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Neuroprotective effects of Astilbin on MPTP-induced Parkinson's disease mice: Glial reaction, α-synuclein expression and oxidative stress

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ABSTRACT

Astilbin (AST), a dihydro-flavonol glycoside, is a major bioactive ingredient in *Astilbe thunbergii*, *Engelhardia roxburghiana*, *Smilax corbularia* and *Erythroxylum gonocladum*, and has been shown to have anti-inflammatory, antioxidative and neuroprotective effects, suggesting potential therapeutic value in the treatment of Parkinson's disease (PD). We explored the neuroprotective effects of AST in 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced Parkinson's disease mice. Mice were administered with MPTP (30 mg/kg, i.p) daily for 5 days, to establish a subacute Parkinson's disease model, followed by daily treatment with AST or saline for 7 days. Pole and traction tests showed that AST ameliorated the impaired motor functions in MPTP-induced Parkinson's disease mice. High performance liquid chromatography analysis revealed that AST treatment prevented MPTP-induced decreases in striatal dopamine levels. Immunofluorescence assays showed that AST reduced the loss of dopaminergic neurons and the activation of microglia and astrocytes in the substantia nigra. Western blot analyses revealed that AST suppressed α-synuclein overexpression and activated PI3K/Akt in the striatum following MPTP treatment. AST also prevented the MPTP-induced reduction in total superoxide dismutase and glutathione activity in the striatum. AST exerts neuroprotective effects on MPTP-induced PD mice by suppressing gliosis, α-synuclein overexpression and oxidative stress, suggesting that AST could serve as a therapeutic drug to ameliorate PD.

1. Introduction

Parkinson disease (PD) is a progressive neurodegenerative disease characterized by loss of dopaminergic neurons in the substantia nigra (SN) and diminished dopamine (DA) content in the striatum [1–3], and is at least partly associated with α-synuclein protein overexpression in striatal neurons [4]. Current drug treatments for PD provide only symptomatic treatment and do not prevent the progressive loss of dopaminergic neurons in PD patients and concomitant decline [5,6]. Thus, discovery of new therapeutic agents, focusing on neuroprotection to block PD progression, is urgently needed.

Although the molecular mechanisms underlying the progressive loss of dopaminergic neurons in PD are not completely clear, accumulating evidence indicates that mitochondrial dysfunction, inflammation, oxidative stress and a high density of microglia in the SN play key roles in the pathogenesis of PD [7,8]. Cellular correlates for loss of dopaminergic neurons in PD at least partly involve overexpression of α-synuclein in the cytoplasm of dopaminergic neurons, as well as dysfunction of the phosphoinositide 3-kinase (PI3K)/Akt signaling pathway [9,10]. The PI3K/Akt signaling pathway is an important signaling pathway in neuronal growth, proliferation, survival and function [11,12]. Activation of the PI3K/Akt pathway increases survival and growth of dopaminergic neurons by inhibiting apoptosis [13,14]. Also, PI3K/Akt signaling can prevent dopaminergic neuron loss in MPTP-induced PD mice [15]. Thus, the activation of PI3K/Akt may represent an operative approach for the prevention and treatment of PD.

Abbreviations: AST, Astilbin; DA, dopamine; DOPAC, 3,4-dihydroxyphenylacetic acid; GFAP, glial fibrillary acidic protein; GSH, glutathione; HVA, homovanillic acid; Iba-1, induction of brown adipocytes 1; IF, immunofluorescence; PI3K, phosphoinositide 3-kinase; PMSF, phenylmethanesulfonyl fluoride; RIPA, radio immunoprecipitation assay; SN, substantia nigra; SOD, superoxide dismutase; TH, tyrosine hydroxylase

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Chinese traditional medicine, known for its low toxicity, can provide good prospects for PD treatment. Recently, some traditional Chinese medicine monomers, such as loganin and ursolic acid, have been reported to alleviate the symptoms of MPTP-induced PD mice [16,17].

Astilbin (AST), a dihydro-flavonol glycoside, is a major bioactive ingredient in *Astilbe thunbergii*, *Engelhardia roxburghiana*, *Smilax corbularia* and *Erythroxylum gonocladum*, and has been shown to possess anti-inflammatory, antioxidative and neuroprotective effects. Previous studies in vitro have shown that AST significantly suppresses the production of nitric oxide (NO) and tumor necrosis factor-α (TNF-α) in lipopolysaccharide-induced RAW264.7 macrophages [18]. Also, AST inhibits the adhesion of T lymphocytes via inhibiting the CD44 expression and TNF-α production in Jurkat cells [19]. In addition, previous in vivo studies reported that AST inhibits Th17 cell differentiation and ameliorates imiquimod-induced psoriasis-like skin lesions in BALB/c mice via inhibiting the Jak3/Stat3 signaling pathway [20]. Moreover, AST improves learning and memory deficits in an APPswe/PS1dE9 transgenic mouse model of Alzheimer's disease, and this was partly mediated by activation of the CREB/BDNF signaling pathway [21]. Together, these provide strong evidence that AST may confer potential neuroprotective effects in PD. In the following experiments, we first investigate the effects of AST on behavioral and neurohistological changes in MPTP-induced PD mice, then seek to determine whether neuroprotection by AST against MPTP is mediated by activation of the PI3K/Akt signaling pathway.

2. Materials and methods

2.1. Animals

Eight-week-old male C57BL/6 mice (18–22 g) were acquired from the Zhaoyan New Drug Research Center (Suzhou, China) Co., LTD. The animals were maintained (5 mice/cage) in an air-conditioned room (24 ± 1 °C) with a 12 h light/12 h dark cycle and ad libitum access to food and water, and were allowed to acclimatize for 1 week before the experiment. All animal procedures were performed in accordance with the Jiangnan University Animal Care Committee's regulations.

2.2. Drugs and treatment

1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine hydrochloride (MPTP-HCl) (23007-85-4) was purchased from Sigma-Aldrich (St. Louis, MO). AST (purity ≥ 98%; 29838-67-3) was purchased from Shanghai ShiFeng Biological Technology Co., LTD (Shanghai, China). DA (62-31-7), DOPAC (102-32-9) and HVA (306-08-1) standards were purchased from Sigma-Aldrich (St. Louis, MO).

