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ORIGINAL PAPER

Dopamine Alters Lipopolysaccharide-Induced Nitric Oxide Production in Microglial Cells via Activation of D1-Like Receptors

Biao Wang[1] · Teng Chen[2] · Guodong Li[1] · Yuwei Jia[1] · Jing Wang[1] · Li Xue[3] · Yanjiong Chen[1]

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Abstract

Dopamine (DA) is important in the maintenance of normal nervous system function. DA is the target of multiple drugs, and it induces critical alterations in immune cells. However, these impacts are controversial, and the mechanism remains unclear. In the present study, we treated BV-2 microglial cells and primary microglia with DA and measured the changes in cytokines. We also identified the expression of DA receptors (DRs) using confocal and immunofluorescent microscopy. Specific agonists and antagonists of D1-like DRs (D1DR and D5DR) were used to observe alterations in cytokines. Western blot and siRNA interference were performed to investigate the involvement of the downstream signaling molecules of DRs. We also measured changes in mitogen-activated protein kinases (MAPKs) and the nuclear factor-kappa B (NF-κB) signaling pathway and assessed their involvement using inhibitors. We found that DA alone produced no effects on IL-6, TNF-α or nitric oxide (NO) production, and it inhibited lipopolysaccharide (LPS)-induced NO in microglial cells. Microglia expressed a high abundance of D1-like DRs (D1DR and D5DR). The agonists inhibited NO production, and antagonists reversed the DA-induced suppression of NO. Adenylatec cyclase (AC), cyclic adenosine monophosphate (cAMP) and protein kinase A (PKA) mediated DA function, and cAMP-response element binding protein (CREB) was not involved. ERK1/2 and NF-κB, but not p-38 or JNK, played roles in DA-suppressed NO generation via altering inducible nitric oxide synthase (iNOS) transcription. These data illustrate that DA modulates LPS-induced NO production via the AC/cAMP-PKA-ERK1/2-NF-κB-iNOS axis in mouse microglia, and D1-like DRs are involved. The present study provides functional evidence for an essential role of DA in immunoregulation.

Keywords Dopamine · Dopamine receptors · Nitric oxide · iNOS · NF-κB

Introduction

Dopamine (DA) is a neurotransmitter in the central and peripheral nervous systems [1]. DA is crucially important in the maintenance of normal functions, such as cognition, motivation, and movement [2]. A considerable amount of evidence revealed that accumulating DA significantly alters the immune system [3]. For example, the administration of psychostimulant drugs, such as methamphetamine, induced a dramatic release and sustained reduction of DA in the brain [4]. Disordered DA induced toxicity directly and triggered an abnormal immune response to deteriorate the disease process [5]. Parkinson's patients exhibit a decrease in the number of $CD4^+$ lymphocytes and reduction in natural killer cell activity [6]. Therefore, the immune regulation of DA is a potential mechanism for its toxicity, and it is attracting more interest and has become a hot research field.

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✉ Yanjiong Chen
chenyanjiong@126.com

[1] Department of Immunology and Pathogenic Biology, College of Basic Medicine, Xi'an Jiaotong University Health Science Center, Xi'an 710061, China

[2] Key Laboratory of the Health Ministry for Forensic Medicine, Forensic Medicine College of Xi'an Jiaotong University, Xi'an 710061, China

[3] Department of Laboratory, The Second Affiliated Hospital of Medical College of Xi'an Jiaotong University, Xi'an 710004, China

DA primarily acts via receptors. Five subtypes of DA receptors (DRs) were identified and are classified into two groups: D1-like DRs (D1DR and D5DR) and D2-like DRs (D2DR, D3DR, and D4DR) [5]. Many immune cells express DRs. For example, mouse NK cells consistently express all five DRs, and T lymphocytes express few DRs [7, 8]. Activation of DRs on the cell surface produces relevant regulatory function [7, 9]. Stimulation of D1/5DRs inhibits the cytotoxic function of $CD8^+$ T cells and impairs the differentiation and function of regulatory T cells [9]. Therefore, a study on the role of DA on immune cells may improve our knowledge of its pivotal role in neuroimmune communications.

Microglia are the resident macrophages of the brain. These cells survey vast extents of the brain and recognize endogenous or exogenous antigens, such as the apoptotic neurons and invading bacteria [10, 11]. Microglia maintain a healthy environment in the central nervous system (CNS) via removal of these antigens and secretion of neurotrophic factors [11]. Microglia also produce significant damage to tissues via excessive inflammation [5]. Microglia are crucial to the development and pathogenesis of multiple neurological disorders, including Parkinson's disease and multiple sclerosis [12]. Microglial cells secrete a wide range of inflammatory factors In vitro and in vivo, and many of these factors have the potential to impact normal neuronal functions, damage brain tissue and worsen disease processes [13–15]. Several papers reported dopaminergic modulation of the microglial immune response, but comparatively little is known about the mechanism of this response [16, 17].

Our previous study revealed the involvement of the downstream signaling of DRs in the microglial immune response, such as cyclic adenosine monophosphate (cAMP), phosphokinase A (PKA) and cAMP-response element binding protein (CREB) [18]. Therefore, we asked whether these molecules also participated in the immune regulation of microglia via exogenous DA exposure. The elucidation of microglia regulation will improve our understanding of the mechanism of DA-induced immune regulation and toxicity. The present study treated microglial cells with DA and found that DA suppressed NO production via inhibition of nitric oxide synthase (iNOS) transcription. We also fully described the D1/D5R-AC/cAMP-PKA-ERK1/2-NF-kB-iNOS signaling pathway as the mechanism for the role of DA using pharmacological and RNAi approaches.

