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REGULAR RESEARCH ARTICLE

The Dopamine Receptor D3 Regulates Lipopolysaccharide-Induced Depressive-Like Behavior in Mice

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Abstract

Background: The altered expression and function of dopamine receptor D3 (D3R) in patients and animal models have been correlated with depression disease severity. However, the morphological alterations and biological effects of D3R in the brain after inflammation-induced depressive-like behavior remain elusive.
Methods: In the present study, we ascertained the changes of D3R expression in the brain regions after depressive-like behavior induced by peripheral administration of lipopolysaccharide (LPS). Protein levels of proinflammatory cytokines, brain-derived neurotrophic factor (BDNF), and extracellular signal-regulated kinase (ERK1/2)-cAMP-response element-binding protein (CREB) signaling pathway after activation or inhibition of D3R in the brain of depressive mice were also investigated.
Results: LPS caused a significant reduction of D3R in the ventral tegmental area (VTA), medial prefrontal cortex (mPFC), and nucleus accumbens (NAc), which are areas related to the mesolimbic dopaminergic system. Pretreatment with pramipexole (PPX), a preferential D3R agonist, showed antidepressant effects on LPS-induced depression-like behavior through preventing changes in LPS-induced proinflammatory cytokines (tumour necrosis factor-α, interleukin-1β, and interleukin-6), BDNF, and ERK1/2-CREB signaling pathway in the VTA and NAc. In opposition, treatment with a D3R selective antagonist NGB 2904 alone made mice susceptible to depression-like effects and caused changes in accordance with the LPS-induced alterations in proinflammatory cytokines, BDNF, and the ERK1/2-CREB signaling pathway in the mPFC and NAc.
Conclusions: These findings provide a relevant mechanism for D3R in LPS-induced depressive-like behavior via its mediation of proinflammatory cytokines and potential cross-effects between BDNF and the ERK1/2-CREB signaling pathway.

Keywords: dopamine receptor D3, inflammation, BDNF, ERK1/2-CREB signaling pathway

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Significance Statement

The dopamine D3 receptor (D3R) is expressed in the brain, targeting the mesolimbic dopaminergic system, and involved in regulating depressive disorders. However, research regarding the role of D3R in an inflammation-induced model of depression has not been reported so far. Here, our study found that the D3R protein expression in inflammation-induced depression was significantly decreased. Further activation of D3R led to antidepressant effects on LPS-induced behavior; on the contrary, inhibition of D3R resulted in depressive-like behavior. Within this context, our findings finally show that D3R regulated inflammation-induced depression through its mediation of proinflammatory cytokines and potential cross-effects between BDNF and the ERK1/2-CREB signaling pathway.

Introduction

A substantial body of evidence has indicated that depression is closely associated with inflammation processes (Crupi and Cuzzocrea, 2016; Kim et al., 2016; Farooq et al., 2017). In this regard, elevated serum and plasma concentrations of interleukin (IL)-6 (Alesci et al., 2005), as well as elevated levels of both peripheral and central IL-1β and tumour necrosis factor (TNF)-α (Kahl et al., 2005), have frequently been observed in patients with depression. In animal studies, exogenous proinflammatory cytokines or administration of a cytokine inducer, lipopolysaccharide (LPS), triggers depressive-like behavior in rodents, resulting in enhanced immobility in the forced swim test (FST) and tail suspension test (TST), decreased consumption of a sweetened solution, and suppression of sexual behavior, which can be attenuated by antidepressant treatments (Kaster et al., 2012; Jiang et al., 2013; Mello et al., 2013; Ming et al., 2015).

The dopamine D3 receptor (D3R) strongly contributes to depression, not only due to its selective expression in the striatum and mesolimbic system, which are involved in cognitive, emotional, and reward functions (Sokoloff et al., 1990; Bouthenet et al., 1991; Xing et al., 2013), but also due to its postsynaptic and presynaptic localization in dopaminergic neurons, making it able to modulate dopamine synthesis and release (Koeltzow et al., 1998; Diaz et al., 2000). Moreover, D3R expression and function have been found to be downregulated following chronic stress, and this change is reversed by antidepressant drugs (Maj et al., 1998) or electroconvulsive therapy (Lammers et al., 2000). Pharmacological evidence performed in the rat has suggested that D3R agonists, such as pramipexole (PPX) (Maj et al., 1997) and 7-hydroxy-2-(di-n-propylamino) tetralin hydrobromide (7-OH-DPAT) (Breuer et al., 2009), have antidepressant actions. A genetic study in $D3R^{-/-}$ mice demonstrated that D3R deficiency results in chronic depressive-like symptoms (Moraga-Amaro et al., 2014), which is congruent with previous pharmacological evidence, all suggesting a potential mediating role of D3R in depressive-like behavior. However, the potential relationship between the mechanisms by which D3R and LPS-induced inflammation may cause depression-like behavior is less clear.

Brain-derived neurotrophic factor (BDNF), a polypeptidic factor, is implicated in the pathophysiology and therapeutic mechanism of depression (Duman et al., 1997; Hashimoto et al., 2004; Martinowich et al., 2007). Importantly, the distinct alteration of BDNF expression in brain regions of rodents is involved in LPS-induced depression (Zhang et al., 2014; Ma et al., 2017). Also, BDNF controls D3R expression in the striatum in drug addiction, behavioral sensitization, and schizophrenia (Guillin et al., 2001; Jeanblanc et al., 2006; Vogel et al., 2006; Leggio et al., 2014). Nevertheless, whether this potential mechanism impacts LPS-mediated depression remains to be determined. The extracellular signal-regulated kinase (ERK1/2)-cAMP-response element-binding protein (CREB) signaling pathway exerts effects on behavioral and biological responses to stressors, such as synaptic plasticity, learning and memory, emotion, and cognitive function (Samuels et al., 2008; Duric et al., 2010; First et al., 2013; Leem et al., 2014). Additionally, alterations in the ERK1/2-CREB signaling pathway are correlated with depression-like behavior induced by chronic mild stress (Zhang et al., 2017), and administration of antidepressants reverses the expression, phosphorylation, and function of this pathway (Thome et al., 2000; First et al., 2011). Under LPS-induced neuroinflammation state, the phosphorylated ERK1/2 (p-ERK1/2) expression in the hippocampus and the CREB expression in the cultured neuronal cells are reduced (Daniele et al., 2015; Tang et al., 2017). A recent study suggested that the ERK1/2-CREB signaling pathway is implicated in changes in the response of $D3R^{-/-}$ mice to passive avoidance conditioning (D'Amico et al., 2013). Currently, there are no reports on the involvement of D3R in the ERK1/2-CREB signaling pathway in LPS-induced depressive-like behavior.

This study aimed to investigate the role of D3R in inflammation-induced depressive-like behavior in mice. We first determined the effects of D3R on LPS-induced depressive-like behavior and proinflammatory cytokines (TNF-α, IL-1β, and IL-6) in selected mesolimbic dopaminergic brain regions, including the ventral tegmental area (VTA), medial prefrontal cortex (mPFC), and nucleus accumbens (NAc). Furthermore, we also examined the relationship between D3R and BDNF or the ERK1/2-CREB signaling pathway in inflammation-mediated depression.

Methods and Materials

Animals

Male C57BL/6 mice (weighing approximately 20–25 g) were obtained from Beijing Vital River Laboratory Animal Technology Co. Ltd. Four animals per cage were housed in a regulated environment (23±1°C, 50±5% humidity) with a 12:12-h light/dark cycle (lights on at 7:00 AM) with food and water available ad libitum. All animal experiments were performed in accordance with the Institutional Animal Care and Use Committee of Xi'an Jiaotong University. Animals were acclimatized to the laboratory conditions 1 week prior to the start of experiments.

Drugs

LPS from *Escherichia coli* (L-3129, serotype 0127:B8), PPX, and NGB 2904 were purchased from Sigma-Aldrich. LPS was dissolved in sterile, endotoxin-free normal saline and administered i.p. at 0.83 mg/kg of body weight (mg/kg b.w.). The selected dose of LPS was chosen based on its ability to induce a proinflammatory cytokine response in the brain and subsequent depressive-like

behavior (O'Connor et al., 2009). PPX and NGB 2904 were dissolved in 0.9% saline and 50% PEG-400 saline, respectively. PPX and NGB 2904, both at the dose of 1 mg/kg b.w., or their respective vehicle were i.p. injected 30 minutes prior to LPS injection. The doses of PPX and NGB 2904 were selected as previously reported (Pritchard et al., 2007; Schulte-Herbruggen et al., 2012; Castro-Hernandez et al., 2015).