Mice were randomly divided into 3 groups (n = 15 each group): normal saline treated (NS), MPTP + NS-treated (MPTP + NS) and MPTP + AST-treated (MPTP + AST). Mice in the normal saline group were treated daily, with 0.1 ml/10 g saline by intraperitoneal (*i.p*) injection over a period of 12 days. Mice in the MPTP + NS-treated group were administered MPTP (dissolved in saline) at 30 mg/kg/d for 5 days, then treated with saline for 7 days, and the dosage of MPTP was chosen in accordance with previous study [22]. Mice in the MPTP + AST-treated group were administered MPTP (30 mg/kg/d), then treated with AST (50 mg/kg/d) for 7 days, and the dosage of AST was chosen in accordance with previous study [21]. AST was dissolved in dimethylsulfoxide (DMSO) as stock solutions. The stock solutions were diluted to the final concentrations with normal saline before application and the final concentration of DMSO did not exceed 0.1%, and AST concentration is 5 mg/ml. Behavioral tests were performed on the first day after the last NS or AST treatment, in a behavioral testing room, and then tissue samples were collected on the first day after completion of the behavioral testing.

2.3. Behavioral tests

Mice were trained once a day for three consecutive days before behavioral testing. On the 4th day of NS or AST treatment following MPTP administration, the following behavior tests were performed.

2.3.1. Pole test

A pole test for assessment of bradykinesia was conducted using a modification of a method reported previously [23]. Mice were placed head-down on a 2 cm diameter spherical protuberance, located at the top of a pole (1 cm diameter and 55 cm height), and the descent back to the floor was timed. Timing began when the experimenter released the animal and ended when one hind-limb reached the floor. For each animal, the pole test was performed the first day after the last NS or AST injection. Three tests were performed, with a 10 min interval between each test, and the average score was taken.

2.3.2. Traction test

Mice were allowed to hold onto a horizontal rope (diameter 5 mm), by their forepaws, and observed for 10 s, while their hind limb placements were scored from 1 to 4, with the lowest score indicating the most severe deficit [24]. Animals were assigned a score of 4 for gripping the wire with both hind paws, 3 for gripping the wire with one hind paw, and 2 for gripping the wire with both front paws, 1 for gripping the wire with one front paw. For each mouse, the traction test was performed the first day after the last NS or AST injection. Three tests were performed, with a 10 min interval between each test, and the average score was taken.

2.4. Assay for striatal DA and its metabolites

Mice in each group were anesthetized with isoflurane and perfused with saline on the first day after completion of the behavioral testing, and the brains were immediately harvested. The right striatum was homogenized in 0.1 M perchloric acid (per milligram of striatum tissue by adding 10 μl perchloric acid), and the homogenate was centrifuged at 13,000*g* (4 °C) for 10 min. 20 μl supernatant was injected into a HPLC system with an Atlantis T3 column (150 mm ∗ 4.6 mm, 5 μm, Waters) and fluorescence detection (Waters 2475). The mobile phase contained acetonitrile, water and 0.01 M PBS (adjusted to pH 4 with phosphoric acid), and the flow rate was 1.0 ml/min. The content of DA and its metabolites were expressed as μg/g tissue.

2.5. Western blot analysis of striatal tyrosine hydroxylase, α-synuclein, PI3K and Akt

Brain tissue stored at −80 °C was homogenized in 0.2 ml of RIPA (radio immunoprecipitation assay) lysis buffer (Vazyme Biotech Co., LTD, China) with 2 μl of 100 mM PMSF (phenylmethanesulfonyl fluoride, Beyotime, China). The homogenate was centrifuged at 13,000 rpm for 10 min at 4 °C, then the supernatants were collected, and 40 μg of protein from each sample was separated electrophoretically on 10% SDS-polyacrylamide gels, and transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, USA). Membranes were blocked with 5% BSA (Amresco, USA), and then incubated with primary antibodies: rabbit polyclonal anti-tyrosine hydroxylase (TH) antibody (1:1000, MAB318, Millipore, USA), mouse monoclonal anti-α-synuclein antibody (1:1000, 610786, BD, USA), rabbit polyclonal anti-PI3K (1:1000, 4257, Cell Signaling Technology, USA), rabbit polyclonal anti-phospho-PI3K (1:1000, 4228, Cell Signaling Technology, USA), rabbit monoclonal anti-Akt (1:1000, 4691, Cell Signaling Technology, USA), rabbit monoclonal anti-phospho-Akt (1:1000, 4060, Cell Signaling Technology, USA) or mouse monoclonal anti-GAPDH antibody (1:8000, 60004-1-Ig, Proteintech, USA) overnight at 4 °C. After washing with TBST, membranes were incubated with corresponding secondary horseradish peroxidase-conjugated goat

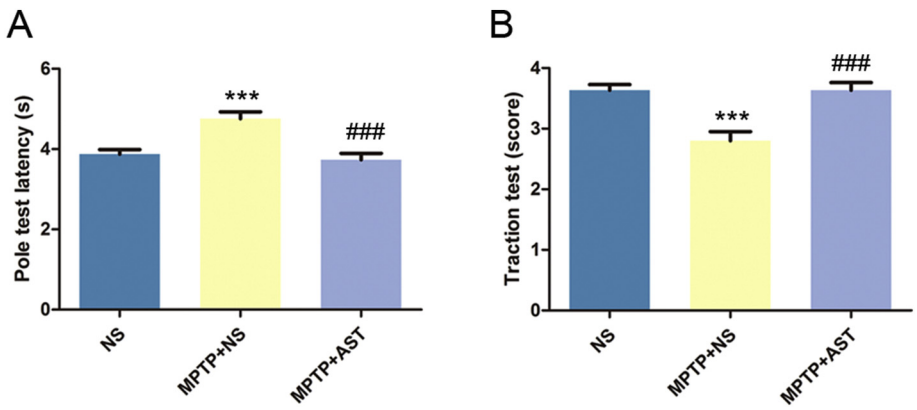

Fig. 1. AST improves motor deficits of MPTP-induced PD mice. (A) Pole test latency. MPTP + AST mice have a significantly shorter descent time compared with MPTP + NS mice, indicating that AST treatment preserves normal behavior. (B) Traction test score. MPTP + AST mice scored higher compared with MPTP + NS mice, showing AST treatment preserves normal behavior. Statistical differences between the means were determined by one-way ANOVA. Data are expressed as mean ± SEM (N = 10). $^{***}P < 0.001$ vs. NS group and $^{###}P < 0.001$ vs. MPTP + NS group.

anti-mouse/rabbit IgG antibody for 2 h at room temperature. Goat anti-rabbit IgG (1:1000, A0208, Beyotime, China) and goat anti-mouse IgG (1:1000, A0216, Beyotime, China) were used as secondary antibodies. After another three washes with TBST, blots were visualized by a Bio-Rad Universal Hood III Detection System (Bio-Rad Laboratories, Inc. U.S.A.). The band intensity of each protein was analyzed using ImageJ (NIH, U.S.A.). Data are expressed as relative intensity following normalization to GAPDH.