Materials and Methods

Regents and Kits

Dopamine (hydrochloride, DA), recombinant mouse IFN-γ (rmIFN-γ), lipopolysaccharide (LPS, Escherichia coli, serotype O55:B5), SKF-38393 (hydrochloride), SCH-23390 (hydrochloride) and MDL-12 330A (hydrochloride) were purchased from Sigma–Aldrich (St. Louis, U.S.A.). RPMI 1640 and Dulbecco's modified Eagle's medium (DMEM)/F12 (1:1) were purchased from HyClone (Logan, U.S.A.). Fetal bovine serum was obtained from SiJiQing (Hangzhou, China). H89 (dihydrochloride) and JSH-23 were purchased from Selleck Chemicals (Houston, U.S.A.). Forskolin was purchased from Beyotime Biotechnology (Wuhan, China). The enzyme-linked immunosorbent assay kits for the quantitative detection of mouse TNF-α and IL-6 were obtained from eBioscience (San Diego, U.S.A.). The cyclic AMP ELISA kit was purchased from Cayman Chemical (Ann Arbor, U.S.A.). PD98059 and primary antibodies for p-p38, p38, p-JNK, JNK, p-ERK1/2, ERK1/2, p-CREB, CREB, p-p65, p65, GAPDH, TLR4, and iNOS were purchased from Cell Signaling Technology (Boston, U.S.A.). Control siRNA, CREB siRNA and primary antibodies for D1DR, D2DR, D3DR, D4DR, and D5DR were purchased from Santa Cruz Biotechnology (Santa Cruz, U.S.A.). Secondary antibodies conjugated with HRP were purchased from Abgent (San Diego, U.S.A.). The Pierce™ ECL Plus Western Blot Substrate detection kit was purchased from Thermo Fisher Scientific (Rockford, U.S.A.).

Cells

BV-2 cells were purchased from the Kunming Institute of Zoology in the Chinese Academy of Sciences (Kunming, China). Primary microglial cells were derived from the brains of postnatal (< 3 days) mice. Briefly, brain tissues were harvested from newborn C57BL/6J mice, and the meninges were carefully removed using a dissecting microscope. The tissues were cut into small pieces and trypsinized for 15 min. Digestion was terminated with DMEM/F12 media containing 10% FBS. Cells were centrifuged at 800 g for 5 min, collected and plated into 25-cm^2 flasks. The medium was replaced every 3 days for 10–15 days. Primary microglial cells were harvested using rapid shaking for 1 h in a reciprocal shaker. The purity of microglial cells was detected prior to use, and 98% purity was considered acceptable. Primary natural killer (NK) cells were derived from the spleens of C57BL/6J mice using an NK Cell Isolation kit (Fisher Scientific, U.S.A.), according to the manufacturer's instructions. The Institutional Animal Care and Use Committee of Xi'an Jiaotong University approved all experimental procedures.

SiRNA and Transfection

Control siRNA and CREB siRNA (50 pmol/well) were transfected into microglial cells using Lipofectamine RNAiMAX (Thermo Fisher, U.S.A.) for 24 h. Transfection efficiency was monitored using Western blotting.

Measurement of Cytokines and NO

Microglial cells were treated with DA for 24 h with or without LPS and H89 (30 μM), forskolin (10 μM), SKF-38393 (10 μM), SCH-23390 (10 μM), MDL-12 330A (10 μM), PD98059 (10 μM) and JSH-23 (10 μM) were added 15–30 min prior to DA when needed. The supernatants were collected and centrifuged at 3000 g for 10 min at 4 °C in a refrigerated centrifuge. Measurements of IL-6 and TNF-α were completed using ELISA assays according to the manufacturer's recommendations. The amount of NO was evaluated as the production of nitrite in the supernatants using a Griess reagent kit (Jiancheng Bioengineering Institute, Nanjing, China) according to the instruction book.

Real-Time PCR for DRs

Total RNA was extracted from cells using a TRIzol kit from Invitrogen (Carlsbad, U.S.A.). Reverse transcription was performed using a Prime Script TMRT reagent kit (Takara Bio Inc., Shiga, Japan). SYBR Green (PowerUp™ SYBR Green Kit, Thermo Fisher, U.S.A.) was used to complete the real-time PCR on a Stratagene Mx 3005p Real-Time PCR Detection System (Agilent Technologies, Santa Clara, U.S.A.). Haining Biotech (Xi'an, China) synthesized all primers. The forward and reverse primers of DRs used in the PCRs were based on Zhao et al. [19] and described as follows (all presented 5′-3′): D1DR, TGTGACACGAGG TTGAGC and GGTGGTCTGGCAGTTCTT; D2DR, CCA TTGTCTG GGTCCTGT and TGCCCTTGAG TGGTGT CT; D3DR, CTACGCCCTGTCCTACTGT and CCACCT GTCACCTCCAAG; D4DR, GTGTTGGACGCCTTTCTT CG and GGGTTGAGGGCACTGTTGA; D5DR, CTGCGA GCATCCATCAAG and CACAAGGGAAGCCAGTCC; GAPDH, TGTGTCCGTCGTGGATCTGA and TTGCTG TTGAAGTCGCAGGAG. Nos2 (the gene encoding iNOS), AGGTTGTCTGCATGGACCAG and GCTGGGACAGTC TCCATTCC. The following qPCR cycle was used: the first step was 95 °C for 30 s; the second step was 40 cycles at 95 °C for 5 s and 60 °C for 30 s; and the last step was 1 cycle at 95 °C for 1 min, 55 °C for 30 s, and 95 °C for 30 s. GAPDH served as the loading control gene. The specificity of the reaction was verified using melting curve analysis, and the $2^{-\Delta\Delta Ct}$ cycle threshold normalized to GAPDH was used to describe the relative quantitative levels of individual mRNA.

Western Blot

Single-cell suspensions were plated in 6-well microtiter plates (1×10^6 cells/well). Cells were incubated with DA for the indicated time with or without LPS or other substrates. Cells were washed three times with precooled phosphate-buffered saline and lysed in RIPA buffer (Solarbio, China) with a 1% protease and phosphorylase inhibitor cocktail (Roche, Switzerland). Protein concentrations were determined using a BCA protein assay kit (Beyotime, China). Samples were separated in 10% SDS gels and transferred to polyvinylidene fluoride membranes (PVDF, 0.22 μm, Millipore, U.S.A.). The membranes were blocked with 5% skim milk for 2 h and incubated with the primary antibodies (1:1000) overnight at 4 °C. Membranes were washed 4 times and incubated with a horseradish peroxidase (HRP)-conjugated secondary antibody (1:1000, Proteintech Group, China) for 1 h. Specific bands were visualized using a Fusion Fx5 machine (Wuzhou, China) with an enhanced chemiluminescent substrate (ECL, Millipore, U.S.A). The results were reviewed using the gel image analysis software ImageJ 2.1.4.7. The content and phosphorylation levels of protein are exhibited as the ratio compared to the control group.