Experimental Design

In the present study, animals were randomly divided into 8 experimental groups (groups I–VIII) for behavioral tests (n = 10 mice/group) and biomolecular assessments (n = 6 mice/group), which were performed by taking different animals.

Groups I and II were treated with saline or vehicle (50% PEG-400 saline) 30 minutes prior to saline injection, these groups served as control groups; Groups III and IV were also treated with saline or vehicle 30 minutes before LPS (0.83 mg/kg, i.p.) administration. These groups were considered as the LPS control groups; Groups V and VI were treated with PPX (1 mg/kg, i.p.) or NGB 2904 (1 mg/kg, i.p.), respectively, 30 minutes prior to saline injection; Groups VII and VIII were treated with PPX (1 mg/kg, i.p.) or NGB 2904 (1 mg/kg, i.p.), respectively, 30 minutes prior to LPS (0.83mg/kg, i.p.) challenge. Various behavioral parameters such as the locomotor activity test and FST were assessed after 24 hours of LPS administration, and the TST was conducted 28 hours following administration of LPS. The animal biomolecular level was estimated 24 hours post-saline or LPS treatment.

Behavioral Experiments

Locomotor Activity Test

The open field test (OFT) was used to assess the spontaneous locomotor activity of the mice. Briefly, each mouse was placed into the center of the experimental apparatus consisting of a black plastic box (45 × 45 × 35 cm) with its white floor divided into 25 equal-sized squares (9 × 9 cm) and recorded for 5 minutes by a camera. The numbers of crossings and rearings were counted by the experienced observers blind to the experimental groups (Zhao et al., 2015).

FST

The FST was performed as described in a previous study (Can et al., 2012), using a cylindrical tank (30 cm height × 20 cm diameter) containing 15 cm of water maintained at 23 ~ 25°C. Each mouse was placed individually in the cylinder and recorded on video for 6 minutes. The water in the cylinder was changed after each mouse during the testing sessions. The immobility time of each mouse during the final 4 minutes of the test was calculated by the experienced observers blind to the treatment conditions.

TST

The TST was carried out as previously explained by O'Connor et al. (2009). Each mouse was individually suspended 30 cm above the floor by hanging on a fixed hook using a small piece of adhesive tape placed approximately 2 cm from the tip of the tail for 10 minutes, and the immobility duration throughout the 10 minutes was recorded.

Immunohistochemistry

Mice were anaesthetized using 5% chloral hydrate and perfused first with 0.9% saline and then with cold 4% paraformaldehyde (0.1 M phosphate buffer, pH 7.4). According to the stereotaxic coordinates of the mouse brain (Paxinos and Franklin 2001), the brain tissue containing the VTA, mPFC, and NAc regions was harvested and postfixed in 4% paraformaldehyde at 4°C for 24 hours, followed by dehydration in 30% sucrose. All brain tissue were embedded in OCT and coronally sectioned into 10-μm-thick sections using an ultramicrotome. One section per 100 μm was picked for the following experiment, and 4 sections were used per animal. After routine treatment with 3% hydrogen peroxide and normal goat serum blocking solution, the sections were incubated with a rabbit anti-mouse D3R antibody (1:100; Boster) overnight at 4°C and then incubated with a horseradish peroxidase (HRP)-labelled goat anti-rabbit IgG for 30 minutes. The positive cells in the sections were subsequently identified through their reaction with diaminobenzidine for staining.

Histological sections were viewed with an Olympus BX-51 microscope, and the images were captured with an Olympus DP71 camera. D3R immunoreactivity was quantified using ImageJ software (National Institute of Health). Briefly, digital images from each mouse were opened in the ImageJ program and converted to 8-bit grayscale, allowing the computer to distinguish between areas of immunoreactivity and background. After a standardized elimination of background through adjusting the threshold, the certain brain areas were selected and the mean optical density (MOD) and area ratio of D3R-positive cells (area of positive signal/area of region of interest) were measured.

Proinflammatory Cytokines and BDNF Assay

The VTA, mPFC, and NAc of mice brain regions were removed and homogenated using a homogenizer. After centrifugation at 3000 rpm/min for 15 min at 4°C in a centrifugal machine, the supernatant was subpackaged and stored at -80°C until further use. Levels of TNF-α, IL-1β, IL-6, and BDNF were quantified using ELISA kits (e-Bioscience, Inc.) according to the manufacturer's protocol, and the minimum detection limits for TNF-α, IL-1β, IL-6, and BDNF were 31.3 pg/mL, 7.8 pg/mL, 31.3 pg/mL, and 31.2 pg/mL, respectively.

Western-Blot Analysis

Mice were immediately sacrificed by cervical dislocation and the VTA, mPFC, and NAc were dissected bilaterally on dry ice. Tissue samples were homogenized in ice-cold RIPA lysis buffer (Beyotime), which contained 1× phosphate-buffered saline, 1% Nonidet P-40, 0.5% sodium deoxycholate, and 1% sodium dodecyl sulfate, supplemented with a protease inhibitor cocktail (Roche). Homogenates were incubated on ice for 20 min and then centrifuged at 12000 × g for 15 min at 4°C. Supernatants were collected, and the protein concentration was determined using a BCA protein assay (Beijing Dingguo Changsheng Biotechnology Co., Ltd). Then, 6 μg of extracts for ERK1/2 and 40 μg of extracts for D3R or CREB were separated by 10% or 12% sodium dodecyl sulfate-PAGE and then transferred to a polyvinylidene difluoride (Millipore) membrane. After incubation in a blocking solution of 5% skim milk in Tris-buffered saline containing 0.1% Tween 20 for 2 h at room temperature, the membrane was probed overnight at 4°C using the following primary antibodies: rabbit anti-mouse D3R at a 1:2000 dilution (Abcam); rabbit anti-mouse p-ERK1/2, ERK1/2, phosphorylated CREB (p-CREB), and CREB at a 1:1000 dilution (Cell Signaling); and rabbit anti-GAPDH at a 1:2000 dilution (Proteintech), followed by incubation with an horseradish peroxidase-coupled anti-rabbit IgG for 1 h at room

temperature. Immunoreactive proteins were visualized by incubation in ECL solution (Millipore), and the images were captured using a Fusion FX5 camera system.

The density of specific bands was measured using ImageJ software. For the expression of D3R, p-ERK1/2, ERK1/2, p-CREB, and CREB, the relative densities were calculated after proteins of interest were normalized to their corresponding loading control (GAPDH). The activation of ERK1/2 and CREB were determined as a ratio of optical density (OD) of phosphorylated protein/OD of total protein.

Statistical Analysis

All data were presented as mean ± SEM. Analysis was performed using IBM SPSS Statistics 20.0. Differences in the MOD and area ratio of positive cells were analyzed by Student's *t* test. Differences in behavior experiment, proinflammatory cytokines, BDNF protein, ERK1/2-CREB signaling pathway after PPX, or NGB 2904 treatment among groups were tested statistically by one-way ANOVA followed by posthoc LSD test. $P < .05$ was considered as statistically significant.

Results

A Reduction of D3R Expression in the Mouse Brain after LPS Administration

To test the relevance of D3R in inflammation-induced depression, we first aimed to confirm whether the levels of D3R expression were altered in the mouse brain after LPS administration. Our results, using both immunohistochemistry and western blotting (supplementary Figure 1) methods, showed a high level of D3R expression in the VTA (Figure 1D), mPFC (Figure 1E), and NAc (Figure 1F) in the saline control group. However, a single dose of LPS significantly decreased D3R expression in the VTA (Figure 1G), mPFC (Figure 1H), and NAc (Figure 1I). Quantitative analysis of immunoreactivity showed that the MOD (Figure 1J) and area ratio of D3R-positive cells (Figure 1K) were markedly decreased in the VTA ($P=.0001$), mPFC ($P=.0001$), and NAc ($P=.0001$) 24 h after LPS injection, suggesting that downregulated endogenous D3R expression in the mesolimbic dopaminergic system may participate in the LPS-induced inflammation that may finally induce depression-like behavior.