2.6. Immunofluorescence of TH, Iba-1 and GFAP in the SN

Mice were anesthetized with isoflurane and perfused with 0.01 M phosphate buffer (pH 7.4) and then 4% paraformaldehyde in 0.01 M phosphate buffer (pH 7.4), then the whole brains were removed. Whole brains were post-fixed with 4% paraformaldehyde in 0.01 M PBS at 4 °C and then successively transferred into 20%, and then 30% sucrose, at 4 °C for 24 h. A series of 8-μm-thick coronal sections of the SN were cut, with a freezing microtome (CM1950, Leica, Germany), for immunofluorescence. Initially, sections were blocked with 5% goat serum for 1 h at 37 °C. Then, sections were incubated at 4 °C overnight with the following primary antibodies: mouse monoclonal anti-tyrosine hydroxylase antibody (1:1000, MAB318, Millipore, USA) and rabbit polyclonal anti-Iba-1 antibody (1:1000, 019-19741, Wako, Japan) or rabbit polyclonal anti-GFAP antibody (1:3000, Z033429, Dako, Denmark). Then, sections were incubated with secondary FITC-conjugated goat anti-mouse IgG (1:1000, A0568, Beyotime, China) and CY3-conjugated goat anti-rabbit IgG (1:1000, A0516, Beyotime, China) for 1 h at 37 °C, respectively, and washed 3 times in PBS. Immunofluorescence images were collected using an epifluorescence microscope (Nikon eclipse 80i, Nikon, Japan), and quantitative analysis of fluorescence images was performed using ImageJ software (NIH, USA). For assessment of dopaminergic neurons number, five midbrain slices of TH-positive cells in the SN was counted from the first (rostral) and every sixth coronal section of a series of 30 frozen sections of each brain, which cover the entire extent of the SN. In order to ensure consistency within and between groups, matched ventral midbrain tissue sections were always processed according to anatomical landmarks provided by the brain atlas. In the double immunofluorescence experiments, 5 representative sections (second (rostral) and every sixth coronal section of a series of 30 frozen sections of each brain) were chosen to be doubly stained with tyrosine hydroxylase (TH) and Iba-1 or GFAP respectively. The cells positive for TH, Iba-1 and GFAP were counted in the SN and the positive cells evaluation was used by the Image J analyzer (NIH, U.S.A.). The above-mentioned 5 representative sections were quantified for each animal, and each group contains 5 animals. These data are present as mean number of cells per section per group. The numbers of TH-positive neurons, iba-1-positive cells and GFAP-positive cells in treatment groups were expressed as a percentage of the NS group.

2.7. ELISA for antioxidative activity assay in the striatum

Striatal tissue was homogenized in normal saline, followed by centrifugation at 13,000 rpm for 10 min at 4 °C. The supernatant was used to test SOD activity and GSH level using an available kit according to the manufacturer's protocol (Nanjing Jiancheng Bioengineering Institute, China). Reaction product was quantified using a microplate reader (BioTek Instruments, USA). SOD activity was expressed as U/mg protein, and the glutathione (GSH) level was expressed as μmol/g protein.

2.8. Statistical analysis

Data were analyzed with SPSS 22.0 software and are presented as the mean ± SEM (standard error of the mean). Statistical analysis was conducted by one-way analysis of variance (one-way ANOVA) with post hoc comparisons of least significant difference (LSD). $P < 0.05$ was considered statistically significant.

3. Results

3.1. AST ameliorates MPTP-induced motor deficits

To evaluate the effects of AST treatment on MPTP-induced motor deficits, we investigated motor function by using the pole test and traction test. In the pole test, MPTP + NS mice took 22.9% longer time than those treated with saline ($P < 0.001$), whereas AST treatment of MPTP mice significantly prevented the MPTP-induced prolongation of descent time by 21.6% during the test compared with MPTP + NS mice ($P < 0.001$) (Fig. 1A). In the traction test, MPTP + NS group mice received a 22.9% lower score than those treated with saline ($P < 0.001$), whereas AST treatment of MPTP mice significantly increased traction score by 29.7% during test ($P < 0.001$ vs. the MPTP + NS group) (Fig. 1B). These results indicate that AST ameliorates MPTP-induced motor deficits.

3.2. AST treatment reduces MPTP-induced loss of SN dopaminergic neurons

To investigate the neuroprotective effects of AST on dopaminergic neurons after MPTP treatment, the number of TH-positive neurons in the SN was assessed by IF (Fig. 2A). MPTP treatment induced an approximate 29.3% reduction in TH-positive neurons. In contrast, the number of tyrosine hydroxylase (TH)-positive neurons in the MPTP + AST group increased by 41.6% ($P < 0.05$ vs. the MPTP + NS group) (Fig. 2B). These results indicate that AST reduces MPTP-induced loss of SN dopaminergic neurons.

