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ORIGINAL ARTICLE

7,8-dihydroxyflavone Ameliorates Motor Deficits Via Suppressing α-synuclein Expression and Oxidative Stress in the MPTP-induced Mouse Model of Parkinson's Disease

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7,8-dihydroxyflavone; Antioxidative activity; Dopaminergic neuron; MPTP; Parkinson disease; α-synuclein.

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SUMMARY

Background: Parkinson disease (PD) is a neurodegenerative disease characterized by the loss of dopaminergic neurons in the substantia nigra (SN) and diminished dopamine content in the striatum, which is at least partly associated with α-synuclein protein overexpression in these neurons. Recent reports show that 7,8-dihydroxyflavone (DHF), a TrkB agonist, has beneficial effects in animal model of PD. However, it is unclear whether the therapeutic effects of DHF are associated with the expression of α-synuclein. **Aims:** In this study, we investigated the protective effects of DHF on 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced deficit of motor functions, the loss of dopaminergic neurons and the expression of α-synuclein as well as antioxidative activity in the C57BL/6 mice. **Results:** Mice were treated with MPTP (30 mg/kg, i.p.) once a day for 5 days to induce dopaminergic neuron death in the SN. DHF (5 mg/kg, i.p.) was administrated once a day from the first day of MPTP injection until 9 days after the last injection of MPTP. Behavioral tests showed that DHF succeeded in ameliorating the impaired motor functions in the MPTP-treated mice. The immunohistochemical assay showed that the amelioration of motor function was accompanied by a reduction in the loss of dopaminergic neurons in the SN and striatum. Western blot analyses showed that DHF prevented the inactivation of TrkB and suppressed α-synuclein overexpression in the SN and striatum following MPTP treatment. Antioxidative activity detection revealed that DHF prevented MPTP-induced reduction in glutathione and total superoxide dismutase activity in the SN and striatum. **Conclusion:** Taken together, these results indicate that DHF treatment may suppress the accumulation of α-synuclein and oxidative stress via activating TrkB and subsequently block the loss of dopaminergic neurons in the SN and striatum, thereby ameliorating MPTP-induced motor deficits in the C57BL/6 mice.

Introduction

Parkinson's disease (PD) is the second most common neurodegenerative disease worldwide affecting 1% at the age of 60 and 5% for people aged over 85 years [1–3]. The pathological feature of PD is characterized by the loss of dopaminergic neurons in the substantia nigra (SN) and the loss of dopamine content in the striatum [1–3]. Previous studies have proposed that the loss of dopaminergic neurons in PD is at least partly due to the overexpression of α-synuclein in the cytoplasm of these neurons. Indeed, nigral α-synuclein expression increases with age in humans and rhesus monkeys [4]. Further genetic studies have shown that the overexpression of wild type or a mutated form of α-synuclein increases copper-induced dopaminergic cell death [5]. On the contrary, downregulation of α-synuclein expression can rescue dopaminergic cells from MPTP-induced cell death both *in vitro* and *in vivo* [6–10]. Alternatively, oxidative injury is also considered as a pivotal role in pathogenesis of PD [11,12]. Human postmortem [13,14] and animal model [15] studies have suggested that oxidative damage occurred in the development of PD. These observations suggest that the inhibition of nigral α-synuclein expression and oxidative stress may be a potential therapeutic for PD.

Brain-derived neurotrophic factor (BDNF) and its receptor tropomyosin-related kinase receptor type B (TrkB) are actively produced and trafficked in multiple regions in the brain, where they influence neuronal activity, survival, synaptic plasticity, and neurogenesis throughout life [16,17]. Given its neurotrophic actions on neuronal populations, BDNF is of particular therapeutic interest involved in neurodegenerative diseases including PD. Indeed, BDNF treatment prevents the loss of dopaminergic neurons induced by MPTP in the SN of rats [18]. Furthermore, intrathecal infusion of BDNF exhibits beneficial anatomical and behavioral effects in MPTP-induced parkinsonism in monkeys, by reducing nigral cell loss and enhancing striatal reinnervation [19].

Unfortunately, the key challenge in the BDNF therapy is drug delivery to the central nervous system, as it is a moderately sized and charged protein, and only minimal amount of BDNF crosses the blood–brain barrier via peripheral administration [20].