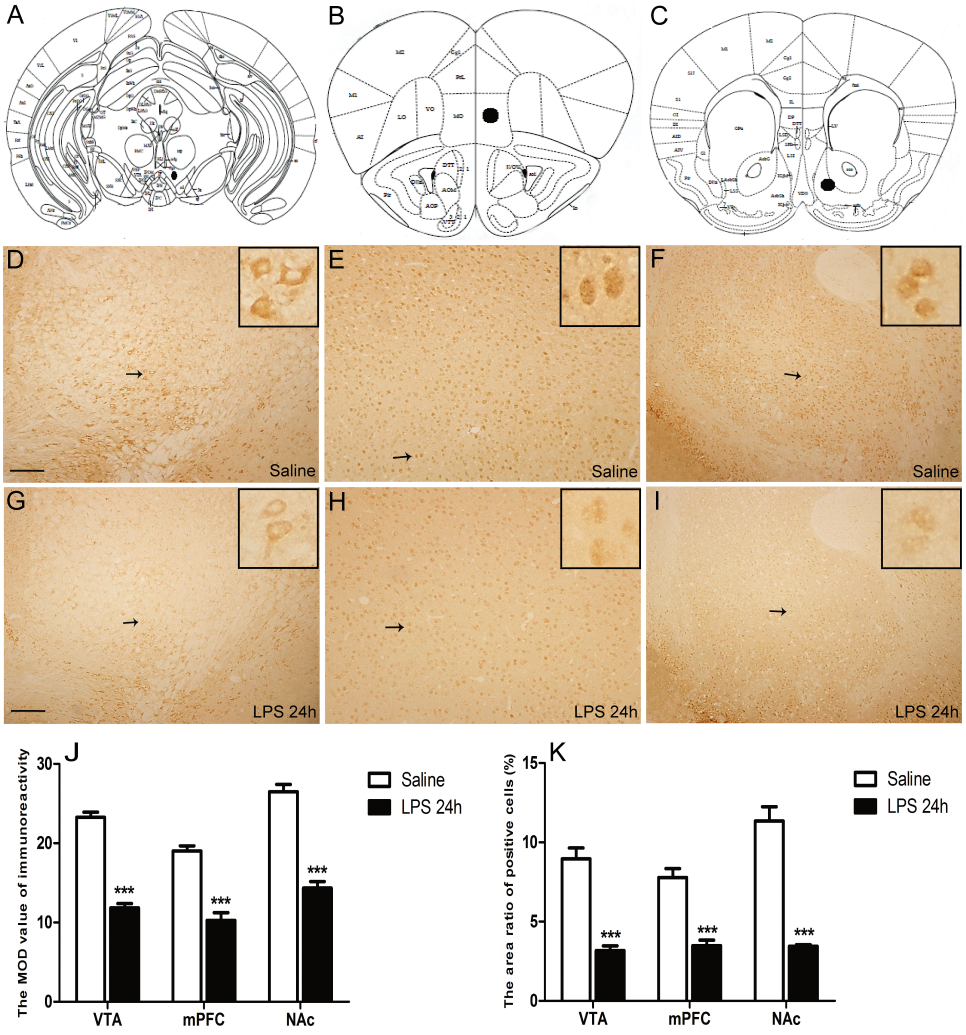

Figure 1. Expression of dopamine receptor D3 (D3R) in the brain of saline and lipopolysaccharide (LPS)-treated mice measured by immunohistochemistry. (A-C) Schematic diagram of brain regions in the ventral tegmental area (VTA), medial prefrontal cortex (mPFC), and nucleus accumbens (NAc). (D-F) A higher expression level of D3R was found in the VTA, mPFC, and NAc of saline-treated mice. (G-I) LPS caused a marked downregulation in D3R expression in the VTA, mPFC, and NAc 24 h after administration. (J-K) Quantitative analysis of immunoreactivity showed that the mean optical density (MOD) and area ratio of D3R-positive cells were significantly lower in the VTA, mPFC, and NAc after LPS administration. The data are presented as the mean ± SEM. ***$P<.001$ vs saline-treated group; scale bar: 100 μm (D-I); $n=6$ mice/group.

D3R Agonist PPX Exerted Antidepressant Effects on LPS-Induced Depressive-Like Behavior

Due to the reduction in D3R expression after LPS administration, we explored the effects of D3R on LPS-induced depressive-like behavior. In agreement with previous studies of LPS-induced depressive-like behavior (O'Connor et al., 2009; Mello et al., 2013; Sekio and Seki, 2014), we found a significant decrease in mouse body weight ($P = .0001$) and an increase in immobility time in the FST ($P = .0001$) 24 h after LPS administration as well as an increase in immobility time in the TST 28 h after administration ($P = .0001$) compared with those in the saline-treated group. PPX (1 mg/kg, i.p.), a preferential D3R agonist that was administered 30 min prior to LPS injection, completely blocked the LPS-induced sickness behavior, significantly facilitated the recovery from weight loss one-way ANOVA, $F(3, 36) = 40.537$, $P = .0001$; Figure 2A], and prevented the extended immobility time in the FST [one-way ANOVA, $F(3, 35) = 10.755$, $P = .0001$; Figure 2C] and TST [one-way ANOVA, $F(3, 36) = 10.672$, $P = .0001$; Figure 2D] induced by LPS administration. Spontaneous locomotor activity in the OFT was not significantly different across all groups [one-way ANOVA, crossings: $F(3, 34) = 0.065$, $P = .9782$; rearings: $F(3, 34) = 0.589$, $P = .6266$; Figure 2B]. Together, these results strongly indicate that activation of D3R plays antidepressant roles in LPS-induced depressive-like behavior.

D3R Antagonist NGB 2904 Treatment Alone Induced Depressive-Like Behavior

Consistent with the above results, the body weight was lower ($P = .0001$) and duration of immobility in the FST ($P = .0004$) and TST ($P = .0026$) were higher after LPS administration than in the vehicle control group. Interestingly, D3R antagonist NGB 2904 treatment alone resulted in a significant reduction in body weight ($P = .0021$; Figure 3A) and increase in immobility time in the FST ($P = .0214$; Figure 3C) compared with those in the vehicle-treated group. However, there was no difference in the spontaneous locomotor activity (crossings: $P = .7074$; rearings: $P = .7886$; Figure 3B) or TST immobility time ($P = .1039$; Figure 3D) between the vehicle control and NGB 2904-treated mice. Furthermore, the combined effects of LPS and NGB 2904 did not further increase the depressive-like behavior (Figure 3), including weight ($P = .4279$), locomotor activity (crossings: $P = .4571$; rearings: $P = .9475$), FST ($P = .3819$), and TST ($P = .9662$). These findings imply that inhibition of D3R in normal mice induces depressive-like behavior.

D3R Regulated Neuroinflammation Induced by LPS Administration

To examine the possible involvement of D3R in the LPS-induced inflammatory response, we measured proinflammatory cytokines (TNF-α, IL-1β, and IL-6) in regions of the mouse brain 24 h after LPS administration via ELISA. As shown in Figure 4, proinflammatory cytokines in the VTA, mPFC, and NAc were significantly higher after LPS treatment than in the respective control groups.

One-way ANOVA revealed that PPX pretreatment significantly inhibited the LPS-induced elevation in TNF-α, IL-1β, and IL-6 expression both in the VTA [TNF-α: $F(3, 20) = 3.701$, $P = .0288$; IL-1β: $F(3, 17) = 7.775$, $P = .0018$; IL-6: $F(3, 18) = 7.966$, $P = .0018$; Figure 4A] and NAc [TNF-α: $F(3, 20) = 3.985$, $P = .0193$; IL-1β: $F(3, 19) = 4.631$, $P = .0288$; IL-6: $F(3, 20) = 20.683$, $P = .0001$; Figure 4C] but had no effect on the levels in the mPFC [TNF-α: $F(3, 17) = 0.476$, $P = .5141$; IL-1β: $F(3, 17) = 2.886$, $P = .0761$; IL-6: $F(3, 18) = 1.039$, $P = .1749$; Figure 4B]. Interestingly, NGB 2904 treatment alone obviously enhanced proinflammatory cytokine expression in the mPFC (TNF-α, $P = .0229$; IL-1β, $P = .0368$; IL-6, $P = .0283$; Figure 4E) and NAc (TNF-α, $P = .0289$; IL-1β, $P = .0298$; IL-6, $P = .0330$; Figure 4F) compared with the levels in the vehicle control groups, yet the levels of those cytokines were not altered in the VTA (TNF-α, $P = .9690$; IL-1β, $P = .9420$; IL-6, $P = .5017$; Figure 4D). Furthermore, co-administration of NGB 2904 with LPS did not result in significantly different levels than in LPS-treated mice (Figure 4D-F).