Confocal and Immunofluorescence Microscopy

Treated cells were fixed in 4% Polyoxymethylene for 15 min and permeabilized with 0.2% Triton X-100 for 20 min. Slides were blocked with 5% BSA for 30 min and incubated overnight at 4 °C with primary antibodies diluted at 1:500. Alexa Fluor 594-conjugated goat anti-mouse IgG was used to visualize bound primary antibodies. DAPI (1 μg/ml) was used to stain the nuclei. Slides were mounted and viewed using a Zeiss Axiovert S100 microscope or an LSM 510 Meta Confocal microscope (Carl Zeiss, Cambridge, UK).

Statistical Analysis

All data are presented as the means ± the standard errors of the mean (SEMs). Normality and homogeneity of equal variance were confirmed prior to statistical analyses. No outlier or missing data were found. Student's t-test and one-way analysis of variance (ANOVA) with Fisher's least-significant differences (LSD) were used to examine differences between groups. Statistical analyses were performed using IBM SPSS Statistics 20.0. Statistical significance was determined at $P < 0.05$.

Results

DA Regulates LPS-Induced NO Production in Microglia

DA regulates inflammation levels in the brain and periphery [20]. However, the role of DA and DRs in the neuroinflammatory response is controversial, and few studies investigated the underlying mechanisms [16, 17]. Actual

changes in immune responses following direct cell exposure to DA in vitro were investigated. We applied DA to resting microglia cells. LPS, as a positive control, induced a significant upregulation in the production of IL-6, TNF-α and NO in BV-2 cells and primary microglia ($P < 0.001$, Fig. 1). However, no significant differences between the DA-treated groups and the vehicle group were observed. We applied DA to microglial cells in the presence of LPS to determine whether DA regulated the immune response in stimulus-challenged cells. DA drastically impeded NO production in a concentration-dependent manner without affecting cell viability (BV-2: $P = 0.655$, $P = 0.049$, $P < 0.001$, $P < 0.001$, respectively; primary microglia: $P = 0.926$, $P = 0.132$, $P < 0.001$, $P < 0.001$, respectively;

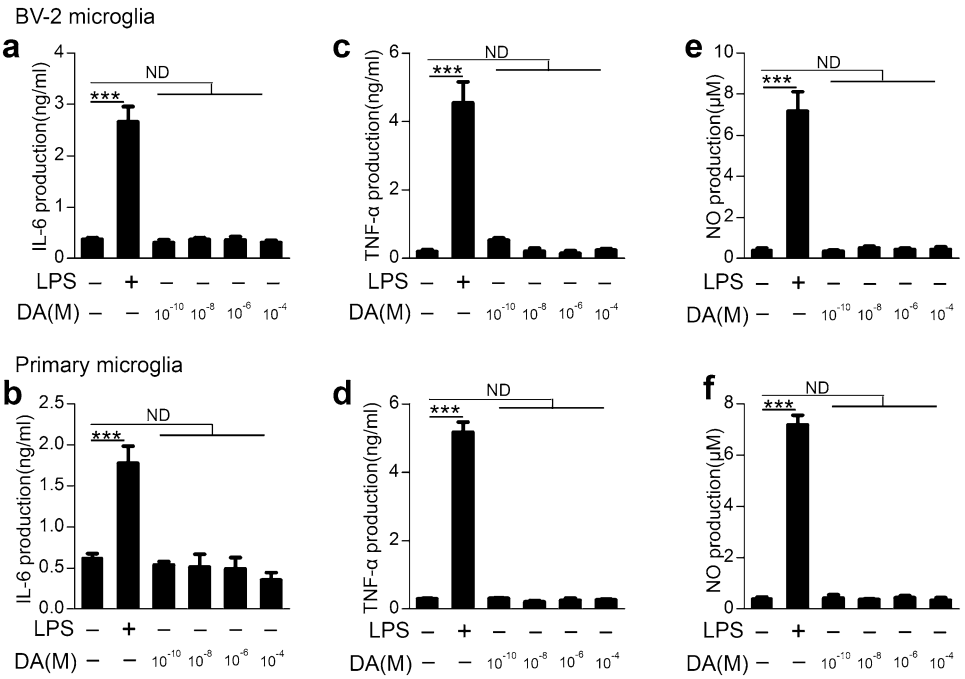

Fig. 1 Dopamine alone produces no effect on microglial inflammation. BV-2 microglial cells (**a**) and primary microglia (**b**) were treated with dopamine (DA) at different concentrations (10^{-10}, 10^{-8}, 10^{-6} and 10^{-4} M, respectively), and LPS (1 μg/ml) was used as a positive control. Supernatants were collected after a 24-h incubation, and specific kits measured IL-6, TNF-α and NO levels. Data are representative of three independent experiments and presented as the means ± SEM, n = 3/group. Statistical analyses were performed using one-way ANOVA and Fisher's LSD test. ND, no difference

