



Full-length Article

Triptolide up-regulates metabotropic glutamate receptor 5 to inhibit microglia activation in the lipopolysaccharide-induced model of Parkinson's disease



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ABSTRACT

Metabotropic glutamate receptor (mGlu)₅ regulates microglia activation, which contributes to inflammation. However, the role of mGlu₅ in neuroinflammation associated with Parkinson's disease (PD) remains unclear. Triptolide (T10) exerts potent immunosuppressive and anti-inflammatory effects and protects neurons by inhibiting microglia activation. In this study, we investigated the role of mGlu₅ in the anti-inflammatory effect of T10 in a lipopolysaccharide (LPS)-induced PD model. In cultured BV2 cells and primary microglia, blocking mGlu₅ activity or knocking down its expression abolished T10-inhibited release of proinflammatory cytokines induced by LPS. Moreover, T10 up-regulated mGlu₅ expression decreased by LPS through enhancing mRNA expression and protein stability. T10 also reversed the reduction in mGlu₅ membrane localization and modulated receptor-mediated mitogen-activated protein kinase activity induced by LPS. Pharmacological inhibition of signaling molecules increased nitric oxide level and inducible nitric oxide synthase (iNOS), tumor necrosis factor- α , and interleukin (IL)-1 β and -6 transcript levels that were downregulated by treatment with T10. Consistent with these *in vitro* findings, blocking mGlu₅ attenuated the anti-inflammatory effects of T10 in an LPS-induced PD model and blocked the decreases in the number and morphology of ionized calcium binding adaptor molecule 1-positive microglia and LPS-induced iNOS protein expression caused by T10 treatment. Besides, mGlu₅ mediated the effect of T10 on microglia-induced astrocyte activation *in vitro* and *in vivo*. The findings provide evidence for a novel mechanism by which mGlu₅ regulates T10-inhibited microglia activation via modulating protein expression of the receptor and its intracellular signaling. The study might contribute to the biological effects of Chinese herbs as an approach for protecting against neurotoxicity in PD.

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Abbreviations: mGlu, Metabotropic glutamate receptor; PD, Parkinson's disease; T10, Triptolide; LPS, lipopolysaccharide; NO, nitric oxide; iNOS, inducible nitric oxide synthase; TNF, tumor necrosis factor; IL, interleukin; SN, substantia nigra; COX, cyclooxygenase; JNK, c-Jun N-terminal kinase; AKT, protein kinase B; NF, nuclear factor; PLC, phospholipase C; PKC, protein kinase C; ERK, extracellular signal-regulated protein kinase; CREB, cyclic AMP response element-binding protein; TH, tyrosine hydroxylase; Iba, ionized calcium binding adaptor molecule; CHPG, (RS)-2-Chloro-5-hydroxyphenylglycine; DHPG, (S)-3,5-dihydroxyphenylglycine; MPEP, 2-methyl-6-(phenylethynyl)-pyridine; MTEP, 3-[(2-methyl-1, 3-thiazol-4-yl) ethynyl]-pyridine; BAPTA-AM, 1,2-bis (o-aminophenoxy) ethane-N,N,N',N'-tetraacetic acid; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; MAPKs, mitogen-activated protein kinases.

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1. Introduction

Parkinson's disease (PD) is a common and debilitating neurodegenerative disease resulting from the massive loss of dopaminergic neurons, particularly in the substantia nigra (SN), leading to motor and non-motor symptoms such as rigidity, bradykinesia, and postural instability. In the normal brain, microglia contribute to the maintenance of homeostasis, disrupting this function induces their activation, which results in changes to their immunological and morphological phenotype (Garden and Möller, 2006) including increased expression of specific inflammatory surface markers, chemokines and their receptors, and of receptors for classical neurotransmitters such as tumor necrosis factor (TNF)- α , interleukin

(IL)-1 β , and nitric oxide (NO) (Chao et al., 1992; Ghoshal et al., 2007). Evidence shows the influence of microglia on the pathogenesis of PD (Long-Smith et al., 2009). Thus, inflammation underlies the pathology of PD, with hyperactivation of microglia being a main contributor and a potential therapeutic target (Lull and Block, 2010; Pessoa et al., 2014; Richardson and Hossain, 2013).

The standard treatment for PD is levodopa (Schapira, 2007), but its efficacy declines with disease progression (Lang and Lozano, 1998; Schapira, 2007). As an alternative therapy, Chinese herbs and herbal extracts have shown potential clinical benefits in attenuating PD progression in humans (Li et al., 2006; Zhou et al., 2005). Triptolide (T10) is the active extract of the traditional Chinese herb *Tripterygium wilfordii* Hook F. Pretreatment with T10 dose-dependently reduced lipopolysaccharide (LPS)-induced NO accumulation and TNF- α and IL-1 β release from microglia (Zhou et al., 2003), which in turn protected neurons. T10 also inhibited cyclooxygenase (COX)-2 expression and prostaglandin E2 release (Gong et al., 2008). The neuroprotective effects of T10 were confirmed in an inflammatory PD model generated by injecting LPS into SN of rats; movement disorder and dopaminergic neuron death were decreased while dopamine absorption was increased by T10 in this model (Li et al., 2006; Zhou et al., 2005). T10 also blocked the activation of c-Jun N-terminal kinase (JNK), protein kinase B (AKT), and nuclear factor (NF)- κ B in LPS-treated microglia (Gong et al., 2008; Liu et al., 2007). Thus, T10 exerts an anti-inflammatory effect via multiple intracellular downstream signaling targets and it is considerable to investigate whether upstream regulation of T10 via a receptor leads to its inhibitory effect on microglia activation in PD.

Glutamate receptors have been implicated in brain injury and neurodegenerative diseases (Ambrosi et al., 2014; Mehta et al., 2013). There are three groups of metabotropic glutamate (mGlu) receptors classified according to pharmacological profiles and downstream signaling mechanisms: group I (mGlu₁ and mGlu₅), group II (mGlu₂ and mGlu₃), and group III (mGlu₄, mGlu₆, mGlu₇, and mGlu₈) (Pin and Duvoisin, 1995). Agonists of group I mGlu receptors activate phospholipase C (PLC), which leads to Ca²⁺ release from the endoplasmic reticulum and protein kinase C (PKC) activation. Group I mGlu receptors are implicated in several neurological disorders, including PD, amyotrophic lateral sclerosis, and Alzheimer's disease (Ma et al., 2006; Ribeiro et al., 2010). mGlu₅ is expressed in microglia (Biber et al., 1999) and activation of mGlu₅ with CHPG was reported to reduce microglia activation in response to LPS via inhibition of TNF- α and production of reactive oxygen species and NO (Byrnes et al., 2009b). T10 treatment reduced LPS-induced microglia activation (Zhou et al., 2003). However, it remains unclear if mGlu₅ plays a role in the suppression of microglia activation by T10 in a PD model.

Based on the above evidence, the aim of this study was to investigate the mechanism underlying T10 targeting on mGlu₅ for the protection against microglia activation in PD. We found that T10 up-regulate the expression of mGlu₅ by increasing mRNA level and protein stability and thereby suppressing inflammation via mGlu₅-mediated signaling. These results provide evidence that T10 activates mGlu₅ to protect against neurotoxicity associated with increased inflammation in neurological disorders.

2. Materials and methods

2.1. Cell cultures

The murine BV2 microglia cell line was provided by Professor Xiao-Min Wang (Capital Medicine University, China) and cultured in Dulbecco's Modified Eagle's Medium (DMEM)/F12 (Corning, Manassas, VA, USA) supplemented with 10% fetal bovine serum

(FBS) and 1% penicillin/streptomycin at 37 °C and 5% CO₂. Rat primary microglia were isolated from whole brains of 0 or 1-day-old Sprague-Dawley rats (from Beijing Weitong Lihua Laboratory Animal Center, SCXK 2012-0001, Beijing, China) as previously described (Gong et al., 2008). Briefly, brain tissue was carefully freed from meninges and blood vessels. Tissue was dissociated by gentle trituration and isolated cells were cultured onto poly-L-lysine-coated 75-cm² flasks (1.5 brains per flask) in DMEM/F12 supplemented with 10% FBS and 1% penicillin/streptomycin at 37 °C and 5% CO₂. After 14 days, microglia were purified by shaking the flasks at 180 rpm, 37 °C for 2 h. Then astrocytes were purified by shaking the flasks at 250 rpm, 37 °C for 18–20 h to remove microglia on the protoplasmic cell layer. The astrocytes were trypsinized and replated onto poly-L-lysine-coated 25-cm² flasks (2.5 $\times$ 10⁶ cells per flask) and cultured for 4 days. These steps were repeated 2 times to further eliminate other cell types. The purity of the microglia cultures was >95%, as determined by immunocytochemical detection of ionized calcium binding adaptor molecule (Iba)-1. The purity of the astrocyte cultures was >98%, as determined by immunocytochemical detection of Glial fibrillary acidic protein (GFAP) and Iba-1. The rabbit anti-Iba-1 antibody (1:500, Wako Pure Chemical Industries, Osaka, Japan) or mouse anti-GFAP antibody (1:200, EMD Millipore, Temecula, CA, USA) was diluted in a solution of phosphate-buffered saline (PBS).

2.2. Drug treatments

T10 provided by Professor Xiao-min Wang (Capital Medical University, Beijing, China) was administered 30 min prior to LPS stimulation. To stimulate or block the activity of mGlu₅, (RS)-2-C hloro-5-hydroxyphenylglycine (CHPG; Tocris Biosciences, Ellisville, MO, USA) was applied to microglia for 1 h prior to stimulation with LPS (Sigma-Aldrich, St. Louis, MO, USA), and 2-methyl-6-(phenylethynyl)-pyridine (MPEP) or 3-[(2-methyl-1, 3-thiazol-4-yl) ethynyl]-pyridine (MTEP) (both from Tocris Biosciences) were applied to microglia for 30 min prior to T10 treatment. U-73122 (Tocris Biosciences), chelerythrine chloride (Cayman Chemical, Ann Arbor, MI, USA), and 1,2-bis(o-aminophenoxy)ethane-N,N,N', N'-tetraacetic acid (BAPTA-AM; Tocris Biosciences) were administered 30 min prior to T10 treatment.

2.3. Measurement of nitrite concentration

BV2 cells (4.0 $\times$ 10⁵/well) or primary rat microglia (1.0 $\times$ 10⁶/well) were seeded in 35-mm culture dishes and treated with the indicated drugs for 24 h. Culture supernatants were collected and the NO concentration was determined using a Griess kit (Promega, Madison, WI, USA) according to the manufacturer's instructions. Briefly, 50 μ l culture supernatant were transferred to a 96-well plate and incubated with an equal volume of the Griess reagent for 10 min. The absorbance at 540 nm was measured on a microplate reader (Elx800; Bio-Tek Instruments, Winooski, VT, USA) and the concentration was determined from a sodium nitrite standard curve.

2.4. Detection of IL-1 β assay levels

BV2 cells (4.0 $\times$ 10⁵/well) were seeded in 35-mm dishes and treated with indicated drugs for 24 h. The level of IL-1 β in the culture supernatant was determined using a mouse IL-1 β enzyme-linked immunosorbent assay (ELISA) kit (ExCell Bio, Shanghai, China) according to the manufacturer's protocol. Briefly, 50 μ l culture supernatant was transferred to a 96-well ELISA plate and equal volumes of assay diluent and biotin conjugate was added. After incubation for 2 h at room temperature, the wells were washed and streptavidin-horseradish peroxidase was added for

1 h at room temperature. After washing, substrate solution was added; the reaction was terminated by adding stop solution. The absorbance at 450 nm was determined on a microplate reader.