Together, these results demonstrate that activation of D3R attenuates the neuroinflammation induced by LPS, which may result in an antidepressant action, whereas preconditioning

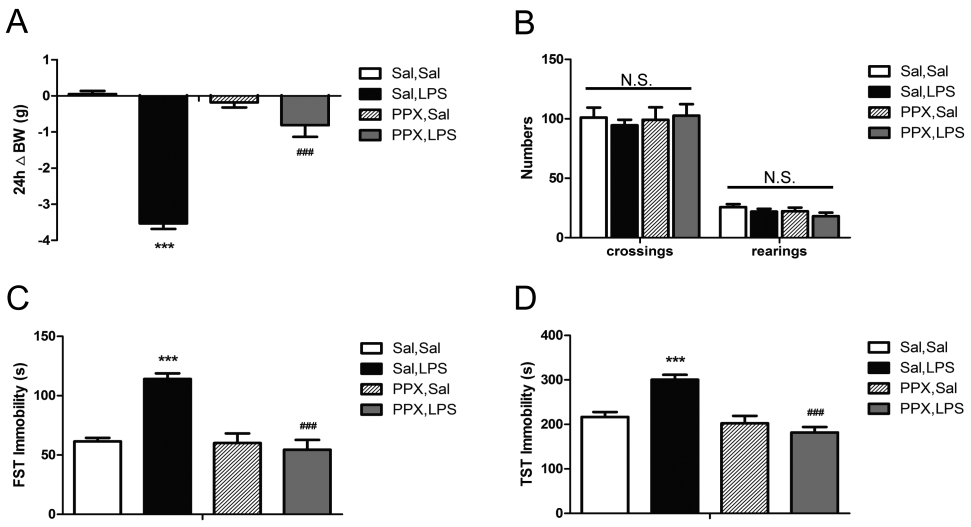

Figure 2. Dopamine receptor D3 (D3R) agonist pramipexole (PPX) exerted antidepressant effects on lipopolysaccharide (LPS)-induced depressive-like behavior. (A) Pretreatment with PPX blocked the change in body weight 24 h following LPS administration. (B) There were no differences in locomotor activity, including crossings and rearings, among all groups. (C) Pretreatment with PPX blocked the immobility time in the forced swim test (FST) recorded 24 h following administration of LPS. (D) Pretreatment with PPX blocked the immobility time during the tail suspension test (TST) 28 h post-LPS. The data are presented as the mean ± SEM. ***$P < .001$ vs Sal, Sal; ###$P < .001$ vs Sal, LPS. N.S., not significant; n = 10 mice/group.

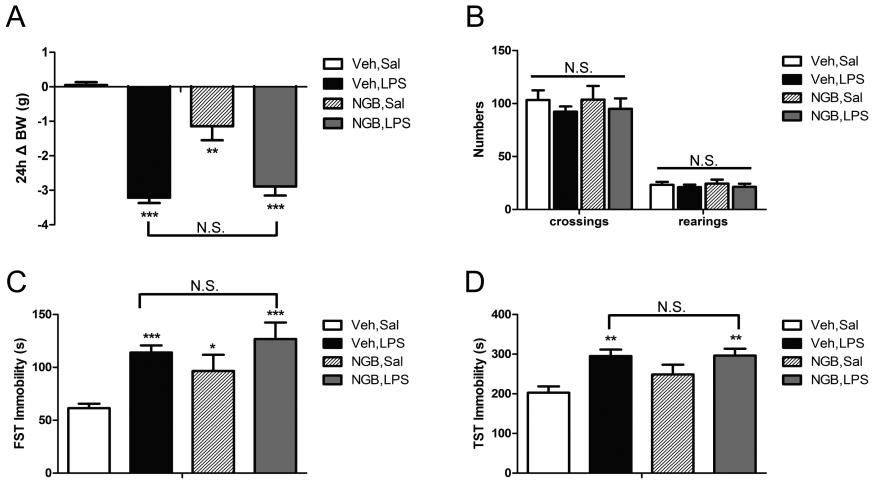

Figure 3. Dopamine receptor D3 (D3R) antagonist NGB 2904 treatment alone induced depressive-like behavior. (A) NGB 2904 treatment alone resulted in a lower body weight than in the vehicle group 24 h after treatment. (B) NGB 2904 treatment alone did not affect the locomotor activity. (C) NGB 2904 treatment alone induced an increase in the immobility time in the forced swim test (FST). (D) NGB 2904 treatment alone failed to affect immobility time in the tail suspension test (TST). The data are presented as the mean ± SEM. $^{*}P<.05$ vs Veh, Sal; $^{**}P<.01$ vs Veh, Sal; $^{***}P<.001$ vs Veh, Sal. N.S.: not significant; n = 10 mice/group.

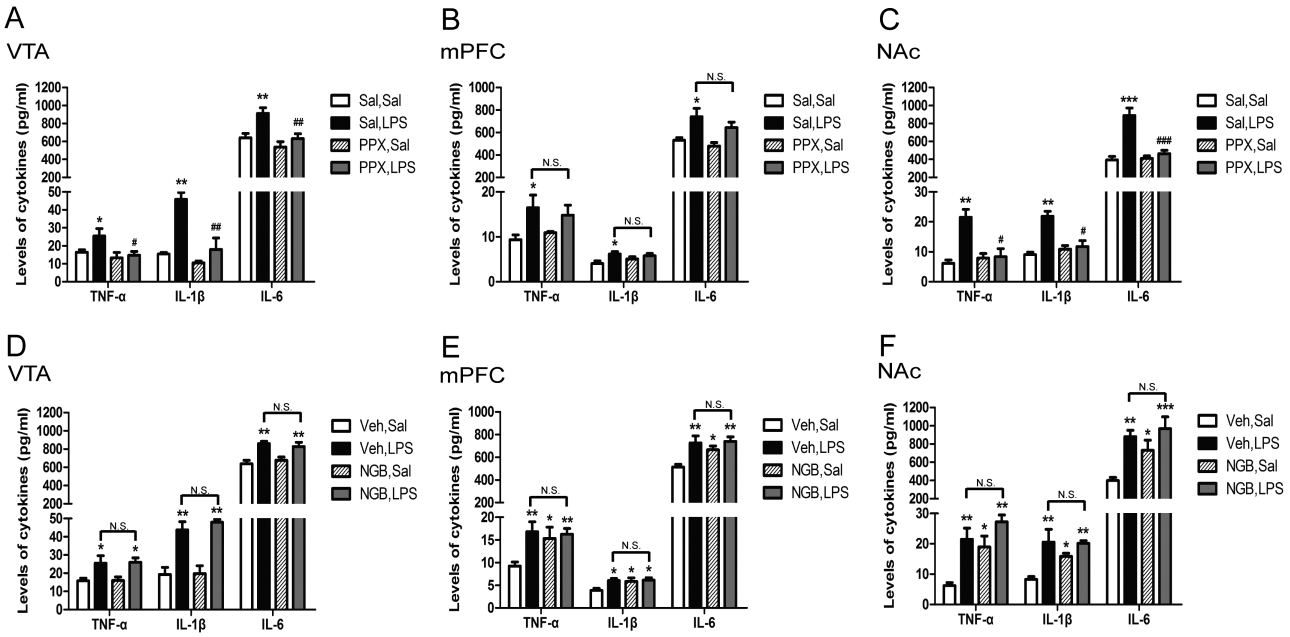

Figure 4. Dopamine receptor D3 (D3R) regulated the neuroinflammation induced by lipopolysaccharide (LPS) administration. (A-C) The proinflammatory cytokines in the ventral tegmental area (VTA) (TNF-α, $P=.0285$; IL-1β, $P=.0016$; IL-6, $P=.0060$), medial prefrontal cortex (mPFC) (TNF-α, $P=.0115$; IL-1β, $P=.0181$; IL-6, $P=.0152$), and nucleus accumbens (NAc) (TNF-α, $P=.0063$; IL-1β, $P=.0048$; IL-6, $P=.0001$) were significantly higher after LPS treatment than in the saline control groups. Pretreatment with pramipexole (PPX) reversed the LPS-induced changes in proinflammatory cytokines tumor necrosis factor (TNF)-α, interleukin (IL)-1β, and IL-6 in the VTA and NAc but not in the mPFC. The data are presented as the mean ± SEM. $^{*}P<.05$ vs Sal, Sal; $^{**}P<.01$ vs Sal, Sal; $^{***}P<.001$ vs Sal, Sal; $^{\#}P<.05$ vs Sal, LPS; $^{\#\#}P<.01$ vs Sal, LPS; $^{\#\#\#}P<.001$ vs Sal, LPS. N.S.: not significant; n = 6 mice/group. (D-F) Following 24 h of LPS injection, the proinflammatory cytokines in the VTA (TNF-α, $P=.0173$; IL-1β, $P=.0022$; IL-6, $P=.0013$), mPFC (TNF-α, $P=.0064$; IL-1β, $P=.0187$; IL-6, $P=.0037$), and NAc (TNF-α, $P=.0056$; IL-1β, $P=.0016$; IL-6, $P=.0016$) were significantly higher than the vehicle control group. Treatment with NGB 2904 alone caused an increase in TNF-α, IL-1β, and IL-6 in the mPFC and NAc but not in the VTA. Furthermore, after co-administration of NGB 2904 with LPS, the proinflammatory cytokines in the brain region of VTA (TNF-α, $P=.8831$; IL-1β, $P=.5688$; IL-6, $P=.5750$), mPFC (TNF-α, $P=.8330$; IL-1β, $P=.8997$; IL-6, $P=.8148$), and NAc (TNF-α, $P=.2807$; IL-1β, $P=.8815$; IL-6, $P=.5005$) did not result in significantly different levels than in LPS-treated mice. The data are presented as the mean ± SEM. $^{*}P<.05$ vs Veh, Sal; $^{**}P<.01$ vs Veh, Sal; $^{***}P<.001$ vs Veh, Sal. N.S.: not significant; n = 6 mice/group.

inhibition of D3R in normal mice may lead to an increase in neuroinflammation correlated with depressive-like behavior.

