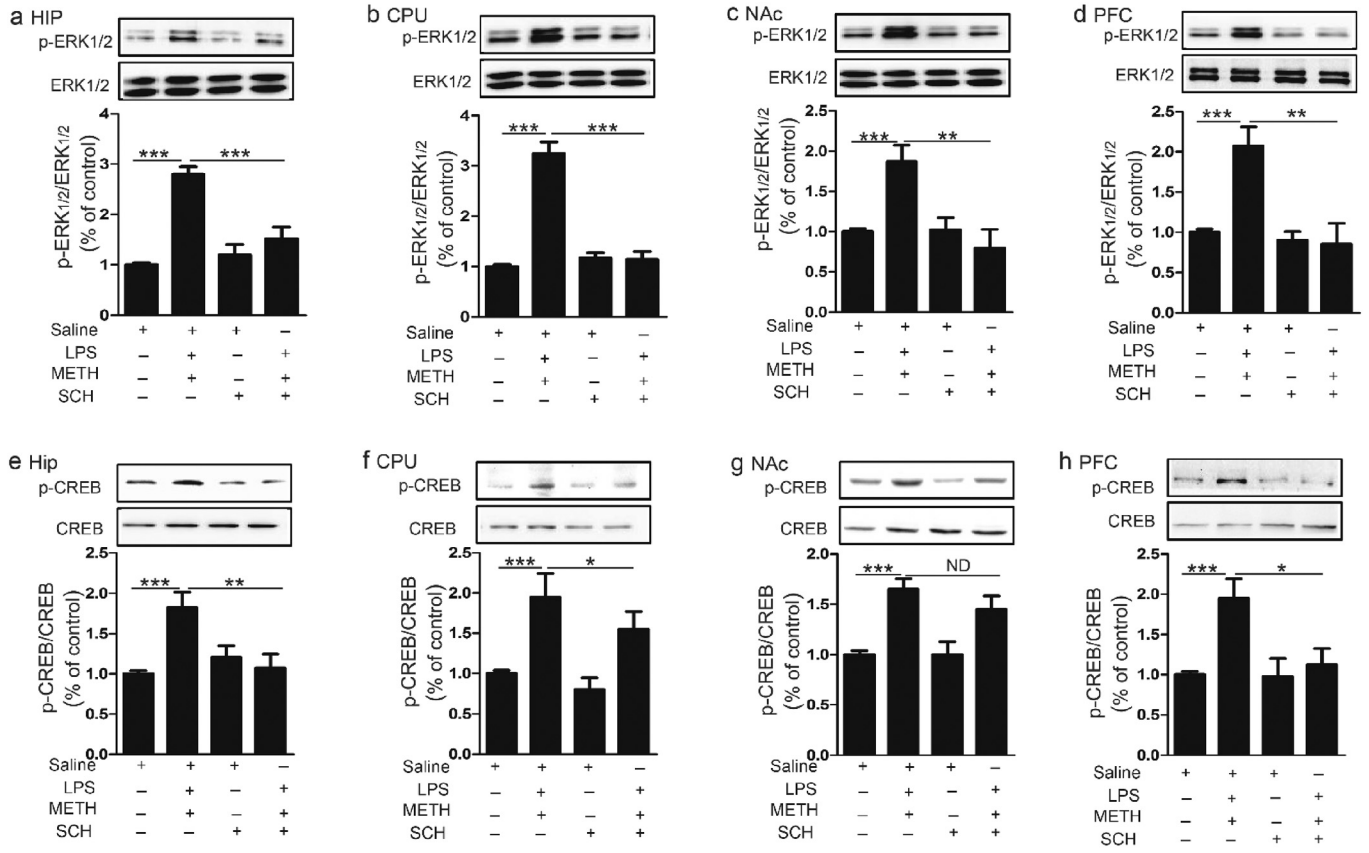

Fig. 6. ERK1/2 and CREB are involved in the D1-like DARs-mediated neuroinflammation in METH/LPS exposure.
(a–d) Western blot of p-ERK1/2 and ERK1/2 in four brain regions. (e–h) Western blot of p-CREB and CREB in four brain regions after the SCH treatment. Mice were subjected to four injections of 5 mg/kg METH at 2 h intervals and then challenged by 330 μg/kg LPS at 24 h after the first METH injection. SCH (0.5 mg/kg) was given to mice 30 min before the injection of METH. Data are represented as the means ± SEM, n = 4 mice/group. Statistical analysis was performed using two-way ANOVA and Fisher's LSD test. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, ND means no significant difference.

CREB regulates the production of a variety of inflammatory factors, which may be due to its altered phosphorylation levels induced by METH [61]. Our data provide new evidence that ERK1/2 and CREB are critically involved in METH-induced inflammatory changes, which is mediated by D1-like DARs. In addition, D2-like DARS and several kinds of signal molecules, such as PI3K and NF-κB are also reported to be regulated by METH [62–64]. In other words, the signaling pathways that participate in the METH-induced regulation are quite complex and interconnected. Therefore, further investigation is required to fully understand the mechanism in future studies.

In conclusion, our results indicate that METH can activate microglia and enhance LPS-induced microglial activation and production of IL-6 and TNF-α in four brain regions (Hip, CPU, NAc and PFC) in mice. D1DR and D5DR are up-regulated by METH to mediate the regulatory effects. Mechanistically, CREB and ERK1/2 are also associated with the immune regulation by METH. Taken together, these findings may improve our understanding of the regulatory role of METH in the CNS immune response by indicating new therapeutic targets.

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Competing interests

All authors claim that there are no conflicts of interest.

Ethics approval and consent to participate

All protocols involving animals were approved by the Animal Ethics Committee of Xi'an Jiaotong University.

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