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TLR4 signaling mediates AP-1 activation in an MPTP-induced mouse model of Parkinson's disease

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ABSTRACT

Objective: To evaluate the effects of Toll-like receptor 4 (TLR4) signaling on the activation of the transcription factor activator protein-1 (AP-1) in a 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced mouse model of Parkinson's disease (PD).
Methods: The following groups were evaluated: normal saline (NS)-treated WT mice, NS-treated TLR4-knockout (KO) mice, MPTP-treated WT mice, and MPTP-treated TLR4-KO mice. After establishing the mouse model, behavioral changes were evaluated. AP-1 expression was detected by RT-PCR, Western blotting, immunohistochemistry and immunofluorescence staining.
Results: Compared to MPTP-treated WT mice, significantly reduced dyskinesia was observed in MPTP-treated TLR4-KO mice. AP-1 mRNA and protein levels were significantly up-regulated in the substantia nigras (SNs) of MPTP-treated WT mice relative to NS-treated mice ($P < 0.01$); these levels were significantly reduced in MPTP-treated TLR4-KO mice relative to MPTP-treated WT mice ($P < 0.01$). Immunohistochemical staining demonstrated that AP-1 was distributed throughout the SN in MPTP-treated mice, and immunofluorescence further showed that AP-1 was expressed in TH-positive neuronal cells and GFAP-positive astrocytes. In addition, immunofluorescence revealed that AP-1 expression was lower in TH-positive neurons and GFAP-positive astrocytes in the SNs of MPTP-treated TLR4-KO mice relative to MPTP-treated WT mice.
Conclusions: The TLR4 pathway may play an important role in regulating AP-1 activation.

1. Introduction

Parkinson's disease (PD) is the second most common neurodegenerative disorder after Alzheimer's disease. PD is characterized by tremor at rest, akinesia, rigidity, postural abnormalities, and episodes of motor arrest [1]. The key feature of PD is a massive loss of dopaminergic neurons in the substantia nigra (SN); as a result, the concentration of striatal dopamine drastically decreases [2]. Despite widespread research efforts, the cause of dopaminergic neuronal loss in the SNs of PD patients remains poorly understood.

Recent evidence has shown that neuroinflammation is involved in the pathogenesis of PD [3]. The presence of activated T-lymphocytes and microglia proximal to the SN has been observed postmortem in PD patients. These cell populations are believed to contribute to the process of neurodegeneration and the release of pro-inflammatory and cytotoxic factors, including interleukin-1β, tumor necrosis factor-α, nitric oxide and reactive oxygen intermediates [3,4]. Toll-like receptors (TLRs) are a family of innate immunity receptors that play a major role in regulating the host immune system [5]. Recent studies have shown that TLR4 and TLR2 are expressed in the brain and may regulate certain physiological processes. Furthermore, these molecules have also been reported to play key roles in neurodegenerative disorders, such as PD and Alzheimer's disease [5,6]. Recent studies have indicated that TLRs are also involved in the pathogenesis of PD [7]. α-Synuclein transgenic mice exhibit significant up-regulation of TLR2. Conversely, TLR-deficient mice are less vulnerable to MPTP toxicity and show decreased microglial activation in the SN following MPTP treatment [6,8,9].

The transcription factor activator protein-1 (AP-1) is a redox-sensitive transcription factor whose activity is controlled by agents that perturb intracellular thiol concentrations [10,11]. AP-1 is mainly composed of Jun, Fos, and ATF protein dimers [12,13]. AP-1 mediates the regulation of numerous genes in response to a variety of physiological and pathological stimuli, including cytokines, oxidative stress, growth factors, bacterial and viral infections, and oncogenic stimuli [14]. AP-1 is activated under oxidative stress conditions in rat cardiomyocytes; thus, this factor exerts additional functions under redox conditions[15]. AP-1 activation is significantly increased in the striatums and midbrains of mice following MPTP administration (98% and 79% higher than controls, respectively) [11]. TLR-mediated signal transduction induces the activation of a large number of immune-response genes, including those encoding

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inflammatory cytokines, which triggers the release of high quantities of pro-inflammatory cytokines that ultimately kill dopaminergic neurons in the SN [8]. In the present study, we investigated TLR4 signaling-mediated AP-1 activation in an MPTP-induced mouse model of PD using reverse-transcriptase polymerase chain reaction (RT-PCR), Western blot analysis and immunohistochemistry.

2. Materials and methods

2.1. Animals

Adult (aged 8–12 weeks) male TLR4-knockout (TLR4-KO) mice [16] and wild type (WT) littermates (Jackson Laboratories, USA) were used in all experiments. All animals were housed under a 12/12-hour light/dark cycle with lights on at 8:00 am, and the room temperature was kept at 23 °C. The animals were provided free access to standard diet and tap water. All experiments were approved by the Chongqing Medical University Animal Studies Committee, and the animals were cared for in accordance with the Guide for the Care and Use of Laboratory Animals (National Research Council, 1996).

2.2. MPTP treatment

A total of 128 mice were randomly divided into the following 4 groups ($n = 30$ to 32 per group): normal saline-treated WT mice (NS + WT); normal saline-treated TLR4-knockout mice (NS + TLR4-KO); MPTP-treated WT mice (MPTP + WT); and MPTP-treated TLR4-knockout mice (MPTP + TLR4 KO). The mice in the MPTP-treated groups were intraperitoneally injected with MPTP (diluted in NS; Sigma-Aldrich, St. Louis, MO, USA) for 5 consecutive days (25 mg/kg per day); the same volume of saline was intraperitoneally injected into the mice for 5 consecutive days. Behavioral experiments were performed after the final administration of MPTP, after which the mice were sacrificed.