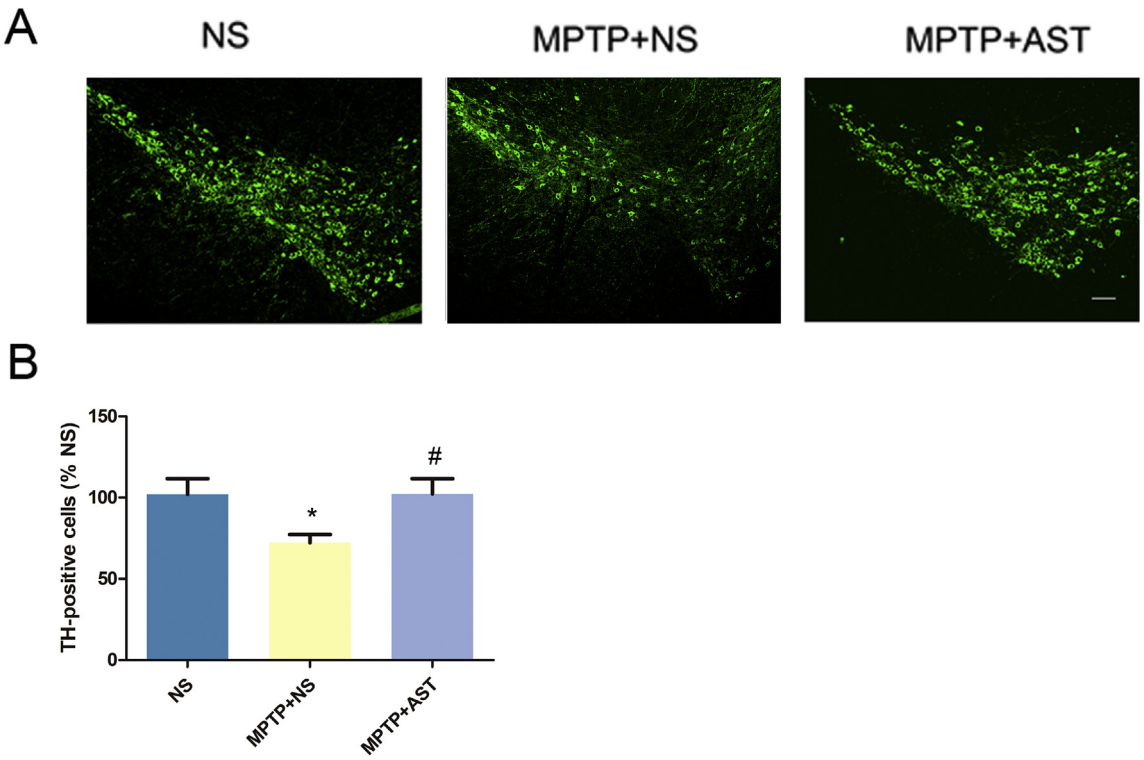

Fig. 2. AST reduces the loss of dopaminergic neurons in the SN of MPTP-induced PD mice. (A) Immunofluorescence staining for the dopaminergic neuron marker TH (green) in the SN (scale bar = 100 μm). (B) Quantitative analysis of the number of TH-labeled dopaminergic neurons in each group. MPTP treatment resulted in a decrease, in the number of dopaminergic neurons in the SN AST treatment reduced the MPTP-mediated decline in dopaminergic neurons. Statistics by one-way ANOVA. Data are expressed as mean ± SEM (N = 5). $^{*}P < 0.05$ vs. NS group and $^{\#}P < 0.05$ vs. MPTP + NS group. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.3. AST treatment blocks MPTP-induced loss of striatal DA and its metabolites

To explore the effects of AST on brain DA, striatal DA and its metabolites, 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA), were measured by high-performance liquid chromatography (HPLC). In accord with previous reports [16], striatal DA, DOPAC and HVA concentrations in mice treated with MPTP markedly decreased by 66.1%, 49.4% and 42.9%, respectively, compared with the NS group ($P < 0.001$). However, mice in the MPTP + AST group had a 13.0% increased level of DA ($P < 0.05$ vs. the MPTP + NS group), a 22.3% increased level of DOPAC ($P < 0.05$ vs. the MPTP + NS group) and a 32.0% increased level of HVA ($P < 0.05$ vs. the MPTP + NS group) (Fig. 3). As the DA level is a marker of dopaminergic synaptic function, these results further indicate that AST has neuroprotective effects on dopaminergic neurons in MPTP-induced PD mice.

3.4. AST reduces the MPTP-induced decrease in TH expression

To further determine the effects of AST treatment on TH expression in the striatum, the TH level was measured by Western blot analysis. Compared with NS mice, the TH level in the MPTP + NS mice significantly decreased by 38.1% ($P < 0.01$). However, AST treatment inhibited the reduction of TH expression by 42.5% compared with the MPTP + NS group (Fig. 4). These results indicate that AST showed neuroprotective effects by inhibiting the MPTP-induced reduction of TH expression in MPTP-induced PD mice.

3.5. AST alleviates the MPTP-induced activation of microglia and astrocytes in the SN

Activation of glial cells is commonly considered to indicate neuroinflammation [25]. We detected the number of activated glial cells, reflective of neuroinflammation in the SN, by double immunofluorescence (IF) staining for TH and Iba-1 (markers for microglia), as well as TH and GFAP (marker for astrocytes). The merged images of TH and Iba-1 indicated that along with the loss of TH-positive neurons, the number of activated microglia in the MPTP + NS group increased by 27.2% compared with the NS group. However, AST treatment alleviated microglia activation by 12.9% compared with the MPTP + NS group (Fig. 5).

Next, we detected co-expression of TH with GFAP (astrocyte marker) by double IF staining. Similarly, there were more astrocytes in the MPTP + NS group than in the NS group, and the number of activated astrocytes in the MPTP + NS group increased by 65.1%. However, AST treatment alleviated astrocyte activation by 28.9% compared with the MPTP + NS group (Fig. 6). These results suggest that AST shows neuroprotective effects by alleviating the activation of microglia and astrocytes in MPTP-treated mice.

3.6. AST inhibits MPTP-induced α-synuclein overexpression

Previous studies have reported that α-synuclein is involved in the formation of Lewy bodies and the loss of dopaminergic neurons in PD [26,27]. In our experiments, the α-synuclein expression was measured by Western blot analysis. Compared with NS group, α-synuclein expression in the MPTP + NS group significantly increased by 101.5% ($P < 0.05$). More importantly, the expression of α-synuclein in the MPTP + AST group was reduced by 42.4% ($P < 0.05$ vs. the MPTP + NS group) (Fig. 7). These results indicate that AST protects