2.5. Cell proliferation assay

The proliferation of microglia was assessed with the 3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay (Cell Titer 96 Aqueous Assay; Promega). Primary rat microglia (1.0×10^5 /well) were seeded in 96-well plate and cultured for 24 h. At 24 h after drug application, MTS solution was added to the cells followed by incubation for 2.5 h at 37 °C with 5% CO₂, and the absorbance at 490 nm was determined on a microplate reader.

2.6. Real-time PCR analysis

BV2 cells (4.0×10^5 /well), primary rat microglia (1.0×10^6 /well) and primary astrocytes (8.0×10^5 /well) were seeded in 6-well plates for 24 h respectively and treated with the indicated drugs for 6 h. RNA was extracted and 1 µg was reverse transcribed using the ImProm-II Reverse Transcription System (Promega) in a total volume of 20 µl. Real-time PCR was performed to amplify mGlu₅, inducible nitric oxide synthase (iNOS), TNF-α, IL-1β and -6, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The 20-µl reaction contained 20 ng cDNA, 200 nM forward and reverse primers (Table 1), and 10 µl SYBR FAST qPCR Kit Master Mix (2×) (Kapa Biosystems, Wilmington, MA, USA). The reaction consisted of 40 cycles of 95 °C for 3 s and 60 °C for 30 s, and was carried out on a CFX96TM real-time PCR detection system (Bio-Rad, Hercules, CA, USA). The signal from each sample was normalized to that of GAPDH. Calculation of Ct, generation of a standard curve, and relative gene expression and quantification were performed using the 'post run data analysis' software provided with the iCycler system (Bio-Rad). Briefly, relative gene expression was analyzed by the ΔCt method. The ΔCt method using a reference gene is a variation of the Livak method (Livak and Schmittgen, 2001) and ratio = (reference/target) = $2^{\text{Ct}_{\text{reference}} - \text{Ct}_{\text{target}}}$.

2.7. Protein extraction and western blot analysis

For whole cell protein extraction, cells were washed twice with ice-cold PBS, and total cellular proteins were extracted with lysis buffer (Xia et al., 2015). For tissue protein extraction, tissue was lysed with RIPA lysis buffer (Solarbio, Beijing, China). For membranous and cytoplasmic protein extraction, 2.5×10^7 BV2 cells were treated with LPS and/or T10 for 24 h, and cytomembrane and cytoplasmic proteins were isolated using the Membrane and Cytosol Protein Extraction kit (Beyotime Institute of Biotechnology, Shanghai, China). Equal amounts of protein were subjected to western blotting as previously described (Xia et al., 2015). The signal was visualized with an enhanced chemiluminescence kit (Applygen Technologies, Beijing, China). Primary antibodies against the following proteins were used: β-actin (1:1000), β-tubulin (1:1000), GAPDH (1:1000), phosphorylated (p)-JNK (1:1000), p-extracellular signal-regulated protein kinase (ERK) (1:1000), p-p38 (1:1000), p-cyclic AMP response element-binding protein (CREB) (1:1000) and COX-2 (1:1000) (all from Cell Signaling Technology, Danvers, MA, USA); and iNOS (1:1000) and mGlu₅ (1:1000) (both from Abcam, Cambridge, MA, USA); tyrosine hydroxylase (TH, 1:5000, Sigma-Aldrich, St. Louis, MO, USA); Na-K ATPase (1:1000, Santa Cruz Biotechnology, Dallas, TX, USA); IL-1β (1:400, R&D Systems, Minneapolis, MN, USA).

Table 1

Real-Time PCR Primers (F: Forward Primer, R: Reverse Primer) and Size of Amplicon.

Gene	Sequence(5' → 3')	Amplicon(bp)
mGlu ₅	F: AGCTCAACTCCATGATGTTGT R: ATCTCTCGGAAGGTCGTCAT	124
iNOS	F: ACATCGACCCGTCCACAGTAT R: CAGAGGGGTAGGCTTGCTC	177
TNF-α	F: ACTCCAGGTTCTCTTCAAG R: CAGAGAGGAGGCTGACTTTC	108
IL-1β	F: TCTGTGACTCGTGGGATGAT R: GGAGAATACCACTGTGTGGC	170
IL-6	F: ACTTCACAGAGGATACCACC R: GCATCATCGCTGTTTATACA	145
rat GAPDH	F: TGACATCAAGAAGGTGCTGAAGC R: GGAAGAATGGGAGTTGCTGTTG	112
mouse GAPDH	GTCTCTGTGACTTCAACAG ACTTGTGTCATTGAGACGAATGC	80

2.8. mGlu₅ knockdown

Two short hairpin (sh)RNAs (Xi et al., 2017) were used to inhibit mGlu₅ expression. A scrambled shRNA was used as a control. The shRNAs were synthesized by Beifang Yitao Co. (Beijing, China). BV2 cells (3.5×10^5 /dish) were seeded in 35-mm dishes for 24 h. The sh-mGlu₅ plasmid mixed with Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA) and 2 ml of culture medium were added to the dishes. After 24 h, the transfection efficiency of cells was evaluated by western blotting.

2.9. Animals and treatment

Male Sprague-Dawley rats (Beijing Weitong Lihua Laboratory Animal Center, SCXK 2012-0001, Beijing, China) weighing 250–300 g were housed under standard conditions on a 12:12-h light/dark cycle with free access to food and water. The animals were allowed to acclimate to their new surroundings for 7 days before experiments. The experimental procedures were approved by the Committee on Animal Care. The sample sizes used in this study were based on estimations by a power analysis. A total of 60 rats were randomly divided into four groups: sham [PBS + intraperitoneal (i.p.) injection of vehicle, n = 15]; LPS (LPS + i.p. injection of vehicle, n = 15); LPS + T10 (LPS + i.p. injection of T10, n = 15); and LPS + T10 + MPEP group (LPS + i.p. injection of T10 and MPEP, n = 15). T10 and MPEP were dissolved in physiological saline containing 5% dimethylsulphoxide, respectively. Rats were injected with 10 µg/kg T10 with or without 3 mg/kg MPEP or vehicle once per day from 3 days before to 21 days after LPS treatment (total treatment of T10 + MPEP for 24 days and LPS treatment for 21 days). The mortality rate of rats in LPS injection group was less than 10%, leaving 13–15 animals per group. The rats were anesthetized with chloral hydrate (350 mg/kg; Tianjin Guangfu Fine Chemical Research Institute, Tianjin, China) and positioned in a stereotaxic apparatus. LPS (Sigma-Aldrich) was dissolved (5 mg/ml) in PBS, and 2 µl PBS or LPS were injected into the right SN pars compacta (5.5 mm posterior, 1.5 mm lateral, and 8.3 mm ventral to bregma) (Sharma and Nehru, 2016) through a drilled hole over a period of 2 min. After injection, the needle was left in place for over 5 min to prevent reflux along the injection track.

2.10. Behavioral test

Rats were tested 3 weeks post-surgery with the rotational and rotarod behavioral tests. Rotational behavior was induced by apomorphine hydrochloride (R-(–)-apomorphine hydrochloride hemihydrates; Sigma-Aldrich), and the rotational rate (number of rotations during the 30-min testing period divided by 30) was

calculated. Motor function was measured using a rotarod treadmill for rats under the accelerating rotor mode (constant acceleration from 5 to 30 rpm for 5 min). The interval from when the animal mounted on the rod to when it fell off was recorded as performance time. Animals were trained for five trials per day for 3 days before surgery. Performance on the rotarod test was measured five times per rat.

2.11. Immunohistochemistry and immunofluorescence analysis

Three weeks after surgery, rats were anesthetized with chloral hydrate and perfused with 4% paraformaldehyde (PFA). The brains were removed and post-fixed overnight in 4% PFA, then sequentially placed in 15% and 30% sucrose at 4 °C until cryostat sectioning. Serial coronal sections were cut at a thickness of 40 μ m on a freezing microtome and a 1:6 series of sections was used

for quantitation as previously described (Xia et al., 2015). For immunohistochemistry, the sections were incubated overnight at 4 °C with mouse anti-TH (1:5000, Sigma-Aldrich), rabbit anti-Iba-1 (1:500; Wako Pure Chemical Industries) and mouse anti-GFAP (1:500; EMD Millipore) antibodies, respectively. The ABC kit (Vector Laboratories, Burlingame, CA, USA) was used to detect immunolabeling. For immunofluorescence, the sections were incubated overnight at 4 °C with mouse anti-TH (1:5000, Sigma-Aldrich) and rabbit anti-Iba-1 (1:500; Wako Pure Chemical Industries) for TH/Iba-1 dual staining; rabbit anti-mGlu₅ (1:200; EMD Millipore) and goat anti-Iba-1 (1:200, Abcam) antibodies for mGlu₅/Iba-1 dual staining; rabbit anti-mGlu₅ (1:200; EMD Millipore) and mouse anti-GFAP (1:500; EMD Millipore) antibodies for mGlu₅/GFAP dual staining. The sections were then incubated with Alexa[®] 594-labeled donkey anti-mouse or -goat IgG and Alexa[®] 488-labeled donkey anti-rabbit IgG (1:200, Invitrogen) cocktails for 1 h at room