2.3. Tissue processing

The NS- and MPTP-treated mice were further divided into two groups. The first group of mice was sacrificed, and their brains were quickly removed for dissection of the SN. These tissues were then snap-frozen by immersion in liquid nitrogen and stored at −80 °C for Western blot and RT-PCR analysis. The second group of mice was anesthetized with ether and transcardially perfused with phosphate-buffered saline (PBS) followed by 4% paraformaldehyde, and collected tissues were postfixed in paraformaldehyde for 24 h. One portion of each collected tissue was transferred into 15–30% sucrose, and the anteroposterior middle area of the SN was coronally sliced with a Microtome-Cryostat at a thickness of 10 μm [17] (−3.5 mm caudal to the bregma, 1.2 mm lateral to the midline, and 4.0 mm ventral to the surface of brain). The sections were stored at −80 °C for immunofluorescence analysis. Another portion of the collected tissue was embedded in paraffin and sectioned into 10-μm-thick sections for immunohistochemistry.

2.4. Western blotting

Western blotting was performed to quantify AP-1 protein levels in the mice ($n = 6$ each group). According to the manufacturer's protocol [18], proteins from the collected brain tissues were isolated in ice-cold RIPA buffer (Biyuntian Technologic Inc., China) containing 1 × PBS, 0.5% sodium deoxycholate, 1% IGEPAL CA-630, 0.1% sodium dodecyl sulfate (SDS), and a protease inhibitor Mini Cocktail (Roche Diagnostics, USA). Protein concentration was measured using a bicinchoninic acid kit (Biyuntian Technologic Inc., China). After boiling in 1 × 6 loading buffer, 20–40 mg of protein was loaded onto a 12–15% SDS–polyacrylamide gel for electrophoresis, transferred to a polyvinylidene fluoride membrane (Millipore, Billerica, MA, USA), and blocked with 5% nonfat dry milk in Tris-buffered saline plus 0.05% Tween 20 (TBST) for 2 h at room temperature. Incubation with primary antibodies was performed overnight at 4 °C; either rabbit anti-AP-1 TLR4 (1:1000; Abcam, Cambridge, U.K.) or an antibody against GAPDH (1:1000; Chemicon, Temecula, CA) was used. On the following day, the sections were incubated with a secondary horseradish peroxidase-conjugated anti-goat antibody (1:10,000; Zhongshan Biotechnology Co., Beijing, China) at room temperature for 40 min. After washing in TBST, the blots were exposed to enhanced chemiluminescence substrate (Biyuntian Technologic Inc., China). Densitometry was performed using ImageJ 1.43 μ (National Institutes of Health, USA); all values were normalized against GAPDH. All experiments were repeated at least three times.

2.5. Real-time polymerase chain reaction (RT-PCR)

Total RNA ($n = 8$ each subunit) was extracted from the SN samples collected from the NS- and MPTP-treated mice using a One-Step RNA PCR Kit (Takara, DaLian Baosheng, China) according to the manufacturer's instructions. RNA quality was determined by measuring the absorbance at 260/280 nm using a NanoDrop 2000 spectrophotometer (Thermo Scientific Ltd., Wilmington, USA) and verified by agarose gel electrophoresis. First-strand cDNA was synthesized from 1 mg of total RNA by reverse transcription using a Transcriptor RevertAid™ First Strand cDNA Synthesis Kit (Thermo Fisher Scientific Inc., USA). The following primer sequences were used: mouse AP-1 (sense: 5′-GCAAGCCCTGAAGGAAGAG-3′; antisense: 5′-AGCATGTTGGCCGTGG AT-3′; 237 bp product) and mouse GAPDH (sense: 5′- ACCACAGTCC ATGCCATCAC -3′; antisense: 5′- ACCTTGCCCACAGCCTTG -3′; 134 bp product). After amplification, the samples were separated on a 2.5% agarose gel via electrophoresis, and the gel was imaged using a Gene Genius Gel Imaging System (Syngene, USA). The sizes of the two PCR products were 190 base pairs (TLR4) and 99 base pairs (GAPDH). Band intensity was quantified using ImageJ 1.43 μ (National Institutes of Health, USA). Real-time PCR was performed according to the manufacturer's protocol provided with Maxima SYBR Green/ROX qPCR Master Mix (Fermentas, Canada). Denaturation was carried out at 94 °C for 30 s, followed by 40 cycles of 95 °C for 1 min, 60 °C for 45 s and 72 °C for 40 s and then annealing at 61 °C. The experimental data were analyzed using the ΔΔCt method.