D3R Controlled LPS-Induced BDNF Expression

A number of studies have demonstrated that BDNF is involved in the pathophysiology of depression, as well as the therapeutic mechanisms of antidepressants (Shirayama et al., 2002; Yu et al., 2012). Thus, we aimed to investigate whether D3R plays an important role in changes in BDNF expression induced by LPS treatment. Our results in Figure 5 demonstrated that the levels of BDNF expression were markedly decreased in the VTA ($P=.0052$; $P=.0025$, respectively) and mPFC ($P=.0013$; $P=.0001$, respectively) but increased in the NAc ($P=.0023$; $P=.0020$, respectively) after

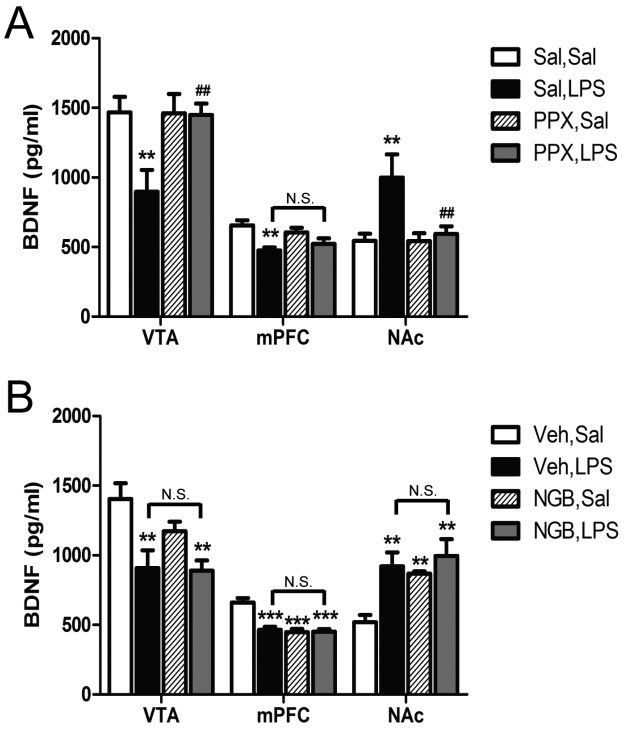

Figure 5. Dopamine receptor D3 (D3R) controlled the lipopolysaccharide (LPS)-induced change in brain-derived neurotrophic factor (BDNF) expression. (A) Pretreatment with pramipexole (PPX) abrogated the LPS-induced decrease in BDNF expression in the ventral tegmental area (VTA) and increase in the nucleus accumbens (NAc), but the levels of BDNF in the medial prefrontal cortex (mPFC) after LPS administration were not altered by PPX pretreatment. The data are presented as the mean ± SEM. **$P < .01$ vs Sal, Sal; ##$P < .01$ vs Sal, LPS. N.S., not significant; n = 6 mice/group. (B) NGB 2904 treatment alone induced a decrease in BDNF levels in the mPFC and an increase in the NAc but failed to affect the VTA. The BDNF expression of the VTA ($P = .9094$), mPFC ($P = .6884$), and NAc ($P = .5287$) did not differ between the group treated with NGB 2904 in combination with LPS and the LPS-treated group. The data are presented as the mean ± SEM. **$P < .01$ vs Veh, Sal; ***$P < .001$ vs Veh, Sal. N.S., not significant; n = 6 mice/group.

LPS administration compared with the levels in their respective control groups.

Nevertheless, one-way ANOVA showed that pretreatment with PPX (Figure 5A) effectively ameliorated the changes in BDNF expression caused by LPS in the VTA [$F(3, 19) = 4.636$, $P = .0143$] and NAc [$F(3, 20) = 5.583$, $P = .0045$] but had no effect in the mPFC [$F(3, 20) = 0.265$, $P = .8611$]. Additionally, the group treated with NGB 2904 alone (Figure 5B) showed significantly less BDNF protein in the mPFC ($P = .0001$) and more in the NAc ($P = .0061$) than in the vehicle control group but did not exhibit any changes in the VTA ($P = .1219$). The results from the ELISA analysis demonstrated that there was no difference between the group treated with NGB 2904 in combination with LPS and the LPS-treated group (Figure 5B).

Taken together, the antidepressant effects of the upregulation of D3R result from an attenuation in the LPS-induced changes in BDNF expression. In contrast, downregulation of D3R leads to changes in BDNF expression in normal mice that are most likely correlated with depressive-like behavior.

Potential Roles of D3R in LPS-Induced Depressive-Like Behavior via the Involvement of p-ERK1/2 and ERK1/2

The involvement of D3R in the regulation of the ERK1/2 signaling pathway caused by LPS-induced depressive-like behavior was evaluated by performing western blotting analysis. As illustrated in Figure 6, compared with control groups, the p-ERK1/2 ($P = .0095$; $P = .0187$, respectively) and ERK1/2 ($P = .0026$; $P = .0017$, respectively) protein levels in the VTA and p-ERK1/2 expression ($P = .0156$; $P = .0105$, respectively) in the mPFC were significantly lower 24 h post-LPS treatment. However, LPS markedly increased the p-ERK1/2 ($P = .0001$; $P = .0004$, respectively) and ERK1/2 ($P = .0014$; $P = .0001$, respectively) protein expression in the NAc compared with control groups. Moreover, the ERK1/2 protein activation ($P = .0169$; $P = .0413$, respectively) was decreased in the mPFC 24 h after LPS administration, but there was no significant difference in the ERK1/2 activation in the VTA ($P = .1132$; $P = .4228$, respectively) and NAc ($P = .7944$; $P = .9118$, respectively) after LPS treatment compared with that in the control groups.

One-way ANOVA (Figure 6A-F) pretreatment with PPX reversed the decline in the protein levels of p-ERK1/2 [$F(3, 19) = 4.516$, $P = .0187$] and ERK1/2 [$F(3, 19) = 6.875$, $P = .0068$] in the VTA and the rise in those [p-ERK1/2: $F(3, 19) = 10.814$, $P = .0001$; ERK1/2: $F(3, 20) = 4.855$, $P = .0059$] in the NAc of the LPS-treated mice. However, such an effect failed to occur in the mPFC [p-ERK1/2: $F(3, 20) = 0.218$, $P = .9347$; ERK1/2: $F(3, 20) = 0.278$, $P = .6889$]. PPX had no effect on the ERK1/2 activity among groups in the VTA [$F(3, 20) = 4.427$, $P = .2766$], mPFC [$F(3, 20) = 2.807$, $P = .0891$], and NAc [$F(3, 20) = 0.349$, $P = .7903$].

Additionally, consistent with the LPS-induced effects on p-ERK1/2 and ERK1/2 protein levels, treatment with D3R antagonist NGB 2904 alone (Figure 6G-L) resulted in significantly lower expression in the mPFC (p-ERK1/2, $P = .0104$; ERK1/2, $P = .8559$) and higher expression in the NAc (p-ERK1/2, $P = .0262$; ERK1/2, $P = .0029$) in normal mice than in the vehicle control groups. Nevertheless, NGB 2904 administration alone had no significant effect on the expression of p-ERK1/2 ($P = .5057$) or ERK1/2 ($P = .6282$) in the VTA. As demonstrated in Figure 6L, the ERK1/2 protein activation in the mPFC ($P = .0157$) was decreased after NGB 2904 administration alone, but the ERK1/2 protein activation did not significantly differ in the VTA ($P = .1355$) and NAc ($P = .2215$). Co-injection of NGB 2904 with LPS failed to bring any further significant change in p-ERK1/2 and ERK1/2 expression and activation compared with those in the LPS control group (Figure 6J-L).