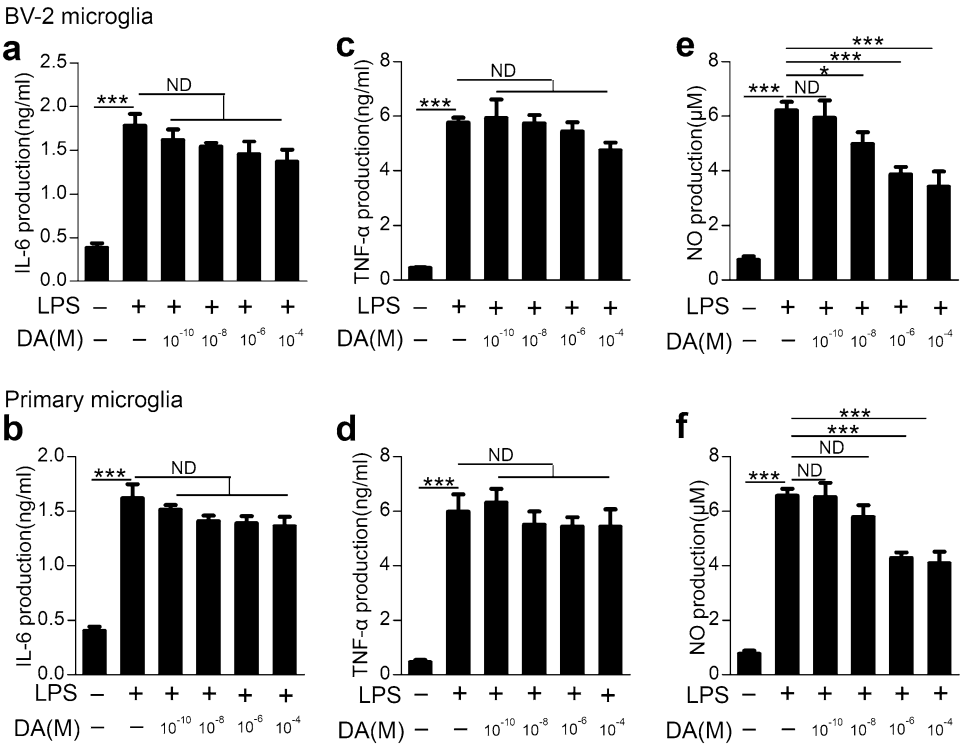

Fig. 2 Dopamine alters LPS-induced NO production in microglial cells. BV-2 microglial cells (**a**) and primary microglia (**b**) were treated with dopamine (DA) at different concentrations (10^{-10}, 10^{-8}, 10^{-6} and 10^{-4} M, respectively) in the presence of LPS (1 μg/ml). Supernatants were collected after a 24-h incubation, and specific kits measured IL-6, TNF-α and NO. Data are representative of three independent experiments and presented as the means ± SEM, n = 3/group. Statistical analyses were performed using one-way ANOVA and Fisher's LSD test. ND, no difference. ***P < 0.001

Fig. 2e, f and Fig. S1a–d). DA also exhibited a suppressive tendency on IL-6 and TNF-α, but the differences were not significant ($P > 0.05$, Fig. 2a–d). The changes in NO suggested that DA regulated the immune response of microglial cells.

D1-Like DRs are Preponderantly Expressed in Microglial Cells

The role of DA on immune regulation prompted further investigation of the function of DRs. We examined the expression of DRs on microglial cells. qPCR revealed that D1-like DRs (D1DR and D5DR) were much more abundant than D2-like DRs (D2DR, D3DR, and D4DR) in primary microglia and BV-2 cells (Fig. 3a, b). We also used confocal laser scanning microscope (CLSM) imaging to visualize the receptors directly and confirm the qPCR results. D1DR and D5DR were clearly observed on the surfaces of microglial cells (Fig. 3c). Normal immunofluorescence also revealed the existence of D1DR and D5DR (Fig. S2). In contrast, D2DR and D3DR exhibited weak spots in CLSM images, and D4DR failed to produce any visible signal in BV-2 cells or primary microglia (Fig. S3). The specificity of the anti-DRs (D2DR, D3DR and D4DR) antibodies was verified using CLSM on NK cells isolated from mouse spleen as positive controls (Fig. S3). These results suggest that the function of DA on microglia is primarily mediated via D1-like DRs because of their high expression levels.

D1-Like DRs and AC/cAMP Regulate NO

The abundance of D1-like DRs suggested that D1/5DRs mediated the supervisory role of DA. Therefore, we measured the involvement of these DRs by pretreating cells with specific agonists and antagonists. The D1/D5DRs agonist SKF-38393 significantly inhibited the LPS-induced production of NO in BV-2 cells and primary microglia ($P = 0.037$, $P = 0.005$, respectively), and it did not affect cell viability (Fig. 4a, b and Fig. S1e, f). We also treated microglia with an antagonist of D1-like DRs, SCH-23390, in the presence of LPS and DA. SCH-23390 completely restored NO production (BV-2: $P = 0.712$, primary microglia: $P = 0.415$; compared to the control group; Fig. 4c, d). These results demonstrated that D1/5DRs mediated DA regulation of NO. D1/5DRs are Gs-coupled receptors that trigger excitation of AC and increase cAMP production [21]. We used the AC activator forskolin to ascertain the role of AC/cAMP. Forskolin impaired LPS-induced NO production similarly to DA, and it did not alter cell viability (BV-2: $P = 0.009$, primary microglia: $P = 0.008$; Fig. 4e, f and Fig. S1g, h). These results supported the activation of AC after DA-induced activation of D1/5DRs. We used the AC inhibitor MDL-12 330A in the presence of DA to further confirmed this mechanism and found the MDL-12 330A significantly restored NO production compared to the DA group (BV-2: $P = 0.049$, primary microglia: $P = 0.027$; Fig. 4g, h). These results suggested that AC/cAMP played a role in DA-induced alteration of NO production.

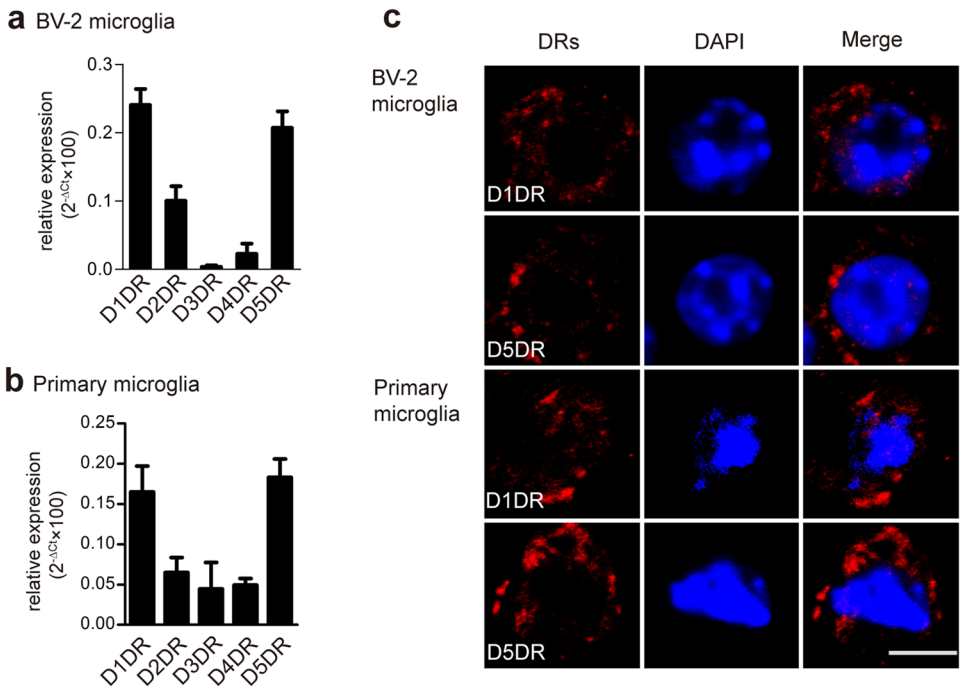

Fig. 3 Microglial cells exhibit basal dopamine receptor expression. **a, b** Total RNA was extracted from BV-2 microglial cell lines and primary microglia, and DRs were measured using specific primers. Data are representative of three independent experiments and presented as the means ± SEM, n = 3/group. (**c**) D1DR and D5DR were labeled using specific antibodies and visualized using a confocal laser scanning microscope. Scale bar = 5 μm