2.6. Immunohistochemistry

For immunostaining, tissue sections were dewaxed ($n = 3$ each subunit), rehydrated through an alcohol gradient and placed into water. Following this, the sections were immersed in 3% H_2O_2/methanol for 8 min, pre-treated in Tris-EDTA antigen retrieval buffer (10 mM Tris Base, 1 mM EDTA solution, and 0.05% Tween 20, pH 9.0), heated in a microwave for 20 min and then cooled. Next, the sections were immersed in a 0.3% Triton X-100 solution for 20 min and incubated with 5% normal goat serum at room temperature for 40 min. The sections were incubated with anti-TH (1:100; Abcam, USA) or anti-AP-1 (1:100; Abcam, USA) primary antibodies overnight at 4 °C followed by incubation with a goat anti-rabbit antibody conjugated to a horseradish peroxidase-labeled dextran polymer (EnVision + System-HRP; Boster, China) for 25 min at 37 °C. Visualization was performed by incubation in 0.5 mg/ml 3,3′-diaminobenzidine tetrahydrochloride (DAB; Sigma), and the sections were counter-stained with hematoxylin. Subsequently, the stained sections were dehydrated in an ethanol gradient, cleared in xylene, and placed on coverslips. Olympus ImageScope Viewer (Olympus Microscope System BX51; Olympus, Tokyo, Japan) was used to capture images. No immunoreactive cells were detected in the control sections, which were processed in a similar manner as above but with the omission of either the primary or secondary antibody. Quantification of the number of tyrosine hydroxylase (TH)-positive neurons (the number of stained nuclei divided by the total number of nuclei

expressed as percentage) was accomplished using ImageJ 1.43 μ (National Institutes of Health, USA).

2.7. Immunofluorescence staining

For all immunostaining procedures, tissue sections were immersed in a 0.3% Triton X-100 solution for 20 min, followed by incubation with 5% donkey serum albumin for 60 min at room temperature. The sections were then incubated with anti-AP-1 (1:100; Abcam, USA), anti-GFAP (1:100; Dako, Germany), or anti-TH (1:100; Millipore, USA) primary antibodies in 0.1 M PBS, pH 7.4, for 48 h at 4 °C on a shaker. As the anti-AP-1 and anti-GFAP antibodies were generated in rabbits and the anti-TH antibody was generated in mice, we used different secondary antibodies for double-labeling after rinsing in PBS. These secondary antibodies included Alexa Fluor 647-conjugated donkey anti-rabbit IgG and Alexa Fluor 488-conjugated donkey anti-mouse IgG (1:500; Life Technologies). The tissue sections were incubated with the secondary antibodies for 20 min at 37 °C. After 3 rinses with PBS, the sections were counterstained with 4′,6-diamidino-2-phenylindole (DAPI) for visualization of nuclei. The sections were examined using confocal microscopy (TCS-TIV; Leica, Nussloch, Germany), and the images were analyzed using ImageJ 1.43 μ (National Institutes of Health, USA).

2.8. Behavioral tests

To evaluate differences in the behaviors of NS-treated vs. MPTP-treated WT and TLR4-KO mice, we employed the pole test. This test was performed 5 days after the final MPTP administration to assess the degree of motor function in each mouse. All tests were performed between 9:00 am and 12:00 am, and the mice were trained twice before each recorded test. The test was assessed as previously described with minor modifications [19,20]. In the test, each mouse was placed head upward near the top of a vertical, wooden, rough-surfaced pole (0.8 cm in diameter and 60 cm in height). The total time needed for a mouse to turn downward and climb to the ground was recorded. Each mouse was required to perform three successive trials at 5-min intervals. All behavioral tests were performed in a double-blinded manner.

2.9. Quantification of double-labeled cells

Three contiguous tissue slices were analyzed per mouse, and three mice were studied per condition. To count the number of double-labeled AP-1^+/TH^+ and AP-1^+/$GFAP^+$ cells, we used Leica Application Suite Advanced Fluorescence Lite Image Analysis software (LAS AF, Leica Mannheim, Germany) with coordinates described by Paxinos and Franklin (2001) [17] (−3.5 mm caudal to the bregma, 1.2 mm lateral to the midline, and 4.0 mm ventral to the surface of brain). After confirming the location of the DAPI layer (blue), the AP-1^+ (green) TH^+ cell layer (red), the $GFAP^+$ cell layer (red), the merged layer of AP-1^+/TH^+ cells (yellow) and the AP-1^+/$GFAP^+$ cells (yellow) in the four different layers, the numbers of AP-1^+ cells, TH^+ cells, $GFAP^+$ cells, and double-labeled AP-1^+/TH^+ cells and AP-1^+/$GFAP^+$ cells were determined by automatic counting in concentric homogeneous areas. During analysis, the investigator who performed the quantification was blinded.

2.10. Statistical analyses

All statistical analyses were performed using SPSS version 18.0 (SPSS Inc., IL, Chicago, USA). The data are expressed as the mean ± SEM. Student's t-test was used for comparisons between two groups, whereas analysis of variance (ANOVA) with Dunnett's test was used for comparisons of more than two groups. Differences were considered significant at $P < 0.05$. All results represent at least three independent replicates. All experiments were examined blind.

3. Results

3.1. TLR4 deficiency attenuated MPTP-induced dyskinesia

To investigate the effects of TLR4 on behavior, pole tests were performed. There were no significant differences in time-of-landing between the NS-treated WT and TLR4-KO mice ($P > 0.05$). MPTP toxicity significantly reduced movement distance, and severe bradykinesia was observed. Compared with the NS-treated WT (2.93 ± 0.45 s) and TLR4-KO mice (2.75 ± 0.37 s), the landing times of the MPTP-treated WT (31.06 ± 5.15 s) and TLR4-KO mice (24.72 ± 4.31 s) were significantly longer ($P < 0.01$; Fig. 1). Our results further showed that the time-of-landing of the MPTP-treated TLR4-KO mice was significantly shorter than that of the MPTP-treated WT mice ($P < 0.01$; Fig. 1). These data suggest that a lack of TLR4 signaling might reduce dyskinesia and protect against MPTP-induced changes in locomotor activity.