In summary, activation of D3R reverses the LPS-induced p-ERK1/2 and ERK1/2 expression, whereas D3R inhibition induces an alteration in p-ERK1/2 and ERK1/2 expression and activation that is similar to that induced by LPS, suggesting that D3R is involved in LPS-induced depressive-like behavior via regulating the ERK1/2 signaling pathway.

Potential Effects of D3R on LPS-Induced Depressive-Like Behavior via Mediated p-CREB Expression and CREB Activation

Finally, to further determine the role of D3R in LPS-induced CREB activation, we examined the effect of PPX and NGB 2904 on LPS-induced CREB transcriptional activity through western blotting analysis. As shown in Figure 7, our results revealed that LPS treatment significantly reduced the expression levels of p-CREB in the VTA ($P = .0007$; $P = .0017$, respectively) and mPFC ($P = .0379$; $P = .0039$, respectively) but increased the p-CREB expression in the NAc ($P = .0063$; $P = .0004$, respectively) compared with the levels in the respective control groups. Consistently, the CREB activation was decreased in the VTA ($P = .0014$; $P = .0076$, respectively) and mPFC ($P = .0003$; $P = .0001$, respectively) but increased in the NAc ($P = .0001$; $P = .0001$, respectively) post-LPS administration. There was no significant difference in the levels of CREB expression after LPS administration among all experimental groups.

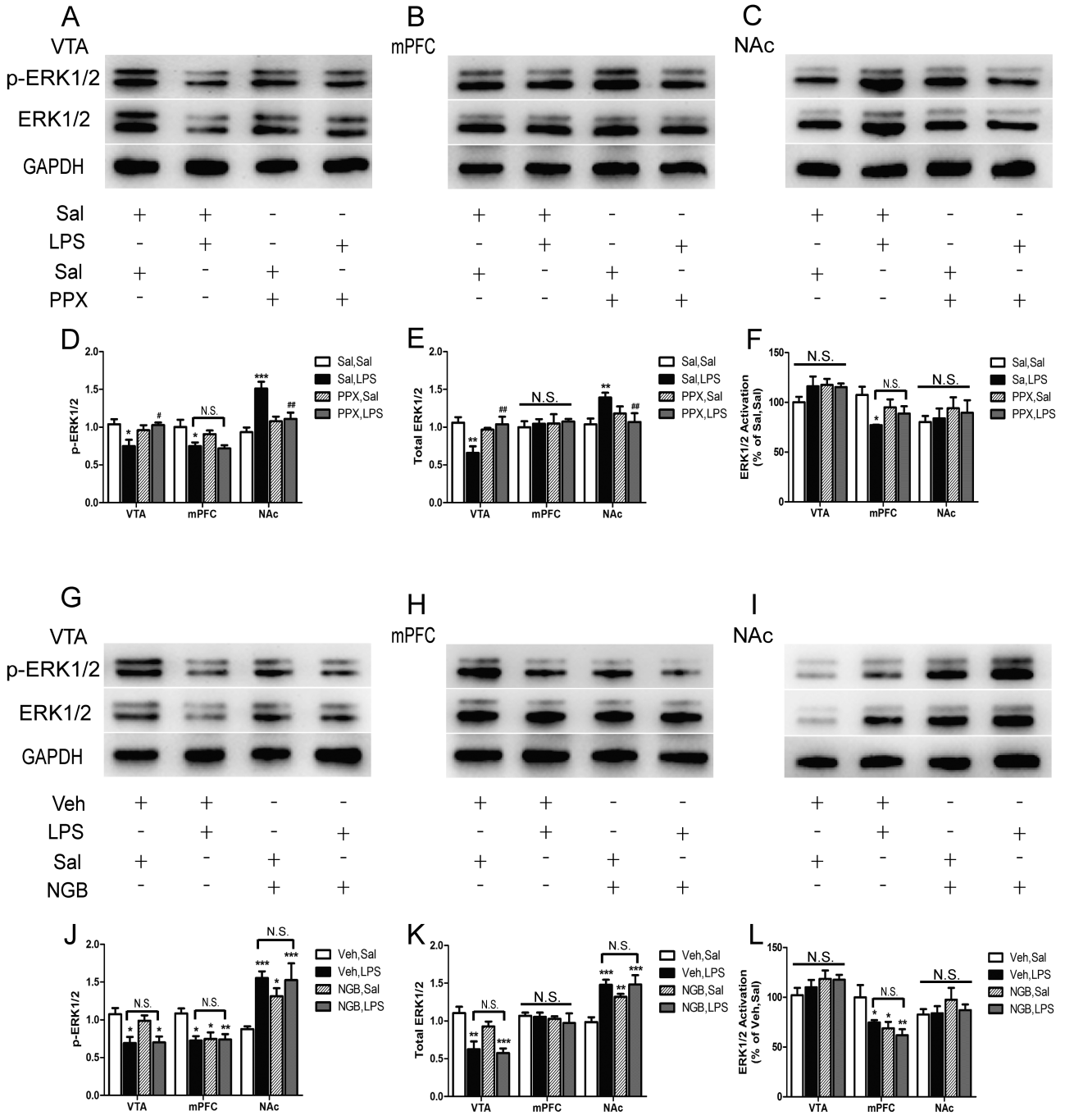

Figure 6. Roles of dopamine receptor D3 (D3R) in lipopolysaccharide (LPS)-induced depressive-like behavior via the involvement of phosphorylated ERK1/2 (p-ERK1/2) and ERK1/2. (A-C) Pretreatment with pramipexole (PPX) reversed the LPS-induced reduction in the expression of p-ERK1/2 and ERK1/2 in the ventral tegmental area (VTA) and increase in the nucleus accumbens (NAc) but had no effect in the medial prefrontal cortex (mPFC); PPX had no effect on the ERK1/2 protein activation among groups in VTA, mPFC, and NAc. (D-F) Quantitative analysis of the effects of PPX pretreatment on the expression and activation of p-ERK1/2 and ERK1/2 in the VTA, mPFC, and NAc. The data are presented as the mean ± SEM. $^{*}P < .05$ vs Sal, Sal; $^{**}P < .01$ vs Sal, Sal; $^{***}P < .001$ vs Sal, Sal; $^{\#}P < .05$ vs Sal, LPS; $^{\#\#}P < .01$ vs Sal, LPS. N.S., not significant; n = 6 mice/group. (G-I) NGB 2904 treatment alone induced a decrease expression in p-ERK1/2 in the mPFC and increased expression in p-ERK1/2 and ERK1/2 in the NAc but had no effect in the VTA. The ERK1/2 protein activation in the mPFC was decreased after NGB 2904 administration alone; however, the ERK1/2 protein activation did not significantly differ in the VTA and NAc. Co-injection of NGB 2904 with LPS failed to bring any further significant change in p-ERK1/2 and ERK1/2 expression in the VTA (p-ERK1/2, $P = .9432$; ERK1/2, $P = .6282$), mPFC (p-ERK1/2, $P = .8996$; ERK1/2, $P = .5173$), and NAc (p-ERK1/2, $P = .8730$; ERK1/2, $P = .9623$) compared with that in the LPS control group, and co-injection of NGB 2904 with LPS also did not affect the ERK1/2 activation in the VTA ($P = .4732$), mPFC ($P = .2995$), and NAc ($P = .7747$) compared with the LPS control group. (J-L) Quantitative analysis of the effect of NGB 2904 treatment alone on the expression and activation of p-ERK1/2 and ERK1/2 in the indicated brain regions. The data are presented as the mean ± SEM. $^{*}P < .05$ vs Veh, Sal; $^{**}P < .01$ or Veh, Sal; $^{***}P < .001$ vs Veh, Sal. N.S., not significant; n = 6 mice/group.