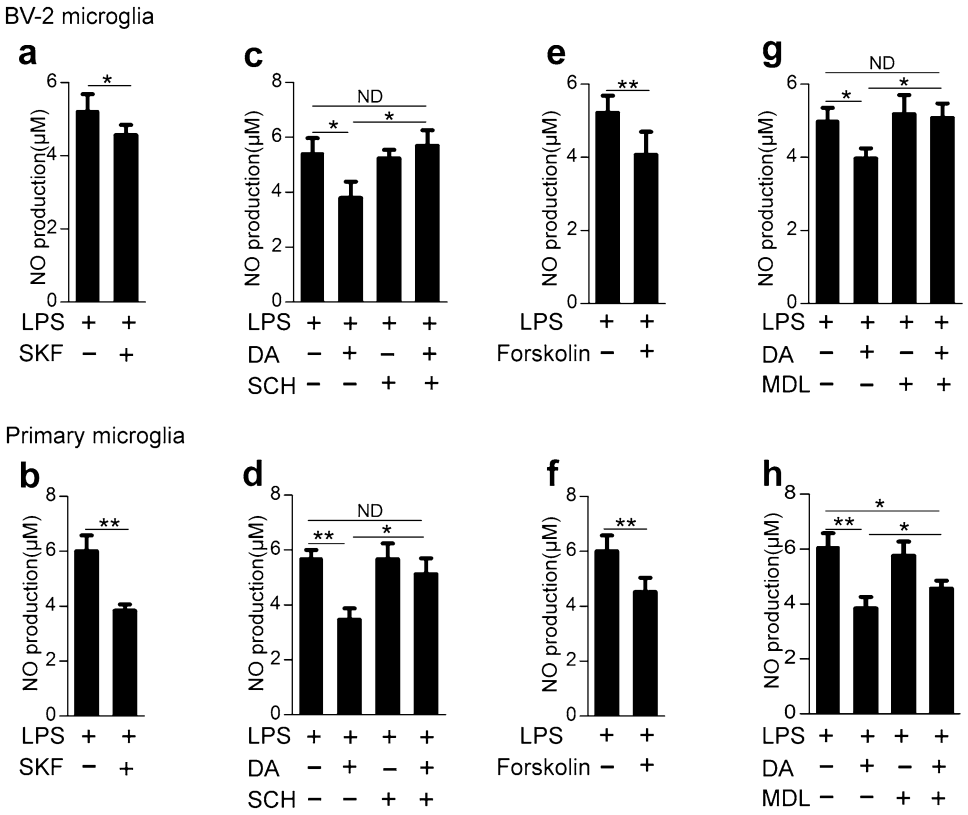

Fig. 4 D1-like receptors and AC/cAMP are involved in dopamine-altered NO production. **a, b** SKF-38393 (hydrochloride, 10 μM) was applied to BV-2 microglial cells and primary microglia in the presence of LPS (1 μg/ml) for 24 h, and the supernatants were collected for NO assays. **c, d** SCH-23390 (hydrochloride, 10 μM) was added in the presence of LPS with or without DA (10^{-6} M). **e, f** Both types of microglial cells were treated with forskolin (10 μM) in the presence of LPS. **g, h** MDL-12 330A (10 μM) was used to treat the cells in the presence of LPS with or without DA. Data are representative of three independent experiments and presented as the means ± SEM, n = 4/group. Statistical analyses were performed using Student's t-test (**a, b, e, f**) or one-way ANOVA and Fisher's LSD test (**c, d, g, h**). *P < 0.05, **P < 0.01, ***P < 0.001

PKA, But Not CREB, is Involved in the Alteration of NO

The AC/cAMP-PKA-CREB pathway is a classical signaling pathway of immunoregulation [18, 19]. Therefore, we hypothesized that PKA and CREB also played roles in DA-altered NO production. Western blot results revealed a DA-induced accumulation of PKA in BV-2 and primary microglia ($P < 0.001$, Fig. 5a, b). We treated cells with an antagonist of PKA, H89, to investigate its role. H89 obviously reversed NO production (BV-2: $P = 0.026$, primary microglia: $P = 0.018$; Fig. 5c, d). We also detected the phosphorylation level of CREB after DA treatment and found elevated p-CREB in microglia ($P < 0.050$, Fig. 5e, f). This increase suggested that CREB participated in DA-altered NO expression. We used specific siRNA to silence CREB in microglial cells to verify its role, and the effectiveness of the silencing was demonstrated using Western blot (Fig. 5g, h). However, there was no significant change in the amount of NO after CREB knock down (BV-2: $P = 0.800$, primary microglia: $P = 0.857$; Fig. 5g, h). These results demonstrated the involvement of PKA, but not CREB, in the DA-activated signaling pathway.