3.2. TLR4 deficiency attenuated MPTP-induced reduction of TH-positive neurons in the SN

The main clinical feature of PD is grievous loss of dopaminergic neurons and depletion of dopamine in the SN. This results in reduced expression of TH, an enzyme that catalyzes the transformation of the amino acid L-tyrosine into L-3,4-dihydroxyphenylalanine. As such, dopamine synthesis diminishes and PD results [21]. MPTP, a neurotoxin, is widely used to produce experimental animal models of PD because it causes selective damage to dopaminergic neurons in the SN and substantially reduces TH expression [22]. Our data showed robustly immunostained fiber networks in the SNs of NS-treated WT and TLR4-KO mice (Fig. 2A–D). The numbers of TH-positive cells in the SNs did not significantly differ between these two groups ($P > 0.05$; Fig. 2A–D). A small number of TH-immunoreactive neuronal bodies were detected in the SNs of the MPTP-treated WT and TLR4-KO mice; there were significantly fewer TH-immunoreactive cells in the SNs of these mice relative to those of the NS-treated WT and TLR4 KO mice (Fig. 2E–H; $P < 0.01$). Furthermore, the number of TH-immunoreactive cells in the SNs of the MPTP-treated WT mice was significantly higher than that in the MPTP-treated TLR4-KO mice ($P < 0.01$).

3.3. TLR4 deficiency attenuated MPTP-induced up-regulation of AP-1 mRNA in the SN

To determine whether TLR4 signaling modulates AP-1 expression, we used RT-PCR to quantify AP-1 mRNA expression in SN tissues collected from NS- and MPTP-treated WT and TLR4-KO mice. Fig. 3A shows representative gel bands produced by AP-1. Bands corresponding to the expected sizes of amplified fragments of AP-1 (237 bp) and GAPDH (134 bp) were observed in all samples. The upper band in

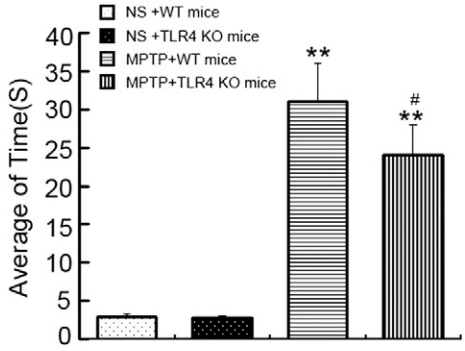

Fig. 1. Changes in movement behavior in NS- and MPTP-treated WT and TLR4-KO mice. Behavioral changes in NS- and MPTP-treated WT and TLR4-KO mice were assessed using a pole test. The values shown are expressed as the mean ± SEM ($n = 8$). $^{**}P < 0.01$ vs. NS-treated mice; $^{\#}P < 0.01$ vs. MPTP-treated WT mice.