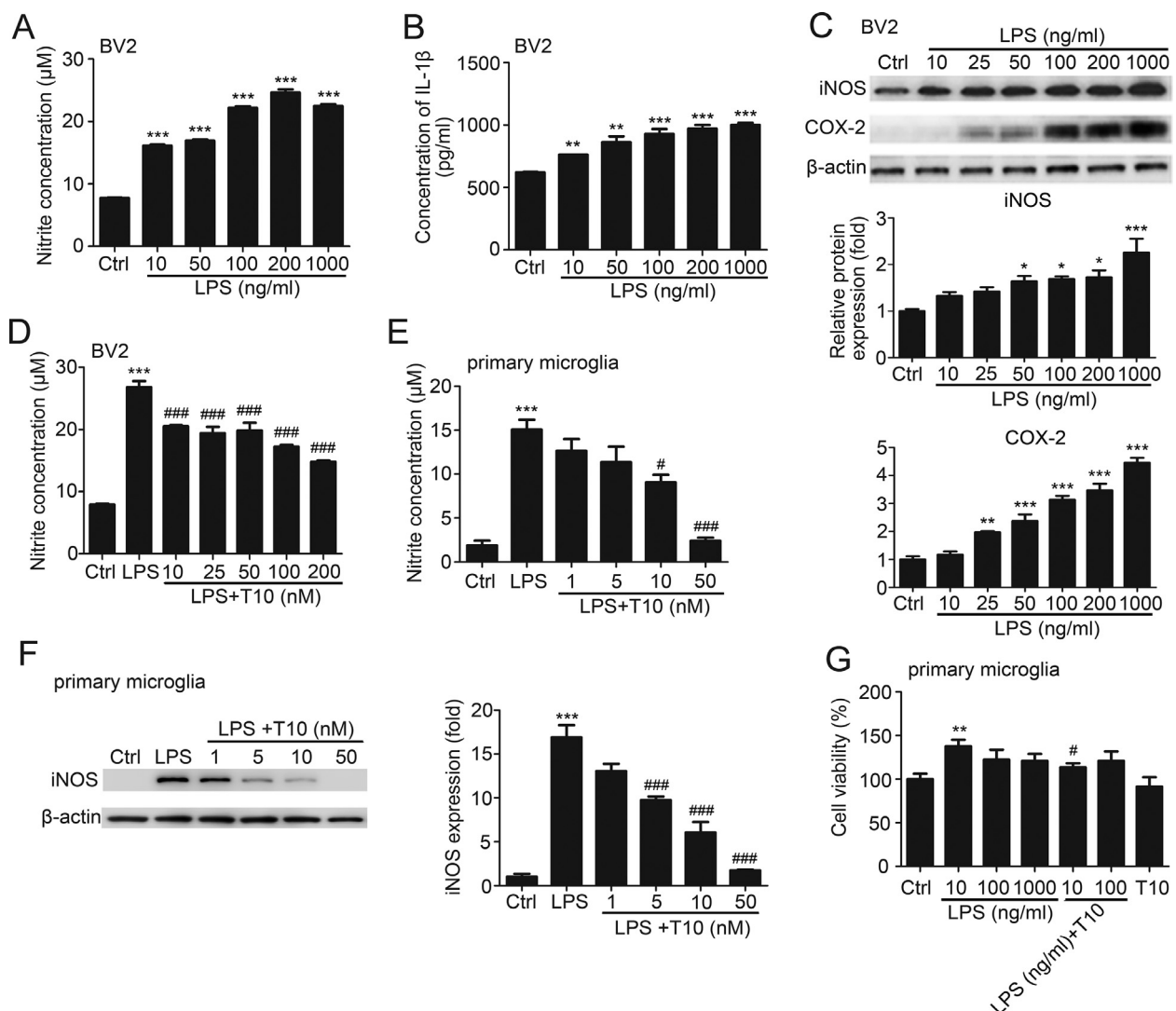


Fig. 1. T10 suppresses LPS-induced activation in microglia. (A and B) BV2 cells were stimulated with different concentrations of LPS (10, 50, 100, 200, and 1000 ng/ml) for 24 h, and culture supernatant was assayed for release of NO (A) and IL-1 β (B). (C) BV2 cells were stimulated with different concentration of LPS (10, 25, 50, 100, 200 and 1000 ng/ml) for 24 h, and iNOS and COX-2 expression was detected by western blotting (upper panel). Protein levels were normalized to β -actin and are expressed as the fold difference relative to the control group (lower panels). (D) BV2 cells were pretreated with different concentrations of T10 (10, 25, 50, 100, and 200 nM) before LPS (100 ng/ml) treatment for 24 h, and culture supernatants were assayed for NO release. (E and F) Rat primary microglia were pretreated with T10 (1, 5, 10, and 50 nM) before treatment with LPS for 24 h, and culture supernatants were collected and assayed for NO release (E). iNOS level in rat primary microglia was analyzed by western blotting (F, left). Protein level was normalized to β -actin and is expressed as a fold difference relative to the control group (F, right). (G) Rat primary microglia were pretreated with T10 (50 nM) followed by LPS (10, 100, and 1000 ng/ml) for 24 h, and cell proliferation was evaluated with the MTS assay. Cells with vehicle treatment served as a control group for each panel. Error bars represent mean $\pm$ SEM ($n \geq 3$). Statistical significances were determined by the one-way ANOVA followed by Dunnett's test. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs. control group; # $P < 0.05$, ### $P < 0.001$ vs. LPS group.

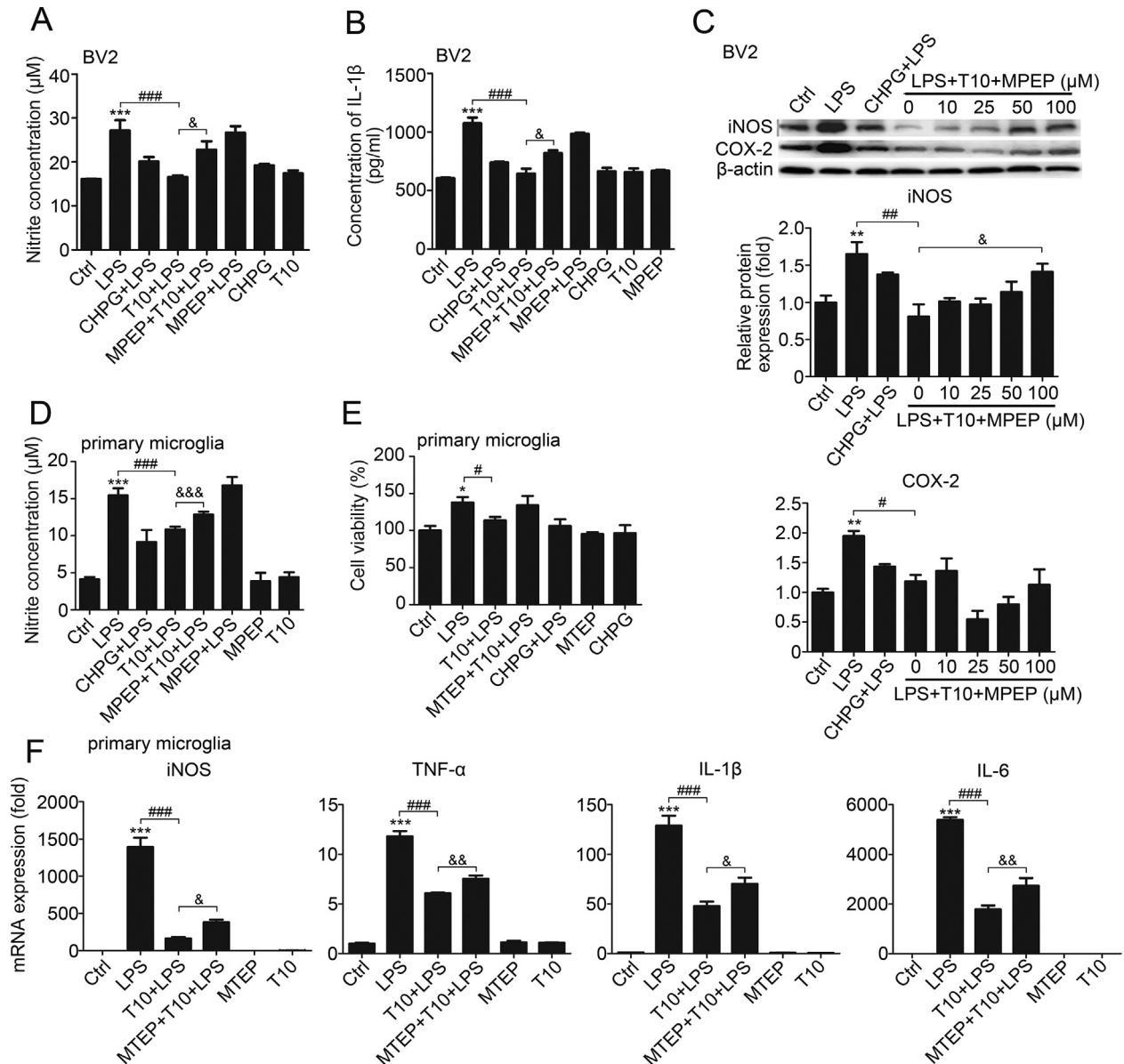


Fig. 2. mGlu₅ inhibition attenuates the anti-inflammatory effects of T10. (A and B) BV2 cells were pretreated with MTEP (100 μM) and T10 (10 nM) before LPS treatment for 24 h, and NO (A) and IL-1β (B) levels were measured. (C) BV2 cells were pretreated with MTEP (10, 25, 50, and 100 μM) and T10 before LPS treatment for 24 h, and iNOS and COX-2 levels were detected by western blotting (upper panel). Protein levels were normalized to β-actin and are expressed as the fold difference relative to the control group (lower panels). (D) Rat primary microglia were pretreated with MTEP (10 μM) and T10 before LPS treatment for 24 h, and NO production was measured. (E) Rat primary microglia were pretreated with MTEP (10 μM) and T10 before LPS treatment for 24 h. Cell proliferation was measured with the MTS assay. (F) Rat primary microglia were pretreated with MTEP and T10 and cultured with LPS for 6 h. mRNA levels were analyzed by quantitative real-time PCR and normalized to that of GAPDH. The fold difference in expression was calculated relative to each control group. CHPG (150 μM, 1 h) treatment before LPS was used as a positive control (A–E). Cells with vehicle treatment served as a control group for each panel. Data represent means ± SEM (n ≥ 3). Statistical significances were determined by the one-way ANOVA followed by Dunnett's test. **P* < 0.05, ***P* < 0.01, ****P* < 0.001 vs. control group; #*P* < 0.05, ##*P* < 0.01, ###*P* < 0.001 vs. LPS group; &*P* < 0.05, &&*P* < 0.01, and &&&*P* < 0.001 vs. T10 + LPS group.

temperature in the dark. Sections were counterstained with 4',6-diamidino-2-phenylindole (DAPI). TH-positive neurons, Iba-1-positive microglia and GFAP-positive astrocytes in the SN were quantified using Image Pro Plus v.5.0 image analysis software (Datacell, London, UK). To quantify the number of branches in microglia, the branches were counted manually using the ImageJ software (NIH). To quantify the Iba-1 intensity in SN or the mGlu₅ intensity in microglia and astrocytes, region of interest (ROI) in the SN were drawn along the region of TH-positive neurons or Iba-1-positive microglia and GFAP-positive astrocytes. Intensity of each ROI was measured by ImageJ software (NIH).

2.12. Statistical analysis

Experiments were performed at least three times. Data were analyzed using Prism 5.0 software (GraphPad Inc., La Jolla, CA, USA) and are expressed as means ± SEM. Where parametric tests were used, we checked normal distribution and difference in variance by the Shapiro-Wilk test and an F-test, respectively. A two-sample unpaired Student's *t*-test was used for two-group comparisons. One-way ANOVA followed by Dunnett's test was used for multiple-group comparisons. *P* values below 0.05 were considered significant throughout the study.

3. Results

3.1. T10 suppresses LPS-induced toxicity in microglia

LPS induces microglia activation and increases pro-inflammatory cytokine production *in vitro* (Awada et al., 2014; Wang et al., 2015; Wu et al., 2015). Meanwhile, T10 dose-dependently inhibits NO accumulation and TNF- α and IL-1 β release from LPS-activated microglia (Zhou et al., 2003). To confirm that T10 inhibits LPS-induced microglia activation, BV2 cells were incubated with different concentrations of LPS for 24 h. NO and IL-1 β were upregulated in a dose-dependent manner by LPS treatment (Fig. 1A and B). NO produced from iNOS promotes the development of neurodegenerative disorders associated with inflammation (Singh et al., 2005). Inducible COX-2 is the rate-limiting enzyme for prostaglandin E2 biosynthesis. Our results showed that LPS increased iNOS and COX-2 protein levels in a dose-dependent manner (Fig. 1C). We investigated the anti-inflammatory effect of T10 by treating BV2 cells and primary rat microglia with T10 followed by stimulation with LPS for 24 h. The results showed that T10 reduced NO production (Fig. 1D and E) and iNOS expression (Fig. 1F). Activated microglia exhibit high rates of proliferation (Byrnes et al., 2009b). We found that LPS stimulated cell proliferation, whereas cell proliferation returned to the normal level after treatment with T10 (Fig. 1G). These results indicate that T10 suppresses microglia activated by LPS, consistent with previous reports (Zhou et al., 2003).