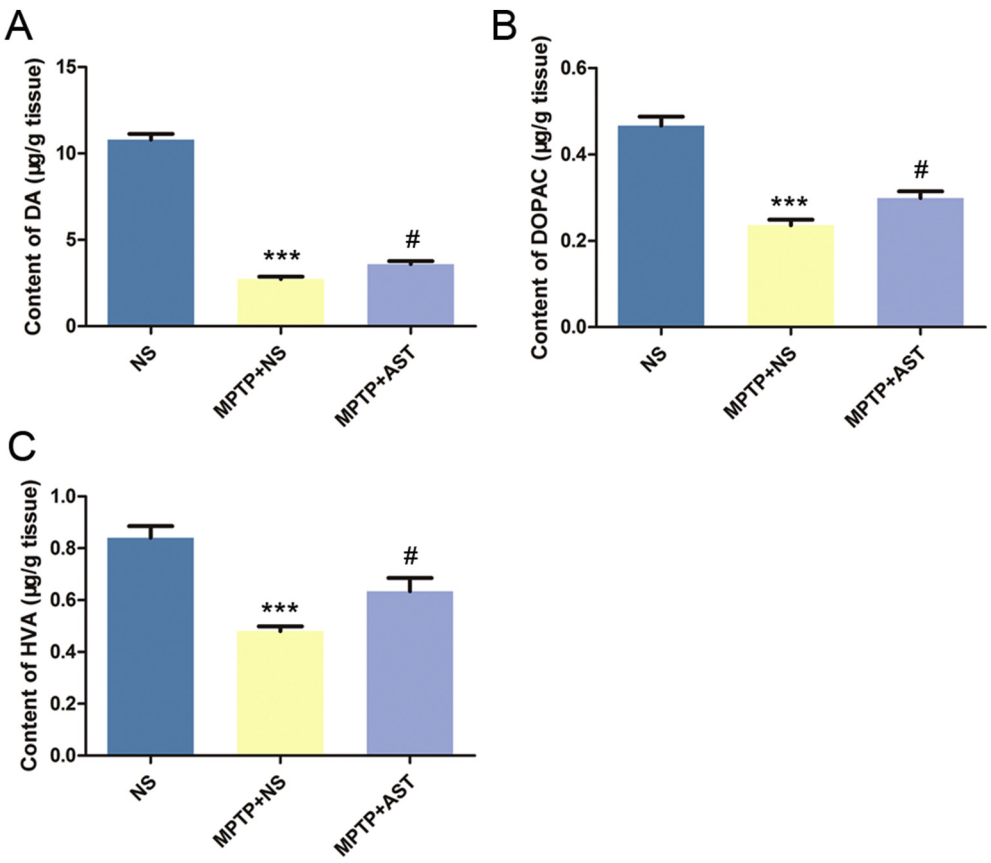

Fig. 3. AST reduces the MPTP-induced loss of striatal DA and its metabolites in the striatum of parkinsonian mice. (A) Relative DA content in the striatum. (B) Relative DOPAC content in the striatum. (C) Relative HVA content in the striatum. Treatment with AST, following MPTP treatment, reduced the MPTP-mediated declines in DA, DOPAC and HVA levels versus the MPTP + NS group. Statistics by one-way ANOVA. Data are expressed as mean ± SEM (N = 8). $^{***}P < 0.001$ vs. NS group and $^{\#}P < 0.05$ vs. MPTP + NS group.

dopaminergic neurons by inhibiting the MPTP-induced α-synuclein overexpression.

3.7. AST reverses the MPTP-induced decrease in SOD and GSH

To determine the mechanisms underlying AST-mediated neuroprotection, the total activity of superoxide dismutase (SOD), a critical antioxidant enzyme in the body, and glutathione (GSH) level in the striatum of treated mice were assessed. We initially examined whether AST could suppress MPTP-induced oxidative stress in vivo. Compared with the NS group, SOD activity in the MPTP + NS group was reduced by 36.8% ($P < 0.05$). However, AST dramatically increased the SOD activity by 57.5% in MPTP-treated mice ($P < 0.05$ vs. the MPTP + NS group) (Fig. 8A). GSH levels are reflective of antioxidant capability in the body. Compared with the NS group, GSH levels in the MPTP + NS group were reduced by 24.9% ($P < 0.05$). However, AST dramatically increased the GSH level by 25.9% ($P < 0.05$ vs. the MPTP + NS group) (Fig. 8B). These results indicate AST treatment strongly enhances antioxidative capacity, in the striatum of MPTP-treated mice, which could be partly responsible for the neuroprotective effects of AST.

3.8. AST induces activation of the PI3K/Akt signaling pathway

To further identify the mechanisms underlying AST-mediated neuroprotection, PI3K and phospho-Akt (Ser473) were measured by Western blot (Fig. 9A). As shown in Fig. 9B, compared with the NS group, the ratio of p-PI3K/PI3K in the MPTP + NS group was markedly decreased by 49.6% ($P < 0.05$). However, compared with the MPTP + NS group, the ratio of p-PI3K/PI3K in the MPTP + AST group increased by 93.3% ($P < 0.05$). Concomitantly, MPTP-treatment markedly decreased the ratio of p-Akt/Akt by 52.4% ($P < 0.05$). In contrast, treatment with AST, following MPTP treatment induced an increase in the p-Akt/Akt ratio compared with the MPTP + NS group ($P < 0.05$) (Fig. 9C). These results suggest that AST induces the

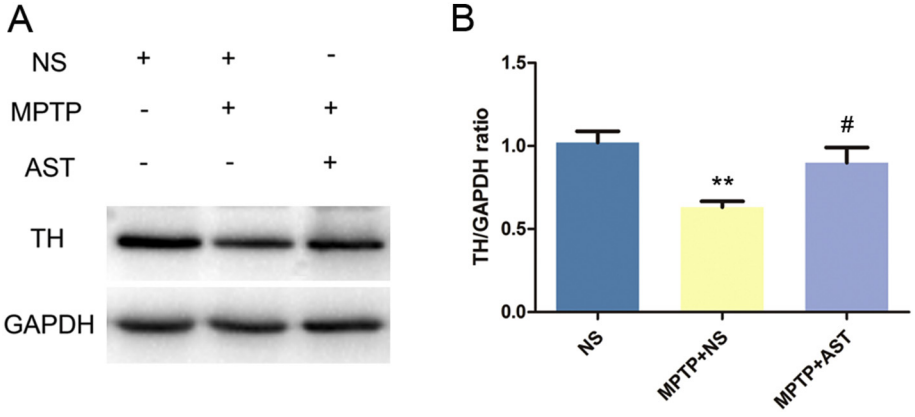

Fig. 4. AST reduces the MPTP-induced decrease in expression in the striatum of parkinsonian mice. (A) Representative Western blot of striatal TH expression. (B) Quantitative data for TH following normalization to GAPDH. Treatment with AST prevented the loss of TH expression following MPTP treatment. Statistical analysis by one-way ANOVA. Data are expressed as mean ± SEM (N = 6). $^{**}P < 0.01$ vs. NS group and $^{\#}P < 0.05$ vs. MPTP + NS group.