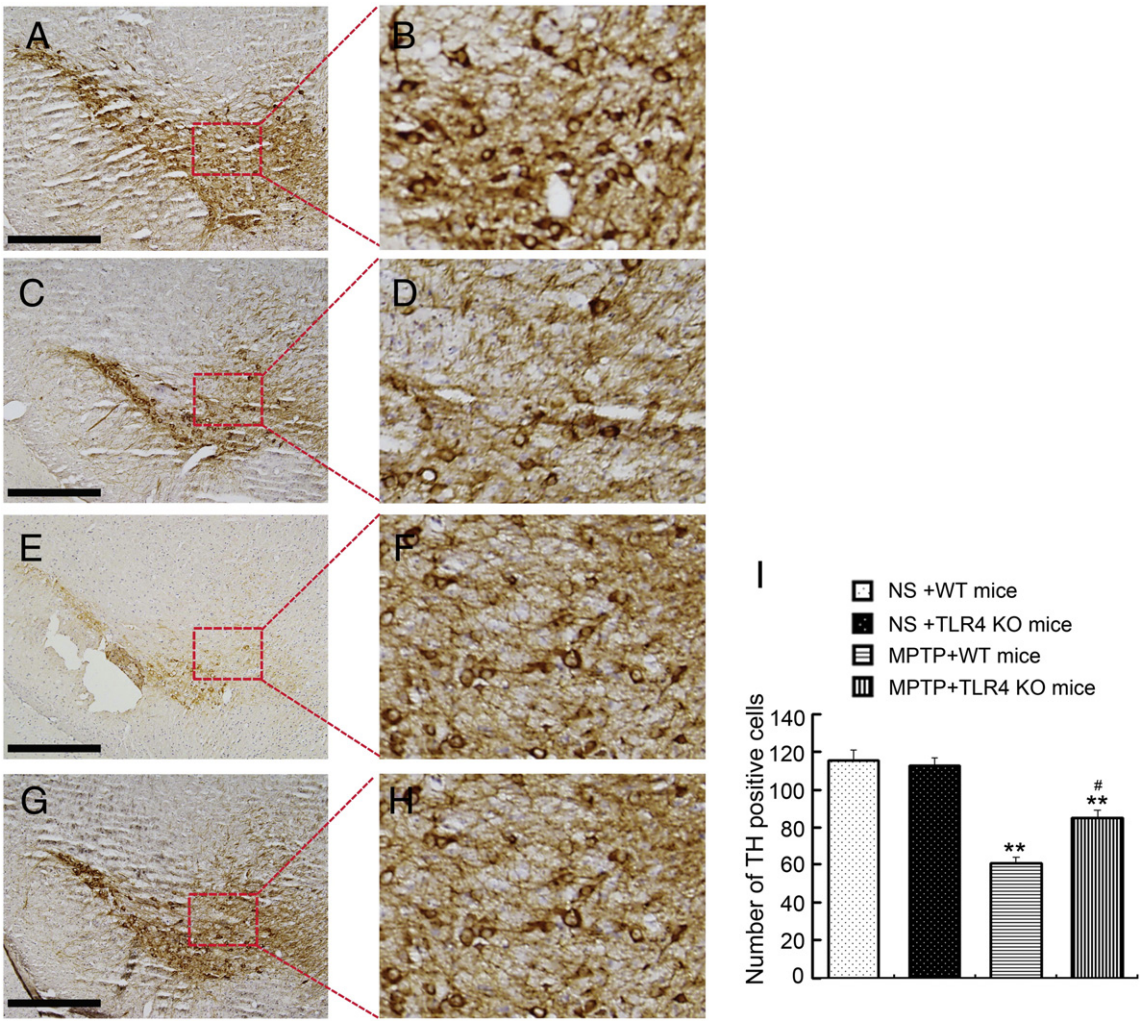

Fig. 2. Loss of TH in MPTP-treated WT and TLR4-KO mice. A–H) Representative photomicrographs of TH expression in NS-treated WT mice (A, B), NS-treated TLR4-KO mice (C, D), MPTP-treated WT mice (E, F), and MPTP-treated TLR4-KO mice (G, H). I) Comparison of the numbers of TH-positive neurons in the different groups. The values are expressed as the mean ± SEM ($n = 8$). $^{**}P < 0.01$ vs. NS-treated mice; $^{\#}P < 0.01$ vs. MPTP-treated WT mice. Scale bars, 20 μm (400×).

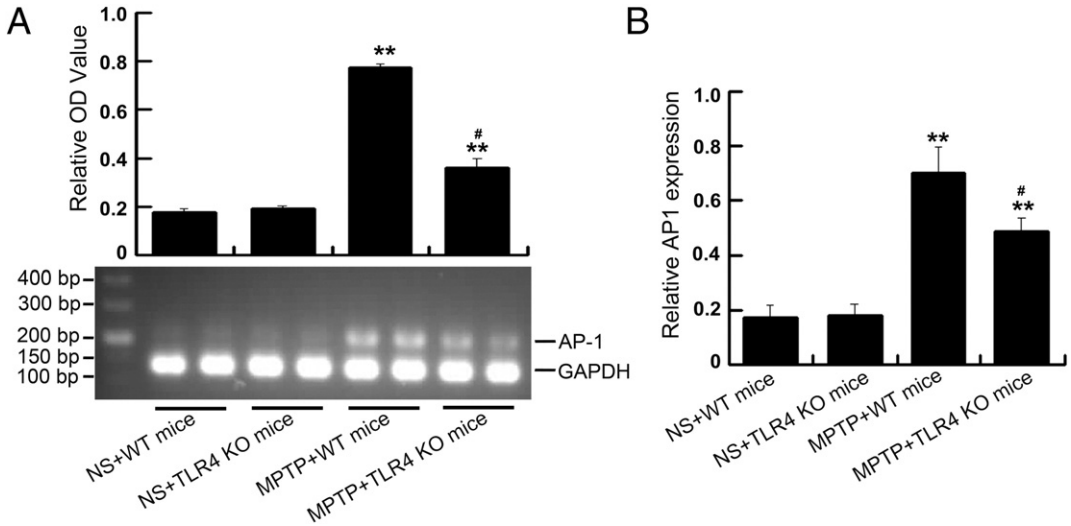

Fig. 3. AP-1 mRNA expression in MPTP-treated WT and TLR4-KO mice. A) Representative RT-PCR results of AP-1 expression. (B) AP-1 mRNA expression in the SNs of the NS-treated WT and TLR4-KO mice and the MPTP-treated WT and TLR4-KO mice. The optical densities of the bands were measured via densitometry and normalized to GAPDH. The values are expressed as the mean ± SEM ($n = 8$). $^{**}P < 0.01$ vs. NS-treated mice; $^{\#}P < 0.01$ vs. MPTP-treated WT mice.

each lane corresponds to AP-1 mRNA, and the lower band in each lane corresponds to GAPDH mRNA. The bands were densitometrically quantified, and the quantities of AP-1 mRNA were normalized against GAPDH. Fig. 3 shows the weak AP-1 expression observed in the NS-treated WT (0.18 ± 0.01) and TLR4-KO mice (0.19 ± 0.01). In contrast, AP-1 mRNA expression was significantly higher in SN tissues from MPTP-treated WT (0.78 ± 0.01) and TLR4-KO mice (0.36 ± 0.04; $P < 0.01$; Fig. 3). Furthermore, AP-1 mRNA expression was significantly decreased in MPTP-treated TLR4-KO mice (4.33 ± 0.15) relative to MPTP-treated WT mice ($P < 0.01$; Fig. 3A). Real-time qPCR data also showed that AP-1 mRNA expression was significantly lower in MPTP-treated TLR4-KO mice relative to MPTP-treated WT mice ($P < 0.01$; Fig. 3B).