3.2. Blocking mGlu₅ partially abolishes the protective effect of T10 against LPS-induced toxicity in microglia

mGlu₅ is thought to modulate neuroinflammation in microglia, which plays an important role in both acute and chronic neurodegenerative disorders (Byrnes et al., 2009b). Consistent with this supposition, we detected that activation of mGlu₅ by treatment with its agonist CHPG suppressed LPS-induced inflammation and proliferation (Fig. 2A–E). Thus, mGlu₅ may be involved in the inhibitory effect of T10 on microglia activation. To examine this possibility, BV2 cells were treated first with the mGlu₅ antagonist MPEP and then with T10 before exposure to LPS for 24 h. Blocking mGlu₅ with MPEP attenuated the effects of T10, including the suppression of NO, IL-1 β , and iNOS levels that were increased by LPS (Fig. 2A–C). However, it did not affect the inhibition of COX-2 expression by T10, suggesting that other mechanisms may be involved in the COX-2-mediated inflammation inhibited by T10 (Fig. 2C). The anti-inflammatory effect of T10 via regulation of mGlu₅ was confirmed in primary microglia, in which MPEP also attenuated the decrease in NO levels to a certain level mediated by T10 (Fig. 2D). MTEP, which is a more potent and selective mGlu₅ antagonist than MPEP, was used to confirm the role of mGlu₅ on microglia activation. Blocking mGlu₅ with MTEP attenuated the inhibitory effect of T10 on LPS-induced cell proliferation (Fig. 2E) and increased iNOS, TNF- α , and IL-1 β and -6 mRNA expression (Fig. 2F). These results indicate that activated mGlu₅ partially mediates the protective effects of T10 against LPS-induced toxicity in microglia.

3.3. mGlu₅ knockdown reduces the anti-inflammatory effects of T10

To further investigate whether the anti-inflammatory effects of T10 are mediated by mGlu₅, BV2 cells were transfected with an shRNA against mGlu₅ (sh-mGlu₅) to inhibit its expression. Following pretreatment with T10, mGlu₅ knockdown and wild-type cells were stimulated by LPS. The protein level of mGlu₅ was reduced by 62.2% relative to the control group by transfection of sh-mGlu₅, as

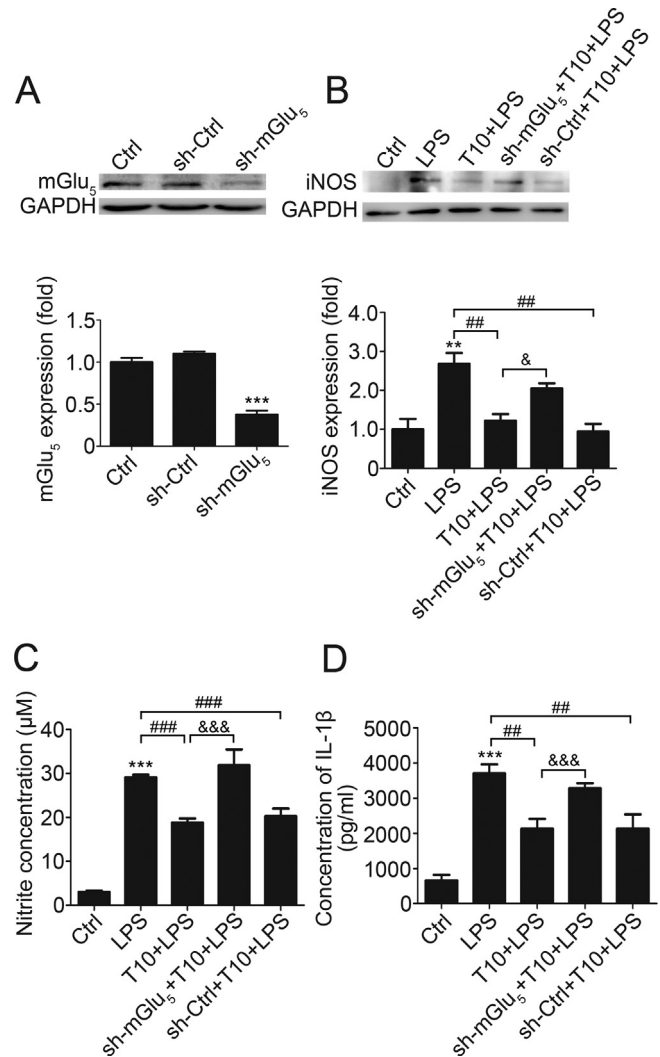


Fig. 3. mGlu₅ knockdown reduces the anti-inflammatory effects of T10. (A) BV2 cells were transfected by non-specific (sh-Ctrl) or mGlu₅-targeted (sh-mGlu₅) shRNA for 24 h, and mGlu₅ level was determined by western blotting. (B–D) Wild-type or mGlu₅ knockdown cells were pretreated with T10 prior to LPS treatment for 24 h. iNOS expression was detected by western blotting (B, up). Protein levels were normalized to β -actin and are expressed as the fold differences relative to the control group (B, down). Culture supernatants were assayed for NO (C) and IL-1 β (D) release. Cells with vehicle treatment served as a control group for each panel. Data represent mean $\pm$ SEM (n = 3). Statistical significances were determined by the one-way ANOVA followed by Dunnett's test. ** P < 0.01, *** P < 0.001 vs. control group; ### P < 0.01, ### P < 0.001 vs. LPS group; & P < 0.05, &&& P < 0.001 vs. T10 + LPS group.

determined by western blotting (Fig. 3A). LPS stimulation increased microglia iNOS synthesis and NO and IL-1 β production in wild-type cells (Fig. 3B–D). However, T10 treatment attenuated the effects of LPS in wild-type microglia but not in mGlu₅ knockdown cells. These results indicate that the anti-inflammatory effect of T10 is mediated by mGlu₅ activation.

3.4. T10 up-regulates mGlu₅ protein expression

LPS was previously shown to inhibit mGlu₅ expression in microglia (Berger et al., 2012). In the present study, we found that blocking mGlu₅ abolished the inhibitory effect of T10 on microglia activation. To determine whether T10 inhibits microglia activation by modulating the level of mGlu₅ protein, BV2 cells were treated with LPS for 24 h in the absence or presence of T10. mGlu₅ expression decreased in a

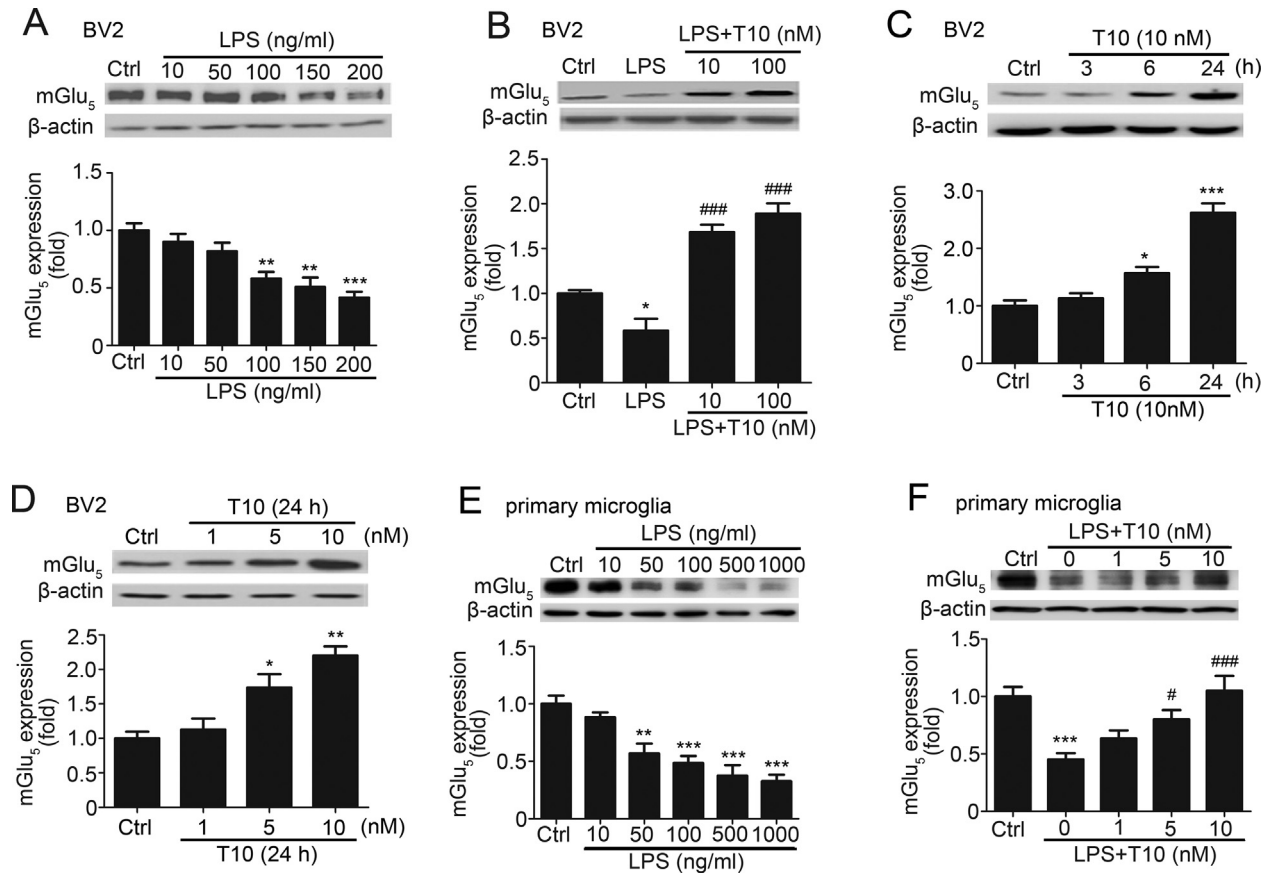


Fig. 4. mGlu₅ expression is upregulated by T10 in LPS-stimulated microglia. (A–D) BV2 cells were treated with LPS (10, 50, 100, 150, and 200 ng/ml) for 24 h (A); with T10 (10 and 100 nM) followed by LPS for 24 h (B); with T10 for the indicated times (C); and with T10 (1, 5, and 10 nM) for 24 h (D). (E and F) Rat primary microglia were treated with LPS (10, 50, 100, 500, and 1000 ng/ml) for 24 h (E) and with T10 (1, 5, and 10 nM) followed by LPS for 24 h (F). mGlu₅ levels were detected by western blotting and normalized to that of β -actin, and are expressed as the fold difference relative to the control group. Cells with vehicle treatment served as a control group for each panel. Data represent mean $\pm$ SEM ($n = 3$). Statistical significances were determined by the one-way ANOVA followed by Dunnett's test. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ vs. control group; # $P < 0.05$, ### $P < 0.001$ vs. LPS treatment group.

dose-dependent manner upon LPS treatment (Fig. 4A), while T10 abolished the downregulation of mGlu₅ expression induced by LPS (Fig. 4B). In addition, we found that mGlu₅ expression was increased by T10 treatment in a time- and concentration-dependent manner (Fig. 4C and D). These results were confirmed in primary microglia, in which T10 prevented the decrease in mGlu₅ level induced by LPS (Fig. 4E and F). Thus, T10 inhibits microglia activation by increasing mGlu₅ protein expression.