ERK1/2, But Not p38 or JNK, is Involved in the Altered NO Production

Mitogen-activated protein kinases (MAPKs) and nuclear factor-kappa B NF-κB are classical signaling molecules, and both proteins are indispensable for the expression of cytokines, hyperoxide and nitrogen oxide after mammalian cell exposure to LPS [22, 23]. These proteins are highly pertinent to the activation of the cAMP/PKA signaling pathway [24, 25]. Therefore, we speculated that the suppressed NO production was due to DA-induced alterations of MAPKs and NF-κB. We detected changes in MAPKs after DA treatment. DA inhibited the phosphorylation of ERK1/2 in BV-2 and primary microglia, but no change in p38, JNK, or TLR4 was observed(BV-2: $P = 0.005$, primary microglia: $P = 0.006$; Fig. 6a–f and Fig. S4a, b). Microglial cells were incubated with the ERK1/2 inhibitor PD98059, which did not alter cell viability (Fig. S1i, j). A significant decrease in NO production was observed following PD98059 treatment (BV-2: $P = 0.007$, primary microglia: $P = 0.001$; Fig. 6g, h). These results suggest the involvement of ERK1/2 in DA-modulated NO expression. Notably, the inhibition of ERK1/2 mimicked the complete inhibition of NO by DA,

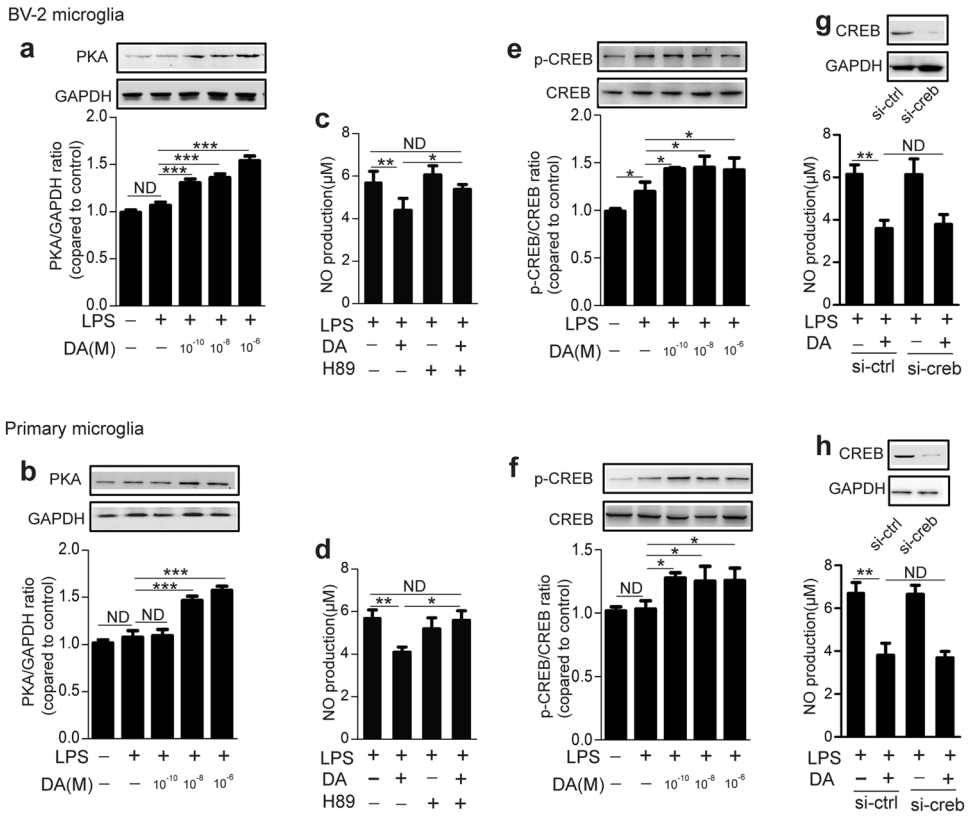

Fig. 5 PKA is involved in dopamine-altered NO production, but CREB is not involved. **a, b** BV-2 microglial cells and primary microglia were treated with DA at different concentrations (10^{-10}, 10^{-8} and 10^{-6} M, respectively) in the presence of LPS (1 μg/ml). Total protein was collected from cell lysates after a 6-h incubation, and PKA content was measured using Western blots. **c, d** The two types of cells were treated with H89 (30 μM) in the presence of LPS with or without DA (10^{-6} M) for 24 h. **e, f** Cells were treated **a** for 1 h to measure p-CREB and CREB. **g, h** Control siRNA and CREB siRNA were applied to cells for 24 h, and the efficiency was measured using Western blot, Supernatants were examined for NO production. Data are representative of three independent experiments and presented as the means ± SEM, n = 3/group. Statistical analyses were performed using one-way ANOVA and Fisher's LSD test. *P < 0.05, **P < 0.01, ***P < 0.001

which indicated the predominant role of ERK1/2 in this event.

DA Abolished NF-κB Nuclear Translocation and Decreased NO

Nuclear ERK1/2 requires transcription factors to initiate the transcription of genes via binding to their promoters directly [26–28]. NF-κB is a key molecule that mediates LPS-induced NO generation via the promotion of iNOS expression [29]. ERK1/2 activates NF-κB via the promotion of IκBβ degradation [30]. Therefore, we investigated whether NF-κB played an ultimate role in DA function. We detected the phosphorylation level of NF-κB after the cells were incubated with DA to test this hypothesis. DA significantly inhibited LPS-induced phosphorylation of NF-κB (p65, BV-2: $P = 0.004$, primary microglia: $P = 0.009$; Fig. 7a, b). CLSM imaging also clearly revealed the suppressed nuclear translocation of NF-κB (Fig. 7c, d). We also treated the cells with JSH-23 to inhibit the nuclear translocation of NF-κB. JSH-23 did not affect cell viability (Fig. S1k, i). Notably, JSH-23 completely downregulated NO production, similarly to DA and the ERK1/2 antagonist (BV-2: $P = 0.008$, primary microglia: $P = 0.002$; Fig. 7e, f). Obvious decreases in *Nos2* mRNA and iNOS content were observed in the presence of DA (BV-2: $P = 0.009$, primary microglia: $P = 0.004$; Fig. 7 g–j). The ERK1/2 antagonist inhibited p65 phosphorylation and iNOS content, but CREB siRNA produced no effect (Fig. S5a, b). Collectively, these findings demonstrated that DA reduced NO production via inhibition of *Nos2* transcription and suppression of NF-κB phosphorylation and nuclear translocation.