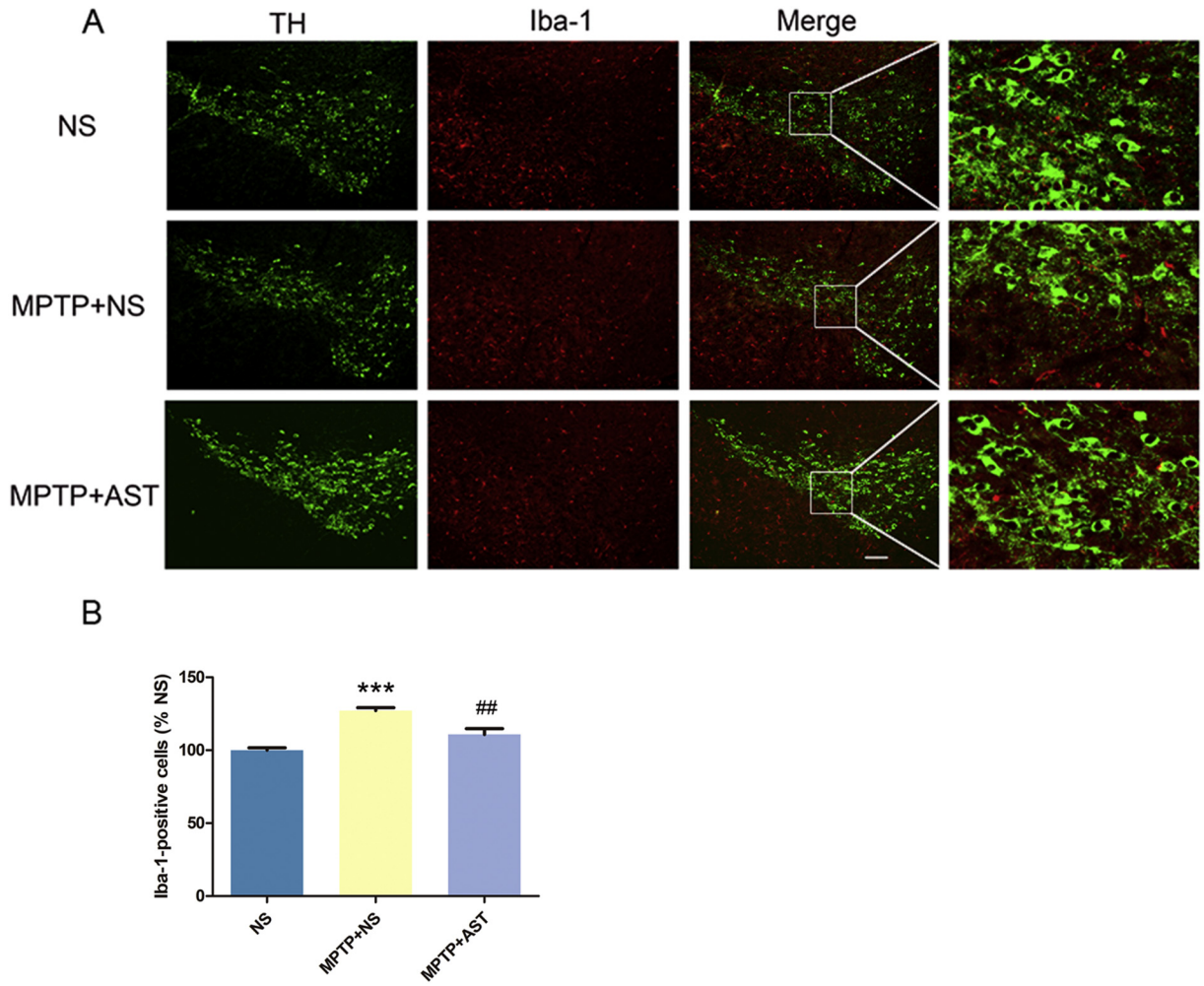

Fig. 5. AST alleviates microglia activation in the SN of MPTP-induced PD mice. (A) Double IF staining was performed, in the SN, for TH (green) and the microglial marker Iba-1 (red). The merged images of TH and Iba-1 indicated AST treatment decreased microglia activation around dopaminergic neurons (scale bar = 100 μm). (B) Quantitative analysis of the number of activated microglia in each group. One-way ANOVA was used for statistical comparison. Data are expressed as mean ± SEM (N = 5). $^{***}P < 0.001$ vs. NS group and $^{\#\#}P < 0.01$ vs. MPTP + NS group. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

activation of the PI3K/Akt signaling pathway in MPTP-treated mice.

4. Discussion

MPTP, a highly lipophilic compound that readily crosses the blood-brain barrier (BBB), has been reported to lead to a selective degeneration of dopaminergic neurons in the nigrostriatal region and cause PD-like neurochemical and histopathological alterations in nonhuman primates and mice [28,29]. Here, MPTP-induced dopaminergic neuron loss in vivo was used in our study to simulate PD. Our motor function assessment showed that MPTP + NS mice exhibit longer descent times in a pole test and lower scores in a traction test compared with mice administered normal saline. These results are consistent with previous studies showing motor impairment following MPTP treatment [30]. However, MPTP-treated mice, which were administered AST, show shorter descent times in the pole test and higher scores in the traction test compared with mice treated with MPTP alone. Thus, based on our behavioral tests, AST improves mobility of parkinsonian mice.

Loss of dopaminergic neurons in the SN is one of the hallmarks of PD [1,3]. The nigrostriatal pathway in the brain is particularly involved in the production of DA, an important neurotransmitter in charge of balance and movement. In addition, TH, an important rate-limiting enzyme, plays a vital role in the rates of synthesis and release of catecholamines (DA and epinephrine) [31]. In our study, mice treated with MPTP alone show a remarkable decline in the number of dopaminergic neurons and levels of striatal DA and TH expression. We further show that AST treatment reduces the MPTP-induced loss of dopaminergic neurons, and blocks the loss of striatal DA, DOPAC and HVA levels in MPTP-induced PD mice. Moreover, AST treatment rescues the MPTP-mediated decrease in TH expression. Results above indicate that AST prevents MPTP-induced decreases in striatal DA and its metabolites by protecting dopaminergic neurons and rescuing the decrease in TH expression in MPTP-induced PD mice.

Recent studies have suggested that glial-derived inflammatory mediators play key roles in the loss of dopaminergic neurons as PD progresses [32,33]. Glial cells comprising microglia and astrocytes are generally beneficial to neuronal function in the brain. However, under neuropathological conditions, reactive glial cells are the primary producers of several neurotoxic factors that cause neuronal cell death and neurodegeneration [34,35]. Also, inhibition of microglia activation has been shown to inhibit neuronal inflammation, and reduce dopaminergic neuronal death [36]. In our studies, MPTP administration significantly induces the activation of microglia in the SN. More importantly, AST alleviates the activation of microglia in the SN. Therefore, AST may reduce neuronal cell death by inhibiting the activation of microglia. Previous studies also found that the first stages of