3.5. T10 increases cell surface mGlu₅ expression at the mRNA level as well as post-translational level

We investigated whether the regulation of mGlu₅ expression by T10 in microglia activation is due to an increase in the mRNA level or in the stability of mGlu₅. Our results showed that mGlu₅ mRNA level in BV2 cells decreased after treatment with LPS relative to the control, which was abrogated by pretreatment with T10 (Fig. 5A). T10 alone also increased mGlu₅ mRNA level in a time- and concentration-dependent manner in BV2 cells (Fig. 5B and C). The effect of T10 on the mRNA levels of mGlu₅ decreased by LPS was further confirmed in primary microglia (Fig. 5D). Cycloheximide (CHX) is a eukaryote protein synthesis inhibitor that blocks translational elongation by interfering with the translocation step of protein synthesis. To investigate whether T10 enhanced the stability of mGlu₅, we used CHX to block mGlu₅ synthesis. CHX treatment reduced mGlu₅ expression levels in a time-dependent manner. However, mGlu₅ degradation was prevented by pretreat-

ment with T10 (Fig. 5E). These results indicate that T10 increases mGlu₅ expression levels at the transcriptional and post-translational levels.

mGlu₅ is expressed on both the cell surface and as well as in intracellular membranes of neurons (Jong et al., 2005; Jong et al., 2009; Purgert et al., 2014). To investigate whether the change in mGlu₅ level after LPS or/and T10 treatment was intracellular or on the cell surface, we extracted the membrane and cytoplasmic fractions of BV2 cells and analyzed mGlu₅ levels by western blotting. Cell membrane mGlu₅ expression was down- and up-regulated by treatment with LPS and T10, respectively (Fig. 5F), which is similar to the results obtained in whole cells. In addition, either LPS or T10 did not significantly affect mGlu₅ expression in the cytoplasm. These results indicate that T10 regulates mGlu₅ cell membrane expression at both the transcriptional and post-translational levels.

3.6. T10 inhibits LPS-induced microglia activation via mGlu₅-mediated signaling

mGlu₅ has been known to modulate mitogen-activated protein kinases (MAPKs) signaling pathways in neurons (Mao et al., 2005). To investigate the effect of T10 on mGlu₅-mediated MAPKs signaling, BV2 cells were pretreated with different concentrations of MPEP prior to T10 and LPS exposure, and the phosphorylation status of JNK, p38 CREB, and ERK was assessed by western blotting. LPS decreased JNK, p38, and ERK phosphorylation 24 h after

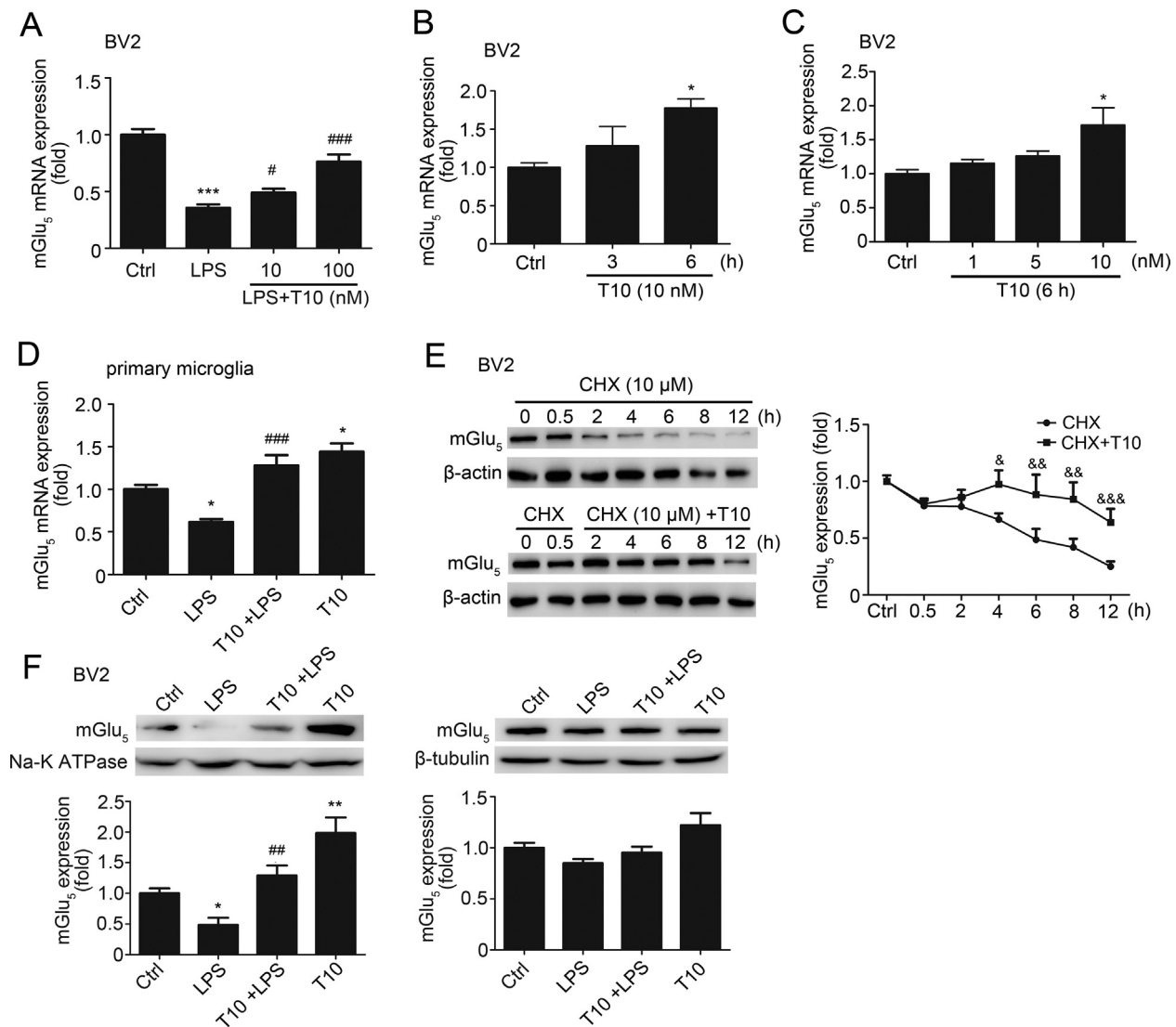


Fig. 5. T10 increases mGlu₅ expression on the cell membrane. (A) BV2 cells were pretreated with T10 (10 and 100 nM) followed by LPS for 6 h. mGlu₅ mRNA levels were analyzed by quantitative real-time PCR and normalized to that of GAPDH. (B, C) BV2 cells were treated with T10 for the indicated times (B) and doses (C), mGlu₅ mRNA levels were analyzed by quantitative real-time PCR and normalized to that of GAPDH. (D) Rat primary microglia were treated with LPS in the presence or absence of T10 for 6 h, and mGlu₅ mRNA levels were analyzed by quantitative real-time PCR and normalized to that of GAPDH. (E) BV2 cells were cultured with 10 μM CHX for 30 min before T10 treatment and mGlu₅ expression was detected by western blotting (right). mGlu₅ levels were normalized to β-actin and were expressed as the fold differences relative to the control groups (left). (F) BV2 cells were pretreated with T10 followed by LPS treatment for 24 h. mGlu₅ levels in the cell membrane and cytoplasmic fractions were evaluated by western blotting and normalized to those of Na-K ATPase or β-tubulin, and are expressed as the fold difference relative to the control group. Cells with vehicle treatment served as a control group for each panel. Data represent means ± SEM (n = 3). Statistical significances were determined by the one-way ANOVA followed by Dunnett's test. **P* < 0.05, ***P* < 0.01, ****P* < 0.001 vs. control group; #*P* < 0.05, ##*P* < 0.01, ###*P* < 0.001 vs. LPS group; &*P* < 0.05, &&*P* < 0.01, &&&*P* < 0.001 vs. CHX group.

treatment. LPS also decreased downstream phosphorylation of CREB, a transcription factor that is targeted by activated ERK, while T10 inhibited this decrease. In contrast, pretreatment with MPEP reversed the increase in p-JNK, p-p38, p-CREB, and p-ERK expression induced by T10 (Fig. 6A). These results indicate that T10 regulates MAPK signaling mediated by mGlu₅.

Activation of mGlu₅ in microglia mediates its downstream signaling pathways such as PLC phosphorylation, phosphatidyl inositol hydrolysis, PKC activation, and calcium release (Byrnes et al., 2009b). To further determine whether the effects of T10 in microglia activation are regulated by mGlu₅ activation, PLC, Ca²⁺, and PKC were inhibited by U-73122, BAPTA-AM, and chelerythrine chloride, respectively. All three inhibitors partially attenuated the decrease in NO levels caused by T10 in BV2 cells (Fig. 6B). These results were confirmed in primary microglia, in which the decreases in mRNA expression of iNOS, TNF-α, and IL-1β and -6

caused by T10 treatment were attenuated (Fig. 6C). These results indicate that the effects of T10 in LPS-induced microglia activation are regulated by mGlu₅-mediated downstream signaling pathways.

3.7. Blocking mGlu₅ partially attenuates the anti-inflammatory effects of T10 in an LPS-induced animal model of PD

Given the above in vitro findings, we investigated whether mGlu₅ plays a role in the neuroprotective effects of T10 in a LPS-induced PD animal model (Fig. 7A). Apomorphine administration produced rotation behavior 21 days after LPS treatment, indicating unilateral damage to the right SN. The reduced time on the rotarod indicated a decrease in motor coordination or fatigue resistance. T10 administration significantly improved LPS-induced rotation behavior and motor coordination, an effect that was partially