3.4. TLR4 deficiency attenuated MPTP-induced up-regulation of AP-1 protein in the SN

To further confirm that TLR4 modulates AP-1 expression in MPTP-induced PD mice, we evaluated AP-1 protein levels via Western blotting. AP-1 protein was detected at the predicted size of 18 kDa. GAPDH (34 kDa) was used as a positive control and was present in each lane. The AP-1 protein expression profiles in the SNs of the NS- and MPTP-treated WT mice and PD mice were consistent with the AP-1 mRNA results shown in Fig. 4. Similarly to the results for AP-1 mRNA expression, AP-1 protein expression was weak in NS-treated WT and TLR4-KO mice. Furthermore, AP-1 protein expression was significantly increased in the MPTP-treated WT and TLR4-KO mice ($P < 0.01$). Moreover, AP-1 protein expression was significantly lower in the MPTP-treated TLR4-KO mice relative to the MPTP-treated WT mice ($P < 0.01$; Fig. 4).

3.5. Distribution of AP-1 expression significantly differed in MPTP-induced TLR4-KO mice

We next investigated AP-1 expression patterns in NS- and MPTP-treated WT and TLR4-KO mice via immunohistochemical staining using the same anti-AP-1 antibody as was used for Western blotting (as described above). The results showed weak to moderate AP-1 staining in cell bodies and dendrites in the SNs of NS-treated WT and TLR4 KO mice (Fig. 5A). However, in MPTP-treated WT mice, strong AP-1 staining was observed in the same regions (Fig. 5B). No immunoreactivity was observed in the negative controls in which the primary antibody was omitted (data not shown). The mean optical densities in the SN showed that AP-1 expression was higher in MPTP-treated WT and TLR4-KO mice than in NS-treated mice. Furthermore, AP-1 expression was significantly lower in the MPTP-treated TLR4-KO mice relative to the MPTP-treated WT mice.

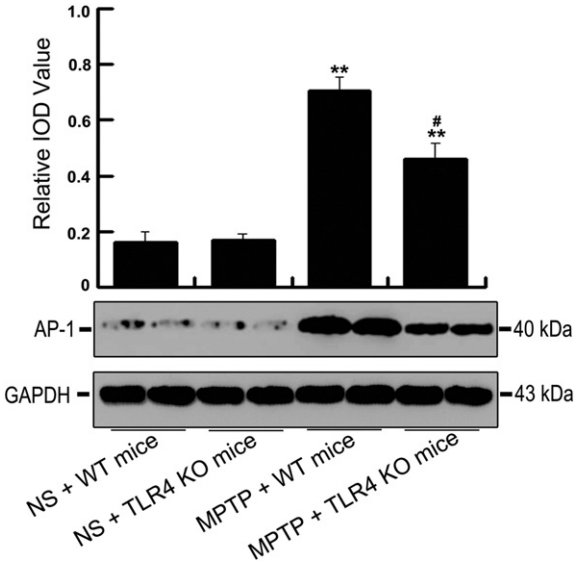

Fig. 4. AP-1 protein expression in MPTP-treated WT and TLR4-KO mice. Representative immunoblot bands showing AP-1 protein expression in NS-treated WT and TLR4-KO mice and MPTP-treated WT and TLR4-KO mice. The optical densities of the bands were measured via densitometry and normalized to GAPDH. The values are expressed as the mean ± SEM ($n = 8$). $^{**}P < 0.01$ vs. NS-treated mice; $^{\#}P < 0.01$ vs. MPTP-treated WT mice.

3.6. TLR4 deficiency attenuated MPTP-induced up-regulation of AP-1 in TH-positive neurons and astrocytes in the SN

To investigate whether AP-1 expression decreased in dopaminergic neurons or astrocytes, we examined AP-1 levels in the SNs of MPTP-treated WT and TLR4-KO mice by immunofluorescence staining. SN slices were prepared on the fifth day after MPTP injection and double-stained with antibodies against TH and AP-1. As shown in Fig. 6A–C, nearly all TH-positive cells exhibited AP-1 expression. Furthermore, as shown in Fig. 6D–H, in MPTP-treated TLR4-KO mice, nearly all TH-positive cells exhibited decreased levels of AP-1, resulting in a decrease in the number of yellow-stained cells. Likewise, astrocytes exhibited decreased levels of AP-1 in MPTP-treated TLR4-KO mice (Fig. 6G–I) compared to MPTP-treated WT mice (Fig. 6J–L). Cell counting showed that the percentage of AP-1-positive neurons in TH-positive cells increased by approximately 80% in MPTP-treated TLR4-KO mice and that the percentage of AP-1-positive astrocytes increased by approximately 70% compared to MPTP-treated WT mice (Fig. 6M). These results indicate that AP-1 becomes selectively inactivated in TH cells following MPTP treatment and that TLR4 deficiency can attenuate AP-1 activation in the SN.

4. Discussion

In the present study, we demonstrated that AP-1 mRNA and protein expression significantly increased in the SNs of MPTP-treated WT mice. Furthermore, AP-1 expression decreased in dopaminergic neurons of MPTP-treated TLR4-KO mice. Our data indicated that AP-1 was activated in PD mice and that AP-1 activation was mediated via TLR4 signaling, which plays a critical role in determining the fate of neural cells in the mouse model of PD.