Recently, 7,8-dihydroxyflavone (DHF) has been identified as a small-molecule compound that binds with high affinity and specificity to the TrkB receptor. As a flavone derivative, DHF has been shown to promote TrkB receptor dimerization and autophosphorylation and activate PI3K/Akt, MAPK, and Erk1/2 signaling cascades just as well as BDNF [21]. A more recent study has shown intervention with DHF blocks further loss of dopaminergic terminals and ameliorates motor deficits in the progressive MPTP mouse model [22]. As aforementioned, the loss of dopaminergic neurons in PD is partly due to the overexpression of α-synuclein and oxidative stress in these neurons, and we therefore propose that DHF may suppress α-synuclein and exert antioxidative activity, and subsequently reduce the loss of dopaminergic neurons, thereby rescuing motor deficits. In this study, we investigated this hypothesis using behavioral, immunohistochemical, and Western blot assessments in the MPTP-induced mouse model of PD.

Materials and Methods

C57BL/6 mice (25–30 g) were obtained from Charles River (Beijing Office, China) and maintained at Children's Hospital of Chongqing Medical University Animal Care Centre. Animals were housed in plastic cages with free access to food and water and maintained in a temperature-controlled room (21°C) with a 12-h/12-h light/dark cycle. All experiments and procedures were approved by Chongqing Medical University Animal Care and Use Committee, and every effort was made to minimize both the animal suffering and the number of animals used.

Drugs and Treatments

MPTP was purchased from Sigma-Aldrich (St. Louis, MO, USA) and dissolved in sterile saline. DHF was purchased from Tokyo Chemical Industry (Tokyo, Japan) and dissolved in sterile saline containing 10% ethanol. Anti-α-synuclein and anti-tyrosine hydroxylase (TH) antibodies were obtained from BD Transduction Laboratories. Anti-TrkB antibody was obtained from Cell Signaling. Anti-phospho-TrkB (p-TrkB) antibody was obtained from Santa Cruz. Anti-β-actin antibody was obtained from Abcam. Glutathione (GSH) and superoxide dismutase (SOD) test Kits were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China).

Mice received intraperitoneal (i.p.) injection of 30 mg/kg parkinsonian toxin MPTP hydrochloride once a day for 5 days to induce dopaminergic neuron death in the substantia nigra, while the control mice received equal amount of saline injection. DHF (5 mg/kg, i.p.) was injected into the MPTP-treated mice once a day from the first day of MPTP injection until 9 days after the last injection of MPTP (Figure 1).

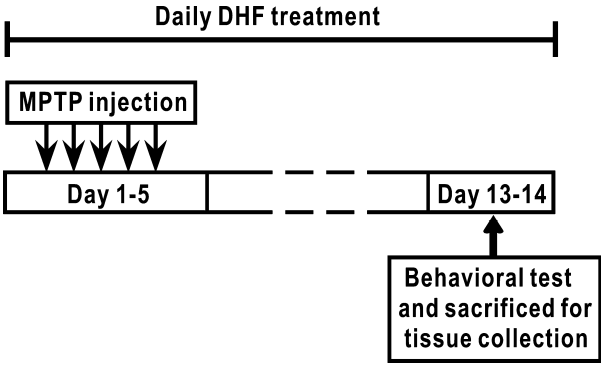

Figure 1 Experimental design. Mice received a chronic MPTP treatment (30 mg/kg per day, i.p.) or the same volume of sterile saline for 5 days, and DHF (5 mg/kg, i.p.) was administrated daily throughout the experiment. Seven days after the last injection of MPTP, behavioral tests were performed, and brain tissues were collected immediately after behavioral tests.

Rotarod Test

The rotarod test was performed as previously described, with modifications [23]. In brief, 1 week after the last MPTP injection, mice received 4 rounds of training on the rotarod (Stoelting Co.). In the first two rounds of training, the rotarod was maintained at constant speed of 20 rpm for 3 min. In the second two rounds of training, the rotarod reversed rotation direction every 3 turns at the constant speed of 20 rpm for 3 min. Twenty-four hours after the last round of training, mice received formal rotarod testing in which the rotarod reversed rotation direction every 3 turns at the constant speed of 20 rpm. Mice were tested 10 times at 20-min intervals, and the time that they remained on the rotarod during each test was recorded. Maximum test time (cut-off limit) was 300 s. The motor performance of the mouse was expressed as the latency to fall off the rotarod. The rotarod was cleaned with 70% ethanol and water between tests.