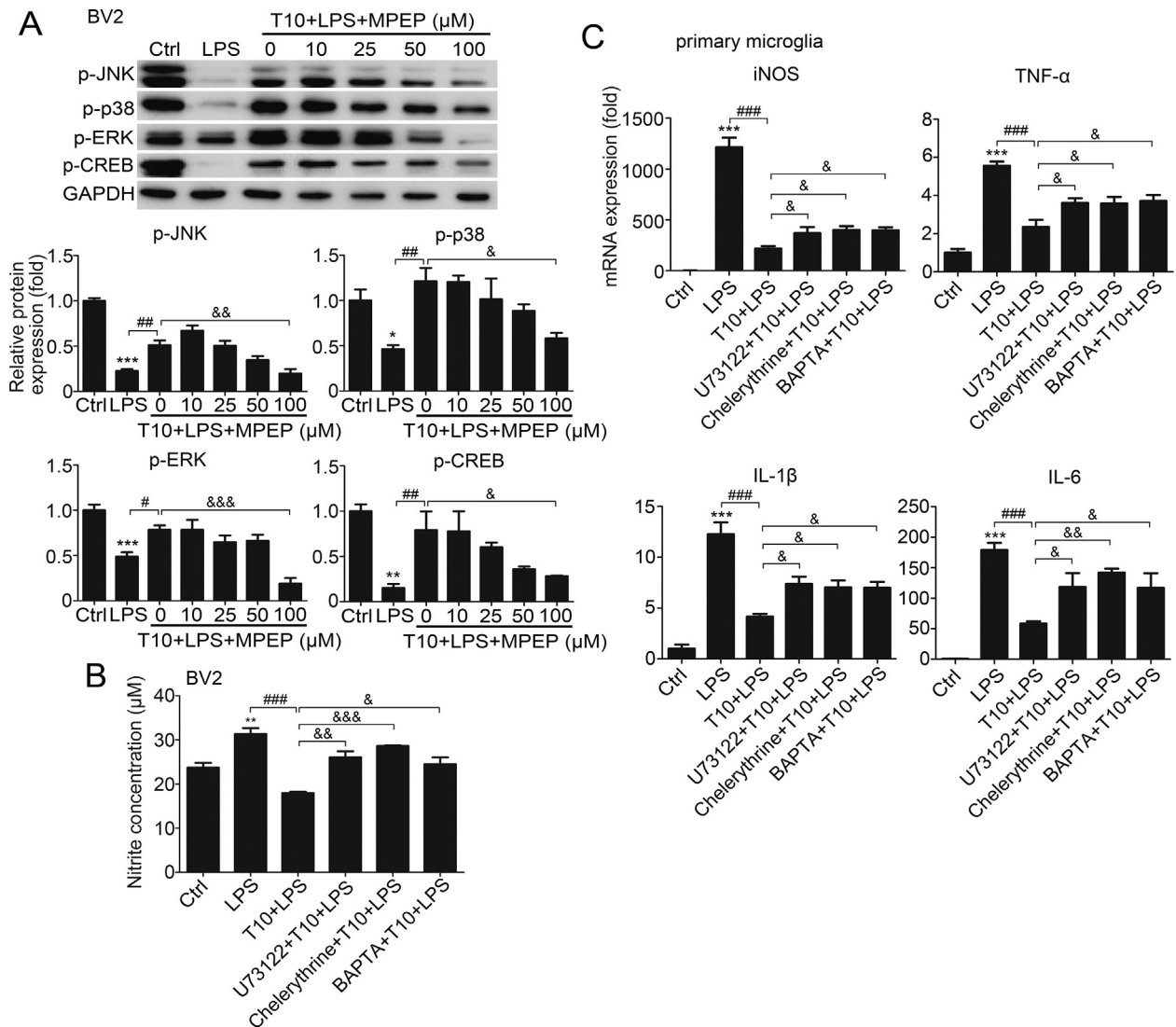


Fig. 6. T10 inhibits mGlu₅-mediated downstream signaling in LPS-treated microglia. (A) BV2 cells were pretreated with different concentrations of MPEP and T10 followed by LPS, and p-JNK, p-p38, p-ERK, and p-CREB levels were analyzed by western blotting (upper panel) and normalized to GAPDH, and are expressed as the fold difference relative to the control group (lower panels). (B) BV2 cells were pretreated with the PLC inhibitor U-73122 (10 μM), calcium chelator BAPTA-AM (10 μM), PKC inhibitor chelerythrine chloride (1.5 μM), and T10 followed by LPS for 24 h, and culture supernatants were assayed for NO release. (C) Rat primary microglia were pretreated with U-73122, BAPTA-AM, chelerythrine chloride, and T10 followed by LPS for 6 h. mRNA levels were analyzed by quantitative real-time PCR and normalized to that of GAPDH, and are expressed as the fold difference relative to the control group. Cells with vehicle treatment served as a control group for each panel. Data represent means ± SEM (n ≥ 3). Statistical significances were determined by the one-way ANOVA followed by Dunnett's test. *P < 0.05, **P < 0.01, ***P < 0.001 vs. control group; #P < 0.05, ##P < 0.01, ###P < 0.001 vs. LPS group; &P < 0.05, &&P < 0.01, &&&P < 0.001 vs. T10 + LPS group.

attenuated by MPEP treatment (Fig. 7B and C). Furthermore, an immunohistochemical analysis showed that the number of TH⁺ neurons in the LPS-injected side of T10-treated rats was increased compared to vehicle-treated rats 21 days after LPS treatment. However, MPEP treatment attenuated this increase (Fig. 7D and E). These results indicate that mGlu₅ partially mediates the neuroprotective effects of T10 in an in vivo model of PD.

To investigate whether mGlu₅ is involved in the anti-inflammatory effects of T10 in vivo, we evaluated microglia activation for detection of microglia marker Iba-1. Immunohistochemistry analysis showed that the number of Iba-1⁺ microglia was increased in the SN of the LPS-injected side in rats treated with LPS only. The number of Iba-1⁺ microglia decreased to the level of the intact side following T10 treatment; however, this effect was partially attenuated in rats treated with T10 + MPEP (Fig. 7F and G). In addition, LPS injection reduced the number of branches in microglia from 5–6 to 2–3, suggesting that microglia were

changed from ramified form to reactive form. T10 treatment increased the number of microglia branches whereas MPEP treatment reversed the effect of T10 (Fig. 7F and H). Finally, confocal microscopy showed that LPS injection increased the fluorescence intensity of Iba-1 in the SN, which was immunostained with TH. T10 treatment decreased it and MPEP attenuated the effect of T10 (Fig. 7I and J). Taken together, the data suggest that activated mGlu₅ play a role in the anti-inflammatory effects of T10 to mediate the neuroprotection in an in vivo model of PD.

3.8. mGlu₅ mediates the anti-inflammatory effects of T10 by regulating its expression in vivo

The above study showed that T10 increased the expression of mGlu₅ in microglia in vitro. We next assessed the expression of mGlu₅ in Iba-1 positive cells by confocal microscopy in vivo. We found that LPS injection decreased the fluorescence intensity of

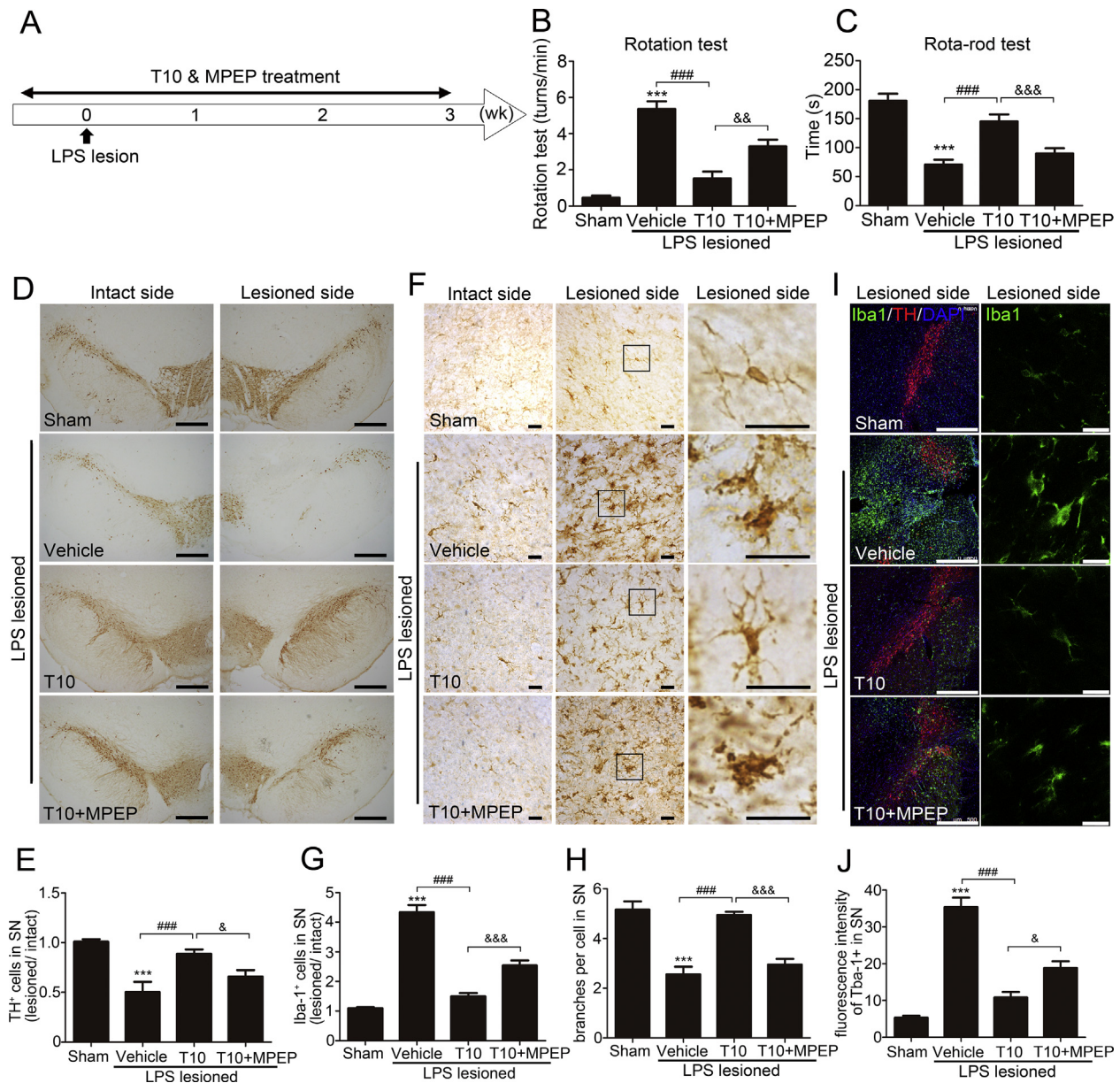


Fig. 7. Blocking mGlu₅ attenuates the anti-inflammatory effects of T10 in an LPS-induced animal model of PD. (A) Schematic illustration of the administration of T10 or T10 along with MPEP to LPS-treated rats. Rats that received unilateral SN injection of LPS were treated with T10 or T10 and MPEP or 5% dimethylsulfoxide for 24 days starting from 3 days before LPS injection. The apomorphine-induced rotational (B) and rotarod (C) tests were performed 3 weeks later. (D) The representative images of TH immunoreactivity in the SN. Scale bar = 500 μ m. (E) The quantification of TH + cells was shown as the ratio of the LPS-injected to the intact side. (F) Brain sections including the SN region were immunolabeled with anti-Iba-1 antibody. Scale bar = 25 μ m. (G) The number of Iba-1 + cells is shown as the ratio of the LPS-injected to the intact side. (H) The number of branches in microglia were counted manually using the ImageJ software. (I) Representative images showing confocal photomicrographs of TH (red) and Iba-1 (green) in the SN. DAPI (blue) was used for nuclear staining. Scale bar = 500 μ m (left) or 20 μ m (right). (J) Fluorescence intensity of microglia in Iba-1 positive cells was quantified in SN immunostained with TH (red). Error bars represent mean $\pm$ SEM ($n \geq 6$). Statistical significances were determined by the one-way ANOVA followed by Dunnett's test. *** $P < 0.001$ vs. sham group; **** $P < 0.001$ vs. LPS lesioned group; & $P < 0.05$, && $P < 0.01$, &&& $P < 0.001$ vs. T10 + LPS group.

mGlu₅ in microglia while T10 treatment attenuated its decrease induced by LPS. MPEP treatment did not significantly affect the mGlu₅ expression although it partially reversed the inflammatory effect of T10 (Fig. 8A and B). Furthermore, western blotting analysis showed that LPS increased iNOS and IL-1 β , and decreased mGlu₅ expression in the LPS-injected side of the SN, which was reversed by T10. Treatment with MPEP blocked the decrease in iNOS and IL-1 β expression induced by T10 and did not significantly change mGlu₅ expression. In addition, the downregulation of TH confirmed that MPEP treatment attenuated the neuroprotective effects of T10 (Fig. 8C). Besides, the results also showed that the house-keeping genes GAPDH, β -actin and β -tubulin were stable in this

experimental condition (Fig. 8C). These results provide evidence that the effects of T10 on microglia activation are mediated by upregulation of mGlu₅ expression in vivo.