AP-1 is a transcription factor formed by dimers composed of members of the Fos and Jun families of proteins [23]. c-Jun is a major component of the transcription factor complex. The mammalian Jun protein family includes the JunB and JunD proteins [24]. Jun proteins can quickly heterodimerize with other transcription factors and other basic zipper-containing transcription factors, including CBP, MyoD, NFat and c-rel [25,26]. The c-Jun protein is a central component of the AP-1 transcription factor complex; over the course of development and into adulthood, c-Jun protein expression typically increases, and high expression levels are also found in injured brains [25]. Our previous study showed that AP-1 expression transiently increases in mice following kainic acid-induced seizures. These results suggest that AP-1 may have an important role in the pathogenesis of epilepsy [27]. Another study showed that AP-1 activation becomes significantly up-regulated in the striatums and midbrains of male mice following MPTP injection [11]. In the current study, we generated an animal model of PD via MPTP injection. This is a well-known animal model that exhibits similar clinical and pathological features to human PD [28]. In accordance with a previous study [11], our results showed that AP-1 transcription and translation were significantly increased in MPTP-induced PD mice. Furthermore, AP-1 expression was significantly increased in TH-positive dopaminergic neurons in MPTP-treated mice. This result suggests that AP-1 has a key role in neurodegeneration following brain injury potentially via its involvement in inflammatory processes.

When levels of extracellular glutamate increase, causing hyperactivation of glutamatergic receptors in the basal ganglia, a cascade of events modulated by changes in intracellular signaling and cell–cell

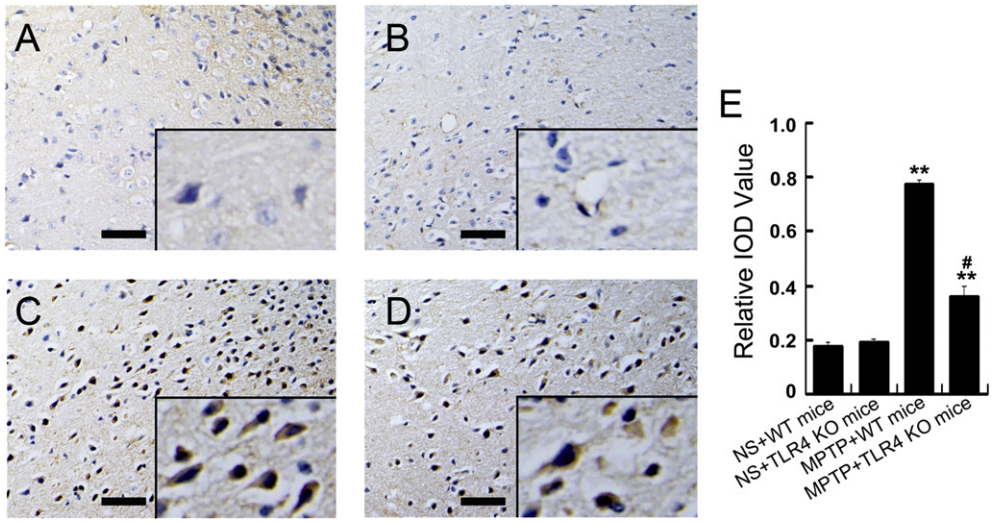

Fig. 5. Histological immunostaining of AP-1 in NS- and MPTP-treated WT and TLR4-KO mice. A–D) Representative photomicrographs illustrating AP-1 immunoreactivity (brown) in the SNs of NS-treated WT mice (A), NS-treated TLR4-KO mice (B), MPTP-treated WT mice (C), and MPTP-treated TLR4-KO mice (D). The insets are amplified images. Quantitative results (E) were obtained by counting AP-1-labeled neurons in the SN. ($n = 6$, each subunit; $^{**}P < 0.01$ vs. NS-treated mice; $^{\#}P < 0.01$ vs. MPTP-treated WT mice). Scale bar, 25 μm (400×).