One-way ANOVA showed that D3R agonist PPX (Figure 7A-F) pretreatment blocked the LPS-induced decrease in p-CREB expression levels in the VTA [$F(3, 20) = 10.814$, $P = .0009$] and increase in the NAc [$F(3, 20) = 5.689$, $P = .0155$] but had no effects on the levels in the mPFC [$F(3, 19) = 1.041$, $P = .1251$]. Similarly, PPX pretreatment reversed the LPS-induced CREB activation in the

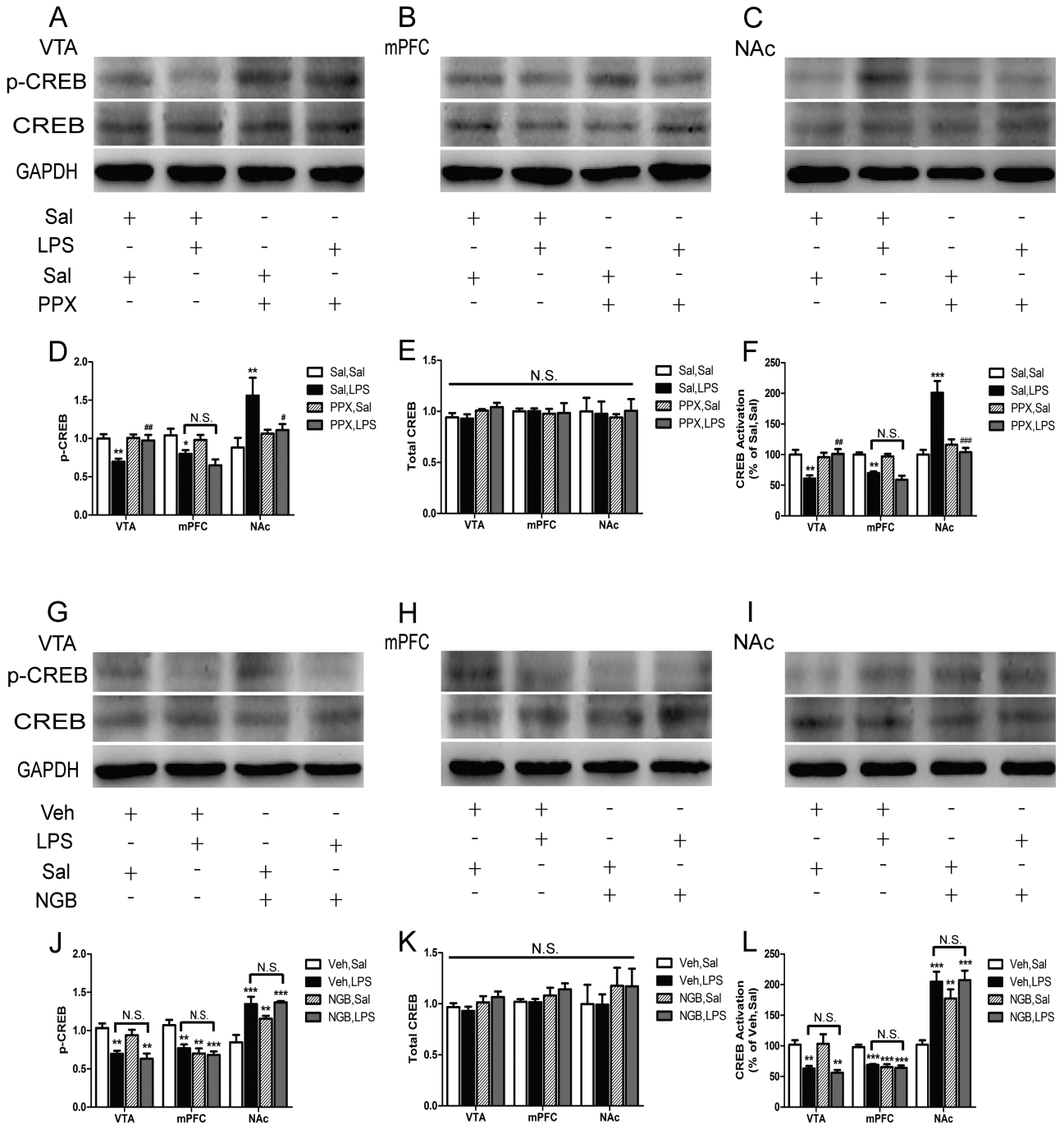

Figure 7. Effects of dopamine receptor D3 (D3R) on lipopolysaccharide (LPS)-induced depressive-like behavior via mediating phosphorylated CREB (p-CREB) expression and CREB activation. (A-C) Pretreatment with pramipexole (PPX) reversed the LPS-induced decrease in p-CREB expression and CREB activation in the ventral tegmental area (VTA) and increase in the nucleus accumbens (NAc), while it had no effect on p-CREB expression and CREB activation in the medial prefrontal cortex (mPFC) after LPS administration. There was no significant difference in LPS-induced CREB expression prior to PPX pretreatment in the VTA [$F(3, 20) = 2.005$, $P = .1839$], mPFC [$F(3, 20) = 1.258$, $P = .3294$], and NAc [$F(3, 20) = 0.296$, $P = .8275$]. (D-F) Quantitative analysis of the effects of PPX pretreatment on the expression and activation of p-CREB and CREB in the VTA, mPFC, and NAc. The data are presented as the mean ± SEM. $^{*}P < .05$ vs Sal, Sal; $^{**}P < .01$ vs Sal, Sal; $^{***}P < .001$; $^{\#}P < .05$ vs Sal, LPS; $^{\#\#}P < .01$ vs Sal, LPS; $^{\#\#\#}P < .001$. N.S., not significant; n = 6 mice/group. (G-I) Treatment with NGB 2904 alone caused a reduction in p-CREB expression and CREB activation in the mPFC and an increase in the NAc, but it failed to alter p-CREB expression and CREB activation in the VTA. Subsequent NGB 2904 treatment in combination with LPS failed to have any significant effect on the p-CREB expression in the VTA ($P = .4697$), mPFC ($P = .3141$), and NAc ($P = .2580$) compared to LPS control group. In addition, co-administration of NGB 2904 and LPS did not alter CREB activation in the VTA ($P = .5980$), mPFC ($P = .3964$), and NAc ($P = .8993$). (J-L) Quantitative analysis of the effects of NGB 2904 on p-CREB and CREB in the VTA, mPFC, and NAc. The data are presented as the mean ± SEM. $^{**}P < .01$ vs Veh, Sal; $^{***}P < .001$ vs Veh, Sal. N.S., not significant; n = 6 mice/group.

VTA [$F(3, 18) = 7.183$, $P = .0029$] and NAc [$F(3, 18) = 16.450$, $P = .0001$], but did not affect activation levels in the mPFC [$F(3, 18) = 1.281$, $P = .0992$]. There was no significant difference in CREB expression between PPX pretreatment group and LPS control group.

Moreover, in agreement with the effects of NGB 2904 treatment alone on p-ERK1/2 expression, the levels of p-CREB expression were suppressed in the mPFC ($P = .0006$) and facilitated in the NAc ($P = .0064$) after NGB 2904 (Figure 7G-L) administration

alone compared with the levels in the vehicle control group, but there were no changes in the VTA ($P = .2991$). The results of the CREB activation, after treatment with NGB 2904 alone, were decreased in the mPFC ($P = .0001$) and increased in the NAc ($P = .0015$); however, it was not altered in the VTA ($P = .9090$). NGB 2904 treatment did not affect the levels of CREB expression among all experimental groups. Subsequent NGB 2904 treatment in combination with LPS failed to have any significant effect on the p-CREB expression and CREB activation compared with those in the group of LPS-treated mice.

These results suggest that activation of D3R blocks LPS-induced p-CREB expression and CREB activation; however, inhibition of D3R produced effects similar to those of LPS on p-CREB expression and CREB activation, suggesting that alterations in CREB signaling pathway mediated by D3R are implicated in LPS-induced depressive-like behavior.

Discussion

Unlike other dopamine receptors, which have broad expression patterns in the brain and periphery, D3R is specifically distributed in the mesolimbic dopaminergic areas associated with the reward- and emotion-related behaviors (Sokoloff et al., 1990; Nestler and Carlezon, 2006; Beaulieu and Gainetdinov, 2011). Consistently, we also observed that D3R was abundantly expressed in the VTA, mPFC, and NAc in the normal condition. However, a single dose of LPS administration significantly decreased the levels of D3R expression in the VTA, mPFC, and NAc compared with those in the saline control group. To the best of our knowledge, this is the first demonstration that LPS-induced inflammation reduced D3R levels of brain regions in the mesolimbic dopaminergic system. Furthermore, our behavioral data demonstrated that PPX, a preferential D3R agonist, reversed the effects of LPS treatment on depressive-like behavior, including inhibiting the weight reduction and shortening the extended immobility time in the FST and TST. A previous study on $D3R^{-/-}$ mice suggested that D3R deficiency results in chronic depression (Moraga-Amaro et al., 2014). In our present study, we surprisingly determined that treatment with the D3R selective antagonist NGB 2904 alone induced depressive-like behavior, manifested as a reduction in weight and enhancement in the immobility time in the FST. It is therefore likely that downregulation of D3R is largely responsible for the inflammation-induced depression and plays a fundamental role in the physiopathology of depression.