3.9. mGlu₅ mediates the effect of T10 on microglia-induced astrocyte activation in vitro and in vivo

It has been reported that microglia activation triggers neurotoxic reactive astrocytes. Inflammatory insult induced reactive astrocytes were abundant in various human neurodegenerative diseases including PD (Liddel et al., 2017). Moreover, the expression of mGlu₅ was also downregulated by inflammatory

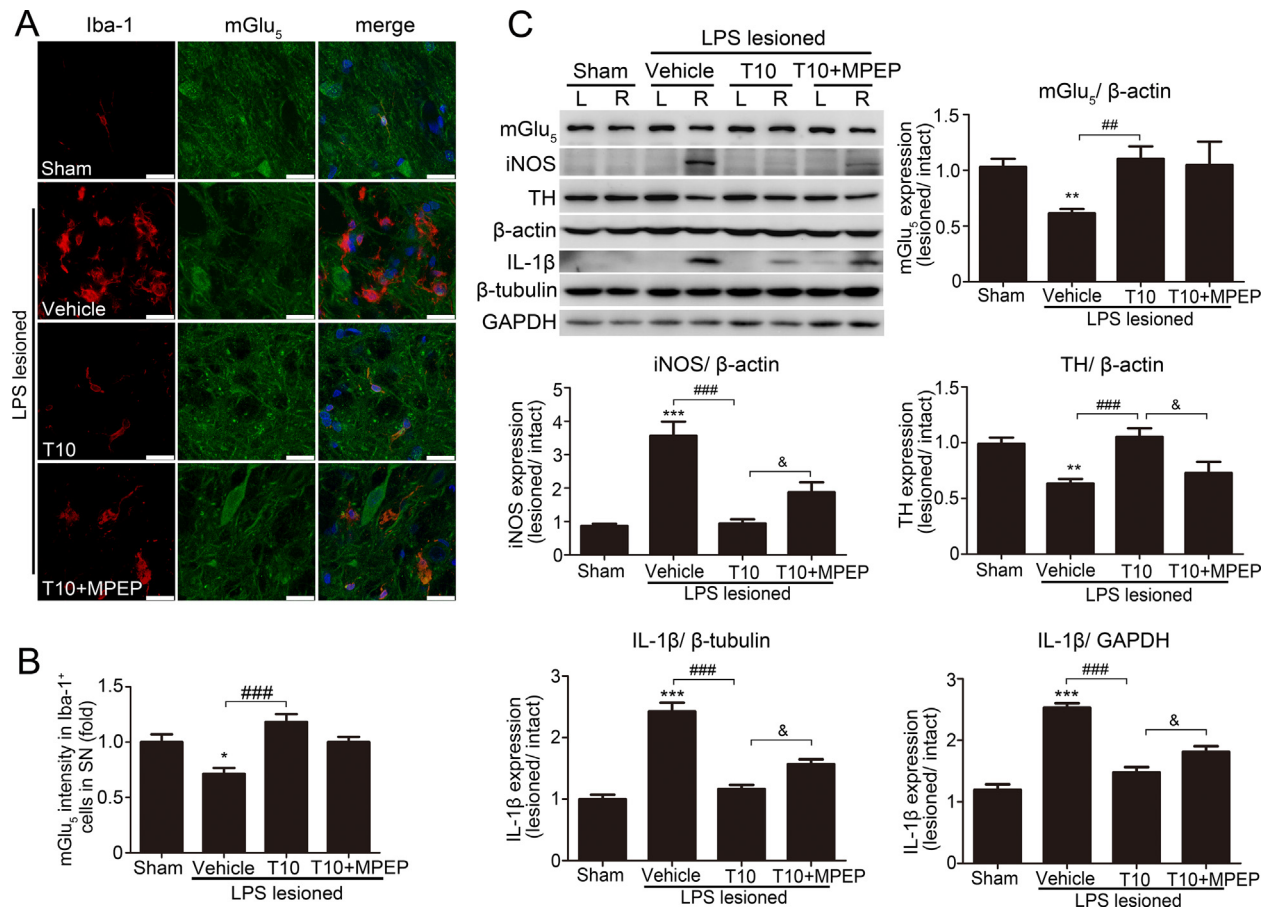


Fig. 8. mGlu₅ mediates the anti-inflammatory effects of T10 by regulating its expression in vivo. (A) Representative images showing confocal microscopy of Iba-1 (red) and mGlu₅ (green) in the SN of lesioned side in the animals subjected to the indicated treatments. DAPI (blue) was used for nuclear staining. Scale bar = 20 μ m. (B) Fluorescence intensity of mGlu₅ in Iba-1 positive cells was quantified in the lesioned SN of animals subjected to the indicated treatments. (C) Protein expression in the SN was assessed in animals subjected to the indicated treatments. Tissue lysates were analyzed for mGlu₅, iNOS, TH, and IL-1 β expression by western blotting. The levels were normalized to β -actin (mGlu₅, iNOS and TH) or β -tubulin and GAPDH (IL-1 β), and quantified as the ratio of the LPS-injected to the intact side as shown in the panel. Error bars represent mean $\pm$ SEM ($n \geq 6$). Statistical significances were determined by the one-way ANOVA followed by Dunnett's test. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs. sham group; ### $P < 0.001$ vs. LPS lesioned group; & $P < 0.05$ vs. T10 + LPS group.

stimuli in astrocytes (Berger et al., 2012). To further test whether astrocytes are also involved in T10 inhibiting microglia activation via mGlu₅, we firstly purified primary astrocytes and immunofluorescence analysis showed that no microglia were present within purified astrocytes (Fig. 9A). Consistent with the previous reports that astrocytes cannot respond to LPS (Liddelow et al., 2017; Pascual et al., 2012), LPS treatments did not significantly affect the mRNA expression of iNOS, TNF α , IL-1 β and IL-6 (Fig. 9B). Next, primary astrocytes were cultured with conditioned medium of microglia. We found that LPS-treated microglia increased the mRNA expression of iNOS, TNF- α , IL-1 β and IL-6 in astrocytes, suggesting that reactive astrocytes were induced by activated microglia via its secreted inflammatory factors. Conditioned medium from LPS + T10 treated microglia inhibited activation of astrocytes whereas conditioned medium from LPS + T10 + MPEP treatment partially returned its activation (Fig. 9C). Unexpectedly, conditioned medium of microglia treated with LPS + T10 + MPEP did not affect the effect of T10 on the mRNA expression of TNF- α in astrocytes. Moreover, T10 attenuated the downregulation of mGlu₅ expression induced by activated microglia in astrocytes and MPEP did not significantly affect the expression of mGlu₅ (Fig. 9D). The above results were further confirmed by immunohistochemical and confocal microscopic analysis in vivo. T10 treatment inhibited the increased number of GFAP + astrocytes, however, MPEP treatment partially reversed its activation state (Fig. 9E and F). Consistent

with the in vitro data, the expression level of mGlu₅ had a decrease in GFAP + astrocytes in LPS-injected group compared to control group and the downregulation of mGlu₅ was attenuated in T10 + LPS group (Fig. 9G and H). Taken together, these results indicate that mGlu₅ regulates the effect of T10 on reactive astrocytes induced by activated microglia.

4. Discussion

T10 has anti-inflammatory and immunosuppressive effects and the excessive production of TNF α , IL-1 β , and NO induced by LPS is abolished by T10 administration (Zhou et al., 2005; Zhou et al., 2003). Meanwhile, mGlu₅ exerts neuroprotective effects in microglia by reducing oxidative stress and inhibiting inflammatory cytokine release in vitro (Byrnes et al., 2009b; Qiu et al., 2015). However, the role of mGlu₅ in the inhibitory effect of T10 on microglia activation and the underlying mechanism are not known. In this study, we found that T10 suppressed microglia activation via upregulation of mGlu₅. Blocking or knocking down mGlu₅ attenuated the anti-inflammatory effects of T10, which increased mGlu₅ expression following LPS treatment by enhancement of mRNA level and protein stability. Moreover, T10 prevented the decrease in mGlu₅ expression at the cell membrane and modulated receptor-mediated MAPK activity induced by LPS. Taken together,