Discussion

DA is the predominant catecholamine neurotransmitter in the mammalian brain [31]. DA affects the functions of peripheral immune cells via DRs, but studies on immune

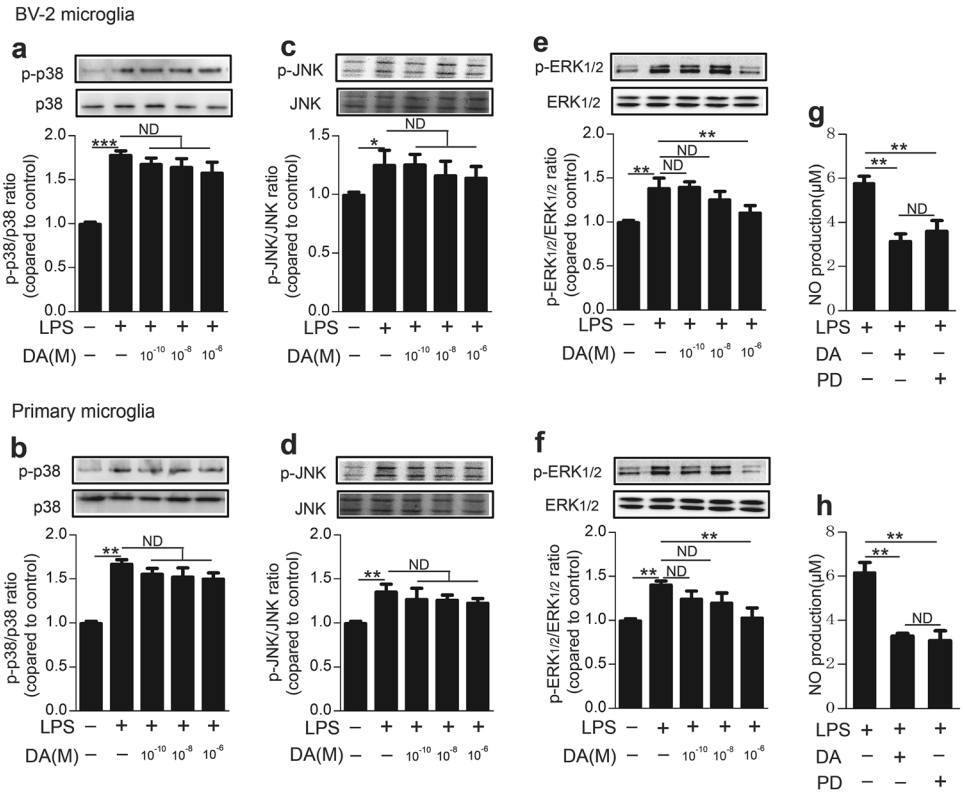

Fig. 6 ERK1/2 mediates the function of DA. **a, b** BV-2 microglial cells and primary microglia were treated with dopamine at different concentrations (10^{-10}, 10^{-8} and 10^{-6} M) in the presence of LPS (1 μg/ml) for 1 h, and the p-p38/p38 ratio was measured in cell lysates. **c, d** The p-JNK/JNK ratio was also detected in cell lysates. **e, f** The p-ERK1/2/ERK1/2 ratio was examined in cell lysates. **g, h** The ERK1/2 inhibitor PD98059 was applied to cells under the condition of LPS, and NO production was measured. Data are representative of three independent experiments and presented as the means ± SEM, n = 3/group. Statistical analyses were performed using one-way ANOVA and Fisher's LSD test. *P < 0.05, **P < 0.01, ***P < 0.001

cells in CNS are limited and contradictory [7, 8]. Therefore, the identification of DR expression on immune cells may elucidate the contribution of DA to the modulation of normal and pathological immune conditions. T-lymphocytes and monocytes exhibit low DR expression, and B cells and NK cells exhibit a higher and more consistent DR expression [32]. DRs also induce inhibitory effects on lymphocyte proliferation and function [21, 33, 34]. However, DRs on microglial cells are poorly characterized. The present study provided detailed information on the expression characteristics of five DA receptor subtypes. Microglial cells expressed high levels of D1-like receptors and a small amount of D2-like receptors in vitro. These data provide a substantial basis for the modulation of microglial function by DA.

The present study found that DA alone produced no effects on IL-6 or TNF-α in microglial cells, and it inhibited LPS-induced NO production. D1-like DRs mediated this process in our study. The two types of DRs receptor subtypes play divergent roles. For example, D1-like receptors reduce Th1 cytokine production, and D2-like receptors augment Th2 cytokines in T-lymphocytes [35]. D1-like DRs antagonists, but not a D2 antagonist, largely abrogated bupropion-induced TNF-α suppression in a mouse LPS model [36]. These results suggest that the D1-like DR-mediated signaling pathway plays a negative regulatory role in the immune response. However, this conclusion needs more evidence. The role of D2-like receptors in microglia should also be elucidated in further studies despite their relatively low expression.

AC/cAMP is a second messenger that plays a critical role in various cellular processes, and any malfunction may lead to pathology [37]. Our study assessed the role of AC/cAMP using a specific agonist (forskolin) and antagonist (MDL-12 330A) that target AC [38, 39]. Activation of AC/cAMP abolished LPS-induced NO production, and the decrement restored it. cAMP promotes the accumulation of PKA, which possesses a cytoplasmic catalytic subunit, and triggers its activation [40]. cAMP-PKA-CREB is a classical signaling pathway to mediate immune regulation, which was also noted in our previous study [18]. The present study demonstrated that PKA was involved in the DA-induced suppression of NO. PKA is a holoenzyme composed of a regulatory subunit dimer and two catalytic subunits. PKA regulates numerous cellular functions, including immune cell activity [41]. PKA upregulates CREB phosphorylation, and this result was also found in our study. We also demonstrated that CREB was not involved in the DA-induced alterations in NO production. However, this finding does not exclude a role for CREB in DA function, and additional work is necessary to elucidate the role of CREB.