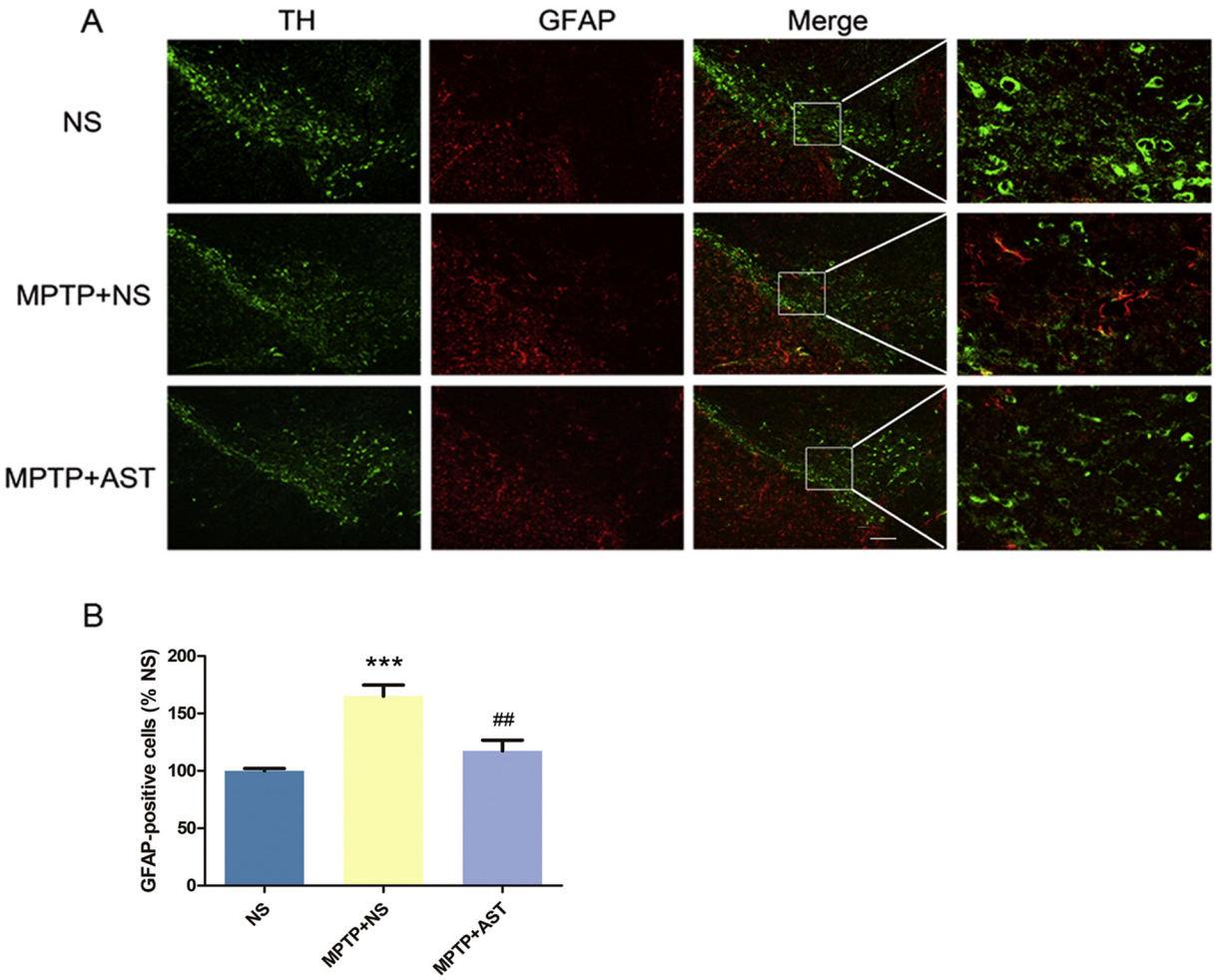

Fig. 6. AST alleviates astrocyte activation in the SN of MPTP-induced PD mice. (A) Double IF staining was performed, in the SN, for TH (green) and the astrocyte marker GFAP (red). Merged images of TH and GFAP indicate decreased astrocyte activation around dopaminergic neurons in the MPTP + AST group (scale bar = 100 μm). (B) Quantitative analysis of the number of activated astrocytes in each group. One-way ANOVA was used for statistical comparison. Data are expressed as mean ± SEM (N = 5). $^{***}P < 0.001$ vs. NS group and $^{\#\#}P < 0.01$ vs. MPTP + NS group. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

PD probably involve reactive astrocytes in the striatum [33]. Similar to the activation of microglia in the SN, the activation of astrocytes in the SN is increased in mice following MPTP-induced parkinsonism. We show AST alleviates the activation of astrocytes in the SN of our parkinsonian mice. The results suggest that inhibiting the activation of microglia and astrocytes may be involved in the mechanism of neuroprotection by AST against MPTP-induced parkinsonism. As glia-derived inflammatory factors, including TNF-α and IL-1β can trigger neuronal cell death, these inflammatory mediators will be analyzed in the MPTP-induced mouse model of PD in future experiments in combination with in vitro studies showing whether AST can block astrocyte and microglia activation.

In addition, previous studies have reported that MPTP-induced dopaminergic neuron loss is related to α-synuclein overexpression [37,38]. In accordance with this observation, we report here that MPTP treatment dramatically increases the expression of α-synuclein in the

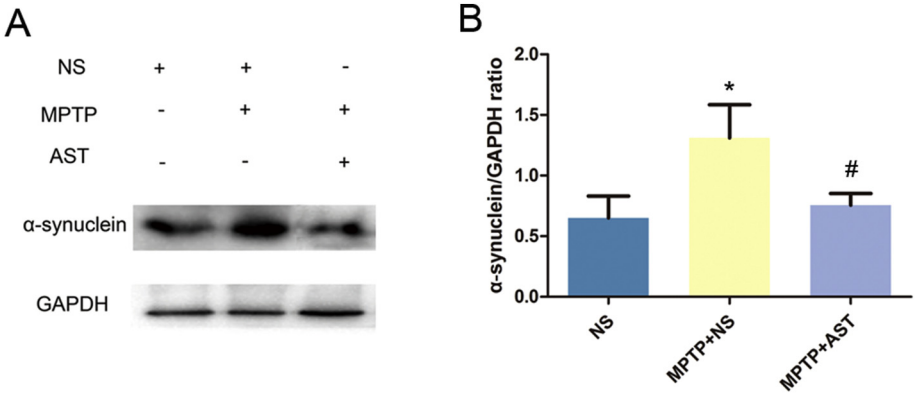

Fig. 7. AST inhibits MPTP-induced α-synuclein overexpression in the striatum of MPTP-induced PD mice. (A) Western blot analysis of striatal α-synuclein expression. (B) Quantitative data for α-synuclein following normalization to GAPDH. Treatment with AST prevented the MPTP-induced increase in α-synuclein expression versus the MPTP + NS group. Statistics by one-way ANOVA. Data are expressed as mean ± SEM (N = 6). $^{*}P < 0.05$ vs. NS group and $^{\#}P < 0.05$ vs. MPTP + NS group.