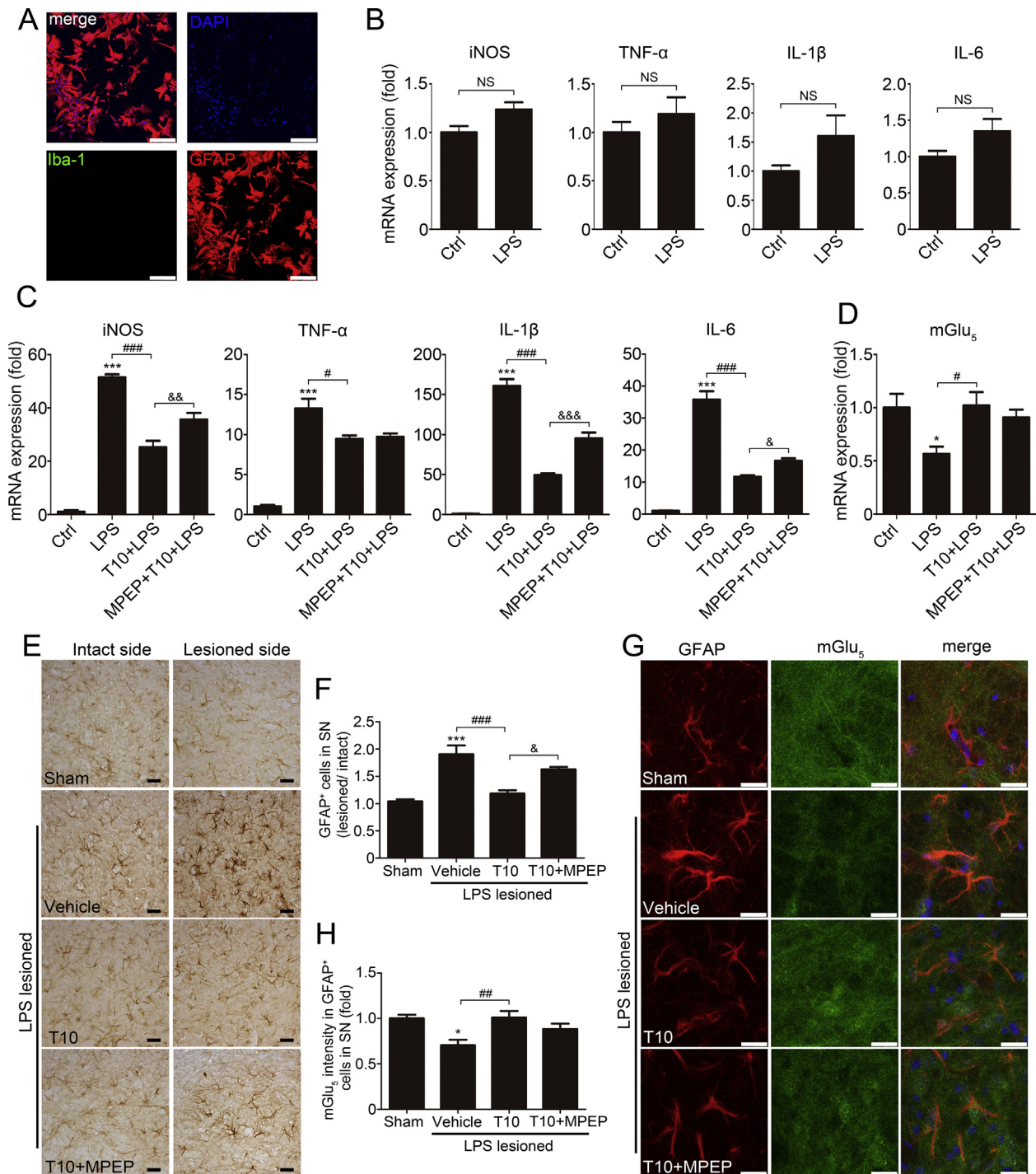


Fig. 9. mGlu₅ mediates the effect of T10 on activation of astrocytes induced by microglia. (A) Representative images of Iba-1 (green) and GFAP (red) immunoreactivity in primary astrocytes in vitro. DAPI (blue) was used for nuclear staining. Scale bar = 200 μm. (B) Rat primary astrocytes were treated with LPS for 6 h and mRNA levels of iNOS, TNF-α, IL-6 and IL-1β were analyzed by quantitative real-time PCR and normalized to that of GAPDH. (C and D) Rat primary astrocytes were cultured for 6 h with conditioned medium of microglia treated with vehicle, LPS, LPS + T10, or MPEP + T10 + LPS for 24 h. mRNA levels of iNOS, TNF-α, IL-1β, IL-6 and mGlu₅ were analyzed by quantitative real-time PCR and normalized to that of GAPDH. (E) Representative images showing immunohistochemistry analysis of GFAP in the SN. Scale bar = 25 μm. (F) Ratio of the number of GFAP⁺ cells in the LPS-injected SN to that of the intact side was quantified. (G) Representative images showing confocal photomicrographs of GFAP (red) and mGlu₅ (green) in the lesioned side of SN in the animals subjected to the indicated treatments. DAPI (blue) was used for nuclear staining. Scale bar = 20 μm. (H) mGlu₅ intensity in GFAP⁺ cells in the lesioned side of SN was quantified in animals subjected to the indicated treatments. Error bars represent mean ± SEM (n = 3–5). Statistical significances were determined by Student's *t*-test (B) and one-way ANOVA followed by Dunnett's test (C, D, F and H). **P* < 0.05, ****P* < 0.001 vs. sham group; #*P* < 0.05, ##*P* < 0.01, ###*P* < 0.001 vs. LPS lesioned group; &*P* < 0.05, &&*P* < 0.01, &&&*P* < 0.001 vs. T10 + LPS group; NS, not significant.

these data indicate that the anti-inflammatory effects of T10 are mediated by increased mGlu₅ expression and activation.

Previous studies have shown that activation of group I mGlu receptor by its agonists inhibited microglia activation and reduced

inflammatory factor release and microglia-induced neurotoxicity both in vitro and in vivo (Byrnes et al., 2009a; Byrnes et al., 2012; Farso et al., 2009; Loane et al., 2013; Loane et al., 2009). mGlu₅ modulates calcium oscillations and the innate immune response

induced by LPS in microglia (Liu et al., 2014). Here, we found that blocking mGlu₅ by treatment with its antagonist MPEP and decreasing its expression by shRNA-mediated knockdown abolished T10-inhibited release of inflammatory cytokines, NO production, and iNOS expression. Thus, the anti-inflammatory effect of T10 may be exerted via mGlu₅. It has been reported that glutamate excitotoxicity contributes to the development of PD (Ambrosi et al., 2014), and pharmacological blockade of mGlu₅ receptors inhibits neurodegeneration in MPTP-, 6-hydroxydopamine-, and rotenone-induced animal models of PD (Ferrigno et al., 2015; Hsieh et al., 2012; Masilamoni et al., 2011; Xia et al., 2015). We found that MPEP treatment attenuated the anti-inflammatory effects of T10 in an LPS-induced rat PD model, indicating that activated mGlu₅ reduces inflammation induced by microglia activation and thus provides protection against neurotoxicity. This suggests that mGlu₅ play different roles in microglia and neurons. That is, T10 inhibits LPS-induced microglia activation via mGlu₅ activation and protects dopaminergic neurons from LPS, whereas neurotoxicity induced by hyperactivation of mGlu₅ can be reduced by receptor blockade.

mGlu₅ gene expression was shown a substantial decrease in microglia exposed to LPS (Berger et al., 2012). Induction of astrogliosis by activated microglia is associated with downregulation of mGlu₅ (Tilleux et al., 2007). Consistent with these reports, we observed an LPS-induced reduction in mGlu₅ expression, suggesting that increased mGlu₅ expression contributes to the suppression of inflammation and microglia activation. Indeed, our results showed that T10 stimulated mGlu₅ expression at both mRNA and protein levels, which were reduced by LPS treatment. An increase in mGlu₅ protein expression may result from regulation at the transcriptional or post-translational levels. For example, T10 may stabilize mGlu₅ expression via a ubiquitin-dependent pathway (Cheng et al., 2010). Enhanced Ten-eleven translocation methylcytosine dioxygenase 1 expression was shown to increase demethylation of the mGlu₅ promoter and induce mGlu₅ transcription in dorsal horn neurons (Hsieh et al., 2017). In addition, activated microglia can also produce the anti-inflammatory factors such as IL-10 and TGF- β to play a protective role (Wolfs et al., 2011) and it would be interesting to further investigate whether T10 mediates mGlu₅ to regulate anti-inflammatory factors. Although the mechanistic details of the T10-mediated increase in mGlu₅ protein expression in LPS-induced inflammation require further study, our results indicate that inhibiting microglia activation by enhancing mGlu₅ expression may be an effective therapeutic strategy for neuroprotection.

Activated mGlu₅ at the cell surface or in the cytosol can stimulate JNK signaling (Jong et al., 2009). ERK1/2 and AKT phosphorylation levels were increased by (S)-3,5-dihydroxyphenylglycine (DHPG)-induced activation of membrane-associated mGlu₅ (Zhang et al., 2016). In our study, T10 increased cell membrane mGlu₅ expression in microglia as well as LPS-induced MAPK/CREB phosphorylation. Blocking mGlu₅ with MPEP and its signaling inhibitors attenuated anti-inflammatory effect of T10. Thus, LPS stimulation may reduce mGlu₅ expression at the plasma membrane, whereas T10 may reverse this effect, resulting in increased cell surface mGlu₅ expression and the activation of downstream anti-inflammatory signaling pathways (Fig. 10). T10 was found to protect dopaminergic neurons against LPS-induced degeneration by suppressing the production of neurotoxic factors (Li et al., 2004). Thus, T10 may inhibit the release of inflammatory cytokines via regulation of mGlu₅ expression and associated MAPK signaling in microglia, thereby protecting against dopaminergic neurotoxicity.

Recent paper showed that microglia emerged as central players in brain diseases (Salter and Stevens, 2017). Microglia activation also triggers neurotoxic reactive astrocytes (Liddel et al.,

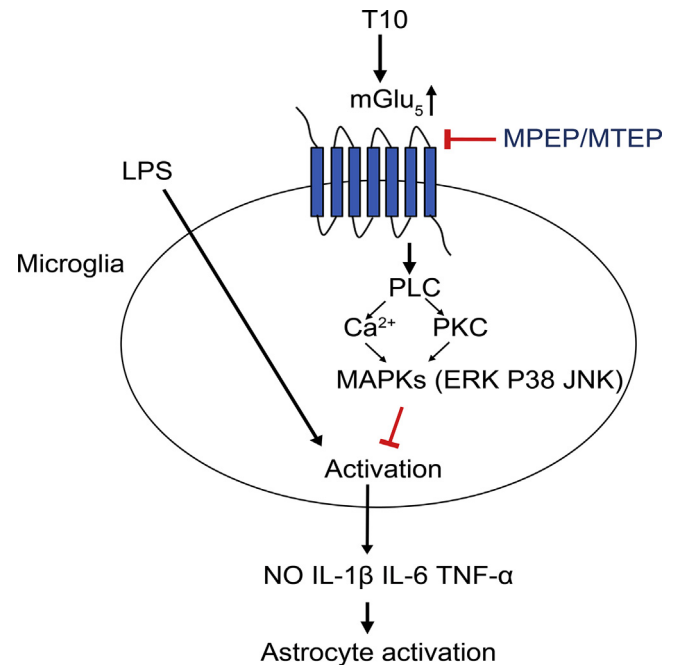


Fig. 10. Schematic illustration of the role of mGlu₅ in the inhibition of microglia activation by T10 in the LPS-induced inflammation model of PD. LPS activates microglia and causes downregulation of mGlu₅ expression. T10 increases mGlu₅ expression on the plasma membrane and activates mGlu₅ to prevent inflammation by microglia activation and further inhibits activated astrocytes via intracellular signaling pathways including MAPKs.

2017), suggesting that microglia might be the first target of T10 on inhibition of inflammation, which subsequently inhibited activation of astrocyte and neuron death. Indeed, our study indicated that T10 inhibited microglia activation via up-regulation of mGlu₅, which further inhibited reactive astrocytes induced by activated microglia and eventually protected dopaminergic neurons in PD model. Interestingly, the study showed that LPS can directly activate primary astrocytes (Tarassishin et al., 2014), which was not the case in our experiment in vitro, and our result is consistent with the findings in the other reports (Liddel et al., 2017; Pascual et al., 2012). However, whether T10 directly modulates astrocyte activation via mGlu₅ might not be excluded in this study. Additionally, our study showed that blockade of mGlu₅ partially attenuated T10-suppressed microglia activation induced by LPS, suggesting other mechanisms might be involved in the process. The study has shown that Toll-like receptor (TLR)4 plays a role in LPS-induced microglia activation (Crews et al., 2017). LPS can bind to Toll-like receptor (TLR)4 in microglia, leading to activation of microglia (Pascual et al., 2012). Deficiency in sterile alpha- and TIR motif-containing 1 acting downstream of TLR3 and TLR4, impairs synaptic function and leads to behavioral deficits, which can be reversed by a mGlu receptor allosteric modulator (Lin et al., 2014). It has been also reported that LPS triggers similar oscillation patterns of intracellular calcium concentration in primary mouse microglia expressing wild-type TLR4 or lacking this receptor to enhance LPS-induced NF- κ B activity, suggesting that LPS-induced oscillations in intracellular calcium concentration are independent of TLR4 (Liu et al., 2014). The above evidence raises the possibility that T10 may regulate TLR4-mediated inflammation via activation of mGlu₅, may directly regulate TLR4 independent of mGlu₅ or only activate mGlu₅ independent of TLR4, which is worthwhile to be further investigated. Notably, T10 treatment alone increased mGlu₅ expression without affecting the anti-inflammatory effects in vitro, suggesting that mGlu₅ regulation by

T10 alone has other physiological functions. It was reported that T10 alone did not cause the inflammatory effect in sham rats when studied in the rat neuropathic pain model (10 µg/kg of T10) (Tang et al., 2012) and the bone cancer pain rats (12 µg/kg of T10) (Hu et al., 2017). These data suggest that the dosage of T10 (10 µg/kg) used in our study might be safe to treat the animals but still needs to be further tested when applied in the further study. As mentioned, in this study we applied BV2 cells, primary microglia and the animal model of PD, which are very different from adult microglia in human as their gene expression can diverge significantly (Gosselin et al., 2017). Despite these limitations, the study provides the experimental evidence that T10 activates mGlu₅ to protect against inflammation mediated-neurotoxicity in neurological disorders.

In summary, we found that T10 suppresses inflammation by modulating mGlu₅ expression in microglia. It is possible that this effect is exerted via direct binding to mGlu₅ on the plasma membrane. The study provides a novel mechanism by which mGlu₅ acts as a target to regulate inflammation in PD, which helps to further clarify signal transduction pathways of T10 inhibiting microglia activation.

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The authors declare that they have no conflict of interest.

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