The LPS-stimulated signaling pathway has been known for decades. TLR4 is the receptor that functions as an initiation factor in this pathway, but MAPKs performed the signal transmission [42]. NF-κB is the most commonly known

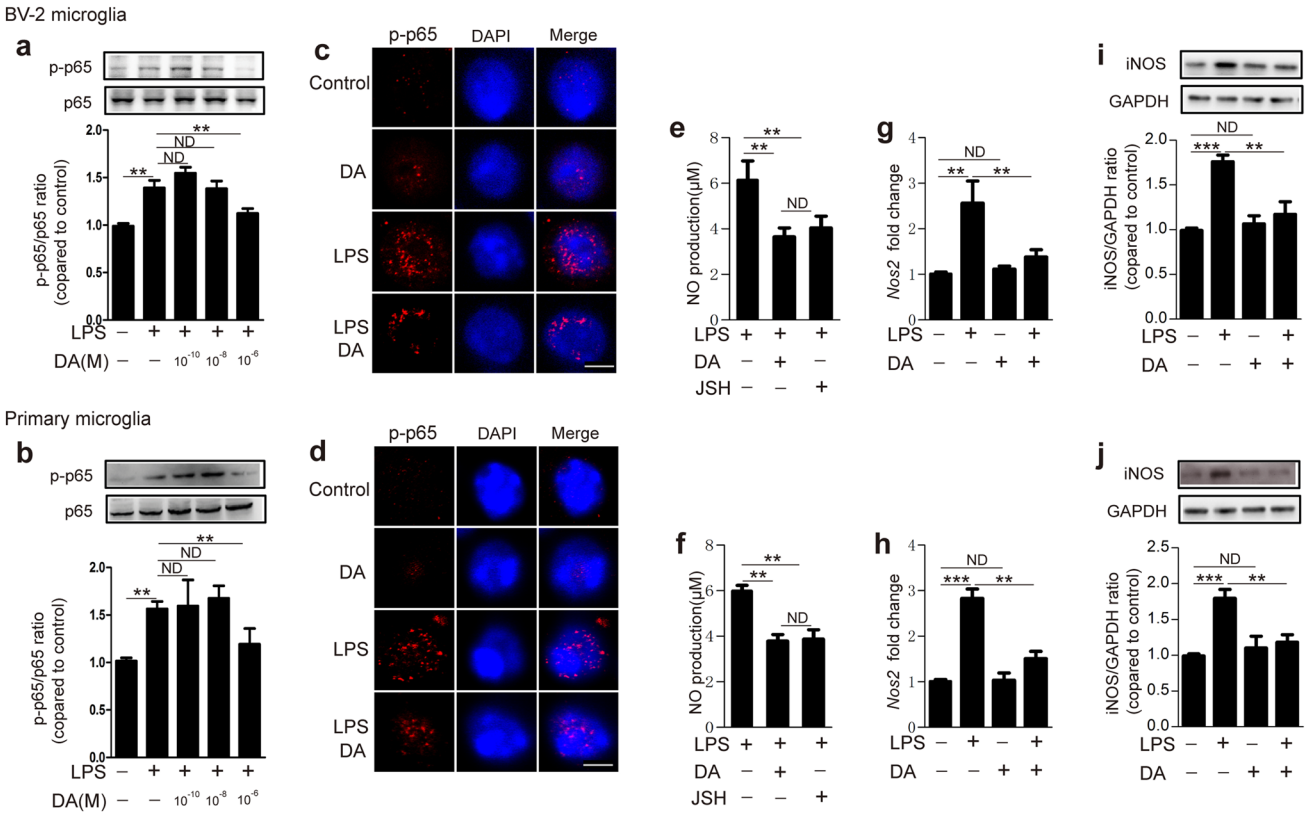

Fig. 7 DA inhibits the nuclear translocation of NF-κB and iNOS transcription. **a, b** BV-2 microglial cells and primary microglia were treated with DA at different concentrations (10^{-10}, 10^{-8} and 10^{-6} M) in the presence of LPS (1 μg/ml) for 1 h, and the phosphorylation of NF-κB (p-p65/p65 ratio) was measured. **c, d** BV-2 microglial cells and primary microglia were treated with DA (10^{-6} M) in the presence of LPS (1 μg/ml) for 1 h, and the nuclear translocation of NF-κB was monitored using a confocal laser scanning microscope. **e, f** BV-2 microglial cells and primary microglia were treated with JSH-23 (10 μM, NF-κB nuclear translocation inhibitor) or DA (10^{-6} M) in the presence of LPS for 24 h, and NO production was measured. **g, h** The two types of cells were treated with DA (10^{-6} M) in the presence of LPS for 6 h, and total RNA was used to examine the transcription level of *Nos2*. **i, j** iNOS levels were evaluated after cells were treated with DA for 12 h. Data are representative of three independent experiments and presented as the means ± SEM, n = 3/group. Statistical analyses were performed using one-way ANOVA and Fisher's LSD test. **P < 0.01, ***P < 0.001

transcription factor that acts as an executor [43]. Therefore, we determined whether the DA-induced suppression of NO was mediated via the inhibition of MAPKs and NF-κB. We demonstrated that DA inhibited the phosphorylation of ERK1/2 and NF-κB and the nuclear translocation of NF-κB. ERK1/2 activated NF-κB via promoting the degradation of IκBβ [30]. NF-κB recognizes the DNA sequence at − 8.2 kb in the Nos2 promoter [44]. The ERK1/2-NF-κB signaling pathway plays a crucial role in many biological processes, such as ischemic processes and nitrosative stress [45, 46]. The data in this study contribute to the knowledge of this signaling pathway.

NO is a gaseous molecule that is associated with the regulation of the cardiovascular, immune and nervous systems, and it exhibits a definite neurotoxic function at high concentrations [47]. There are several ways to facilitate NO-performed toxic effects [48, 49]. NO triggers stimulatory or inhibitory signals via direct interactions with intracellular targets [48]. Therefore, inordinate concentrations of NO induce an array of messy signal transduction in cells and organs. NO is also processed into reactive nitrogen species that causes serious cellular damage directly [49]. Therefore, the suppression of NO by DA suggests its protective effect. NO is a ubiquitous molecule, and it is regulated by diverse mechanisms. For example, IFN-γ triggers the generation of NO in immune cells [50]. We found that DA also suppressed IFN-γ-induced NO production (Fig. S6). This result suggests the universality of the mechanisms of DA-induced regulation in cellular process.

In conclusion, the present study provides a detailed description of DR expression in microglial cells. Our results indicate that DA inhibited LPS-induced NO production via activation of D1-like DRs and the AC/cAMP-PKA-ERK1/2-NF-κB signaling pathway. This observation suggests an endogenous physiology role of D1-like DRs on the microglial immune response to stimuli. Taken together, this study

elucidated a variety of DA functions and supports DA as a potential therapeutic target.

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Data Availability The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflict of interests.

Ethics Approval and Consent to Participate All protocols were approved by the Ethics Committee of Xi'an Jiaotong University.

Informed consent Informed consent was obtained from all individual participants included in the study.

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