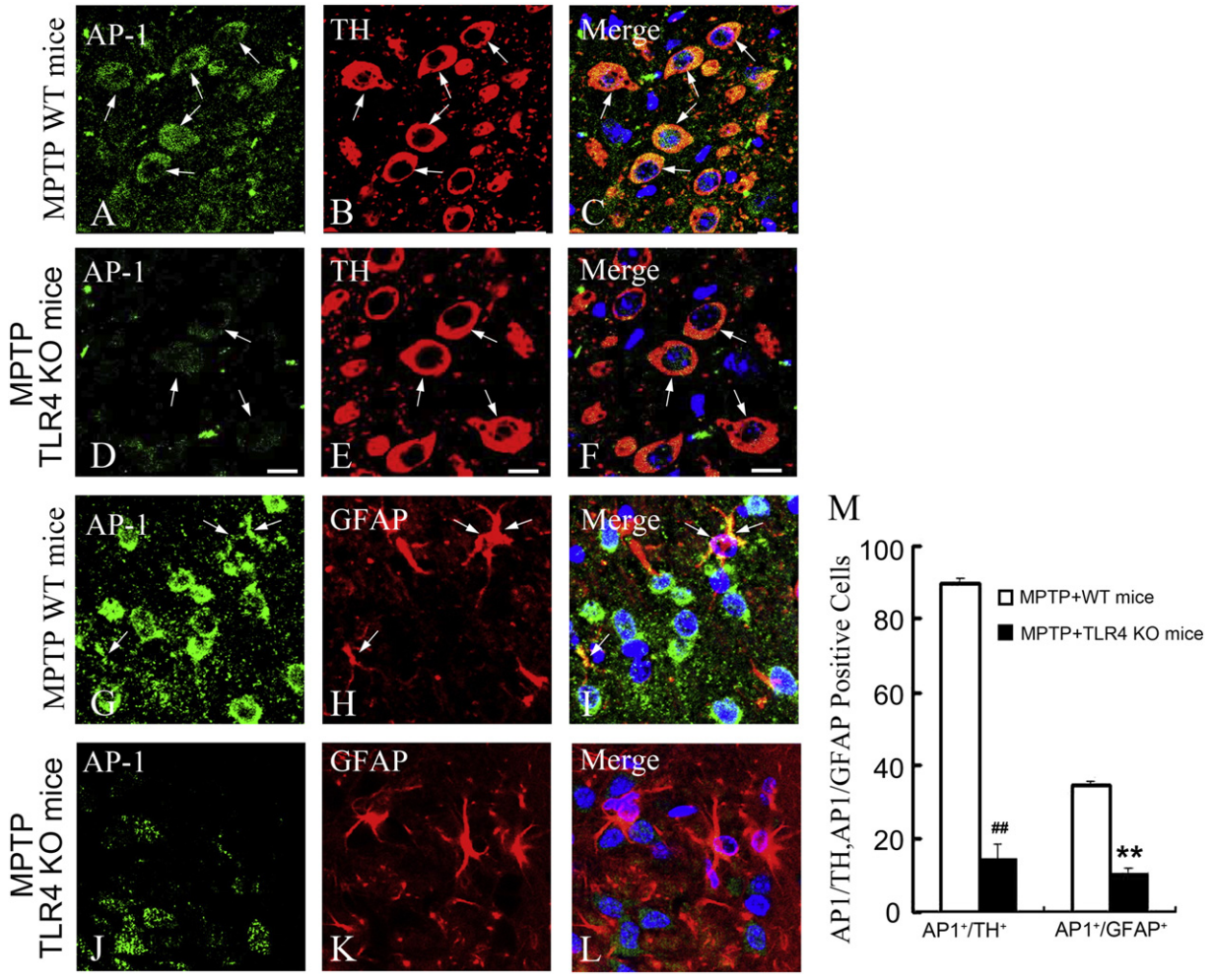

Fig. 6. Reduced colocalization of AP-1 expression in TH-positive dopaminergic neurons and GFAP-positive astrocytes in MPTP-treated TLR4-KO mice. A–F) Representative photomicrographs illustrating cells double-labeled for visualization of AP-1 (A, D; green) and TH (B, E; red) in MPTP-treated WT (A–C) and TLR4-KO mice (D–F) treated over 5 consecutive days. G–L) Representative photomicrographs illustrating cells double-labeled for visualization of AP-1 (G, J; green) and GFAP (H, K; red) in MPTP-treated WT mice (G-I) and TLR4-KO mice (J-L) treated over 5 consecutive days. M) Comparison of AP-1/TH and AP-1/GFAP cell numbers in MPTP-treated WT and TLR4-KO mice. Compared to the MPTP-treated WT mice, the MPTP-treated TLR4-KO mice had significantly lower numbers of AP-1/TH cells and AP-1/GFAP cells (mean ± SEM, $n = 6$; $^{**}P < 0.01$ vs. MPTP-treated WT mice; $^{\#\#}P < 0.01$ vs. MPTP-treated WT mice).

interactions occurs that affects cell viability and contributes to neuronal death [29]. These glutamate-triggered events cause excitotoxicity, a phenomenon involved in several pathological conditions affecting the central nervous system, including neurodegenerative diseases such as PD [29]. Glutamate-mediated excitotoxicity severely augments neural degeneration in the SN in PD patients and can even cause neuronal cell death [29]. Neuronal cell death caused by glutamate excitotoxicity is ubiquitous in neurodegenerative disorders, including PD, and has been associated with AP-1 activation [30]. Conversely, the inhibition of AP-1 has been shown to exert neuroprotective effects in primary cortical neuronal cultures under acute glutamate excitotoxicity [30]. As mentioned above, recent studies have indicated that TLR signaling is also involved in the pathogenesis of PD [7]. TLR4-deficient mice are less vulnerable to MPTP toxicity and exhibit higher numbers of surviving TH-positive neurons following MPTP challenge. Another study suggested that the TLR4 pathway plays an important role in neuronal death in PD [4]. In the current study, AP-1 expression was found to decrease in TH-positive neurons in the SNs of TLR4-deficient mice. This result suggests that TLR4 signaling-mediated activation of AP-1 may result in neuronal cell death.

Dopaminergic neurons are vulnerable to oxidative products and inflammatory responses; these processes may play important roles in the etiology of PD. Because glial cells are the main generators of inflammatory responses in the CNS, the possibility that PD results from glial cell dysfunction must be considered [31]. Reactive astrocytes are abundant in the SNs of PD cases and in the 6-hydroxydopamine and MPTP PD animal models [31–33]. Astrocytes normally exert neuroprotective functions; however, they may contribute to harmful chronic inflammation under adverse stimulation [31]. To date, the roles that astrocytes play in various parkinsonian syndromes are not sufficiently understood because astroglial reactions occur in late-stage disease and therefore do not directly participate in initiating cell death [34]. Our results showed that AP-1 expression significantly increased in astrocytes in MPTP-treated mice. This result suggests that astroglial AP-1 can stimulate sterile inflammation in PD. Furthermore, AP-1 expression was decreased in GFAP-positive astrocytes in the SNs of TLR4-deficient mice, suggesting that TLR4 signaling mediated the astroglial activation of AP-1 and enhanced neuronal cell death.

Taken together, our results indicate that AP-1 was over-expressed in TH-positive neurons and astrocytes in the SNs of MPTP-treated mice and that TLR4 mediated the expression of AP-1, which may be associated with the pathogenesis of PD.

Disclosure

None of the authors have any conflicts of interest to disclose. All authors confirm reading the journal's position on issues related to ethical publication and affirm that this report is consistent with those guidelines.

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