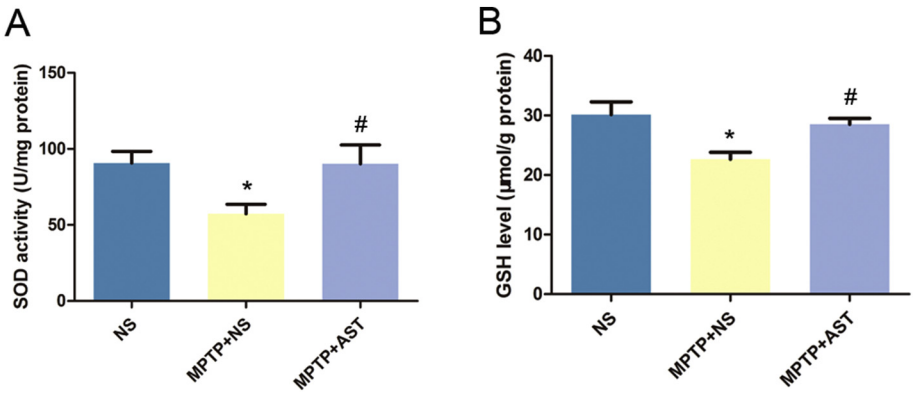

Fig. 8. AST reverses the MPTP-induced decrease in total superoxide dismutase (SOD) activity and glutathione (GSH) levels in the striatum of parkinsonian mice. (A) ELISA for SOD activity. (B) GSH level. Treatment with AST, following MPTP treatment, reduced the MPTP-mediated declines in SOD activity and GSH levels versus the MPTP + NS group. Statistical comparisons by one-way ANOVA. Data are expressed as mean ± SEM (N = 6). $^{*}P < 0.05$ vs. NS group and $^{\#}P < 0.05$ vs. MPTP + NS group.

striatum. Indeed, these findings support the hypothesis that MPTP administration can increase α-synuclein expression and α-synuclein-mediated motor impairment, eventually leading to oxidative stress [39,40]. And also previous studies reported suppressing oxidative stress may ameliorate motor deficits in MPTP-induced PD mice [41,42]. In our studies, AST treatment significantly inhibits MPTP-induced α-synuclein overexpression in the striatum, which may reduce oxidative stress, thereby contributing to the amelioration of motor impairment. Consistent with this, we show AST reduces the decrease in SOD activity and the GSH levels in mice with MPTP-induced parkinsonism. These results suggest that AST reduces dopaminergic neurons loss by inhibiting α-synuclein overexpression and reducing oxidative stress. In addition, other antioxidant capacity markers, such as catalase (CAT) and malondialdehyde (MDA) will be analyzed in the MPTP-induced mouse model of PD in future experiments.

Previous studies reported that activation of PI3K/Akt can prevent the loss of dopaminergic neurons in MPTP-induced PD mice [43]. In our study, we show that MPTP treatment induces a dramatic reduction in p-PI3K/PI3K and p-Akt/Akt ratios. In contrast, AST treatment prevents this reduction. Hence, our present study suggests that treatment with AST prevents the loss of dopaminergic neurons in MPTP-induced PD mice by inducing the activation of the PI3K/Akt signaling pathway.

In conclusion, AST exerts neuroprotective effects on MPTP-induced PD mice by suppressing gliosis, α-synuclein overexpression and oxidative stress, suggesting that AST could serve as a therapeutic drug to ameliorate PD.

Conflict of interest

The authors declare that there are no conflicts of interest.

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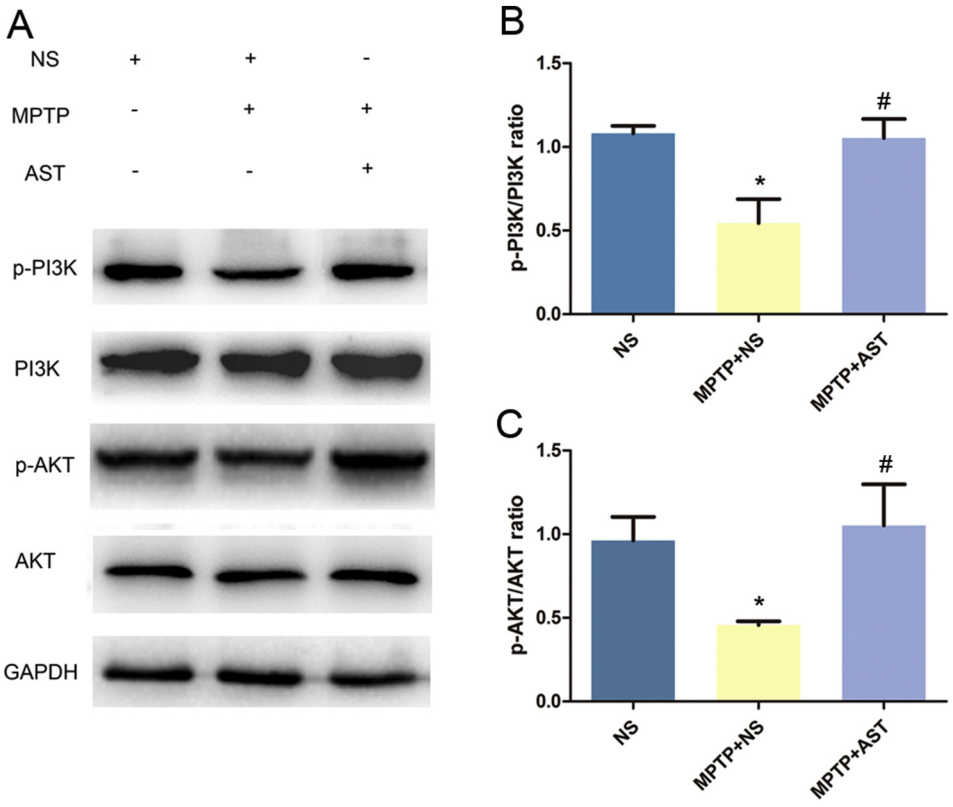

Fig. 9. AST induces activation of the PI3K/Akt signaling pathway. (A) Western blot analysis of striatal PI3K, p-PI3K, Akt and p-Akt expression. (B) Quantitative data for p-PI3K following normalization to total PI3K. (C) Quantitative data for p-Akt following normalization to total Akt. Mice administered AST, following MPTP treatment, showed elevated ratios of p-PI3K/PI3K and p-Akt/Akt compared with the MPTP + NS group. Statistical comparisons by one-way ANOVA. Data are expressed as mean ± SEM (N = 6). $^{*}P < 0.05$ vs. NS group and $^{\#}P < 0.05$ vs. MPTP + NS group.

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