Among the inflammatory animal models of depression, peripheral administration of LPS is an experimental approach that causes the activation of microglia and proinflammatory cytokines (TNF-α, IL-1β, and IL-6) (Andre et al., 2008; O'Connor et al., 2009). This increase in the expression of proinflammation cytokines in the brain is implicated in facilitating corticotrophin-releasing hormone production and subsequent activation of the hypothalamic-pituitary-adrenal axis; in turn, the activation of the hypothalamic-pituitary-adrenal axis has been shown to trigger symptoms of depression, resulting in fatigue, difficulties with sleep, and anhedonia (McKay and Zakzanis, 2010; Euteneuer et al., 2011). As expected, we showed a remarkable stimulation of TNF-α, IL-1β, and IL-6 in the VTA, mPFC, and NAc after LPS administration, which is important in inducing depression-like behavior. In our study, we highlighted pretreatment with PPX inhibited LPS-stimulated expression of TNF-α, IL-1β, and IL-6 in the VTA and NAc but not the mPFC. In agreement with our observation, a recent study also showed that repeated PPX treatment attenuated the LPS-induced levels of IL-1β increased in the hippocampus of mice (Lieberknecht et al., 2017). Thus, we speculate that upregulation of D3R plays antidepressant roles via suppressing the activation of proinflammatory cytokines signaling in the brain. Interestingly, treatment with the D3R antagonist NGB 2904 alone increased the levels of proinflammatory cytokines TNF-α, IL-1β, and IL-6 in the mPFC and NAc but not in the VTA, which perhaps results in depressive-like behavior. Accordingly, these results imply that D3R regulates LPS-induced inflammation and further controls depression-like behavior.

Our present study using ELISA analysis verified that BDNF expression was inhibited in the mPFC and elevated in the NAc in LPS-induced depression with mice. These findings are consistent with reduced BDNF expression in the prefrontal cortex (Dwivedi et al., 2003; Karege et al., 2005; Zhang et al., 2014; Muller et al., 2015), and increased in the NAc (Krishnan et al., 2007; Zhang et al., 2014; Ma et al., 2017) has also been reported in patients with depression and inflammation-related depressive animal models. Importantly, our results also determined that BDNF was expressed in the VTA in the normal condition; however, LPS caused a reduction in BDNF in the VTA. Consequently, as Sun et al. (2013) reported, abnormal BDNF levels in the prefrontal cortex and the VTA-NAc pathway seem to play a causative role in the pathophysiology of depression. However, the correlation between D3R and BDNF in different cerebral nuclei in LPS-induced depressive-like behavior is yet to be determined. From our results, pretreatment with PPX abrogated LPS-induced BDNF levels in the VTA and NAc but not in the mPFC, whereas NGB 2904 treatment alone directly contributed to the reduction in BDNF expression in the mPFC and increase in the NAc but not in the VTA. Several studies in vivo and in vitro have proven that dopamine regulates BDNF expression (Kuppers and Beyer, 2001; Ohta et al., 2003; Chiba et al., 2010). Moreover, Du et al. (2005) found that PPX modulates the production of endogenous BDNF in cultured dopamine neurons, which may participate in their neuroprotection. Thus, an intriguing possibility is that D3R controls dopamine synthesis and release, as it is located both pre- and postsynaptically, and the activation/inhibition of dopamine mediated by D3R further modulates BDNF expression. Another possibility is that the altered expression of D3R could change the induction of inflammation, as we have shown, which would then influence BDNF expression in brain. According to these two hypotheses, we conclude that activation of D3R ameliorates LPS-induced BDNF expression and then plays an antidepressant-like role. Significantly, the alterations in BDNF expression directly induced by D3R suppression may be involved in depression-like behavior.

Growing evidence suggests animal models of depression induced by chronic mild stress are correlated with decreased levels of ERK1/2 and CREB in the prefrontal cortex, which are then increased by antidepressant-like treatment (Qi et al., 2008; Lu et al., 2013; Liu et al., 2014). Rats implanted with cannulas in the mPFC and treated with a subchronic regime of ERK1/2 inhibition results in depressive-like behavior and decreases CREB protein in the mPFC (Qi et al., 2009). Our current results in an LPS-induced inflammatory model demonstrated that p-ERK1/2 expression and ERK1/2 activation were decreased, as well as p-CREB expression and CREB activation were reduced in the mPFC, which is in accordance with previous research. Conversely, a strong increase in p-ERK1/2 levels in the NAc is observed with chronic defeat stress (Krishnan et al., 2007; Sun et al., 2013). A study concerning CREB showed that an elevation in CREB in the NAc increases immobility time in the FST, an effect that is opposite to the effect of chronic antidepressant

treatment (Pallis et al., 2001). Similarly, overexpression of CREB in the NAc of inducible transgenic mice leads to a depression-like phenotype (Newton et al., 2002). Our results from an LPS-induced inflammation model, supporting previous studies, showed that not only the expression of p-ERK1/2 and ERK1/2, but p-CREB expression and CREB activation were facilitated in the NAc. In addition, the expression of p-ERK1/2 and ERK1/2 was decreased and concomitant suppression of p-CREB expression and CREB activation in the VTA after LPS exposure. In this context, a single injection of LPS induces depressive-like behavior, possibly mediated by the ERK1/2-CREB signaling pathway.

Recent studies have demonstrated that the BDNF/ERK1/2/CREB signaling pathway in rodents is sufficient to elicit depression, and pharmaceutical drugs display antidepressant effects via this pathway (Yi et al., 2014; Li et al., 2015; Luo et al., 2017). Conversely, there is a conflicting concept that BDNF is considered to be a downstream factor affected by CREB (Shieh et al., 1998; Tao et al., 1998), and the ERK1/2/CREB/BDNF signaling pathway is involved in chronic mild stress (Thome et al., 2000; Guo et al., 2014; Yan et al., 2016). The effects observed from previous findings may be specific to the interaction of BDNF and ERK1/2-CREB that may contribute to the modulation of depression. Here, we mainly focused on the effects of D3R on the ERK1/2-CREB signaling pathway in LPS-induced depressive-like behavior. We found that pretreatment with PPX reversed the levels of ERK1/2-CREB in the VTA and NAc after LPS injection; by contrast, NGB 2904 treatment alone produced the effects on ERK1/2-CERB expression in the mPFC and NAc that are consistent with the changes caused by LPS induction. These findings demonstrate that D3R directly or indirectly regulates the ERK1/2-CREB signaling pathway in inflammation-induced depression.

In our current study, treatment with D3R antagonist alone can trigger depressive-like phenotype, while D3R antagonist failed to worsen the LPS-induced depressive behavior. One explanation is that D3R protein expression has been remarkably inhibited in LPS-induced inflammation, which may lead to negligible influence of D3R antagonist under combined action of two reagents in brain of mice. Another possibility is that functional interactions of D3R with glutamatergic system may together be involved in the development of depressive-like behavior after inflammation. Studies have suggested that the N-methyl-D-aspartate (NMDA) receptor and α amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor are implicated in the emergence of depressive-like behavior after inflammation (Walker et al., 2013; Sekio and Seki, 2014). Moreover, the VTA-NAc pathway receives not only dopamine inputs, but strong glutamatergic inputs from several brain regions (Nestler and Carlezon, 2006). It has been reported that AMPA receptor-mediated neurotoxicity can be modulated by dopamine receptor D2 (Zou et al., 2005), which is characterized for its high sequence homology with D3R and co-localized with D3R in dopamine neuron (Fiorentini et al., 2015). Therefore, it seems that other neurotransmitter systems, such as glutamatergic system, interact with D3R in depressive-like behavior induced by inflammation, although future detail studies are needed to support this hypothesis.

In conclusion, our work found LPS-induced downregulation of D3R protein levels in the reward system is associated with induction of depressive-like behavior. We also showed pretreatment of D3R agonist PPX alleviated LPS-induced depressive behaviors, reduced LPS induced proinflammatory cytokine levels, and reversed LPS-induced BDNF and ERK1/2-CREB signaling pathway changes in certain brain regions. Further, our findings demonstrated selective D3R antagonist mimicked the phenotypes of LPS-induced depression model as well as produced effects similar to those of LPS on proinflammatory cytokines and BDNF expression and the ERK1/2-CERB signaling pathway changes in indicated brain regions.

Supplementary Material

Supplementary data are available at *International Journal of Neuropsychopharmacology* online.

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Statement of Interest

None.

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