Pole Test

The pole consisted of a thin wooden cylinder (length: 50 cm, diameter: 1.5 cm) and a cross-shaped wooden base placed in a clean cage. Rubber bands were wrapped around the cylinder at intervals of approximately 1.5 inches to increase traction. Mice were placed at the top of the pole facing downwards, and latency to descend the pole was measured. Trials were excluded if the mouse jumped or slid down the pole rather than climbed down. On the first day, each mouse was trained with two trials. On the second day, each mouse was given five trials before the rotarod test and the lowest latency to descend the pole was analyzed. The pole was cleaned with 70% ethanol and water between tests.

Wire Suspension Test

The wire (length: 80 cm, height: 25 cm) was fixed horizontally between two platforms. Each animal was hung with its paws on the middle of the wire, and the time needed to reach one platform was recorded. The maximal time allowed was set at 120 s. On the first day, each mouse was trained with two trials. On the second day, each mouse was given five trials before the rotarod test and the lowest latency to reach the platform was analyzed.

Western Immunoblotting

Brain tissues were lysed on ice in the lysis buffer, and then the solution was centrifuged at 18,000 × ***g*** for 10 min at 4°C. Supernatant was collected, and protein concentration was determined by BCA protein assay kit (Thermo Fisher Scientific, Waltham, MA, USA). Equal amount of protein samples was mixed with 4 × sample buffer, boiled at 95°C for 5 min, and separated on 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were then transferred to Immobilon-PTM polyvinylidene fluoride (PVDF) membranes (Bio-Rad, Hercules, CA, USA). The membranes were blocked with 5% nonfat milk in Tris-buffered saline containing 0.1% Tween-20 (TBST) for 1 h at room temperature and then incubated overnight at 4°C with primary antibody. After washing 3 × 5 min in TBST, membranes were incubated with horseradish peroxidase-conjugated secondary antibody for one hour at room temperature. After another three washes with TBST, protein was visualized in the Bio-Rad Imager using ECL Western blotting substrate (Pierce). Immunoblotting with anti-β-actin was used to control equal loading and protein quality. The band intensity of each protein was quantified by the Bio-Rad Quantity One software.

TH Staining and Quantification

Mice were deeply anesthetized with urethane (1.5 g/kg, i.p.) and then perfused with 0.9% saline and 4% paraformaldehyde. Brains were taken out and fixed in 4% paraformaldehyde for one more day before they were transferred to 30% sucrose/PBS solution for cryoprotection. After the brains had sunk to the bottom of sucrose solution, they were sectioned into 30-μm slices using Leica cryostat. After blocking and permeabilization using PBS solution containing 1% BSA and 0.2% Triton X-100 for 30 min at room temperature, the brain slices were incubated with anti-tyrosine hydroxylase antibody (1:800 dilution) for 24 hours at 4 degree. Finally, the tyrosine hydroxylase-positive neurons on the slices were stained and visualized under light microscope using the anti-mouse Ig HRP detection kit according to the manufacturer's instruction.

The number of TH-positive cells was quantified in brain sections with typical morphology of the SN, as described previously [24]. Briefly, the number of TH-positive neurons was counted manually at six-section intervals throughout the entire extent of SN by bright-field microscopy using ImageJ software. To quantify changes in the number of TH-positive neurons in the SN, the number of TH-positive neurons in control mice was normalized to 100%, and the number of TH-positive neurons in other groups was expressed as a percentage of the control.

The optical density of the TH-positive neuronal terminal staining in the mouse dorsolateral striatum was quantified using NIH ImageJ software. The optical density from the overlying corpus callosum was used as a background and subtracted from every measurement in the striatum [25]. The optical density in the experimental group was normalized to the value from the control group.

Antioxidative Activity Analysis

Brain tissues of SN or striatum were homogenized with PBS or SOD detection buffer, followed by centrifugation at 4000 g for 10 min at 4°C. The supernatant was used to test glutathione (GSH) level and total superoxide dismutase (SOD) activity using dithio-binitrobenzoic acid method and xanthine/xanthine oxidase method, respectively. Reaction product was determined by microplate reader (BioTek Instruments).

Statistical Analysis

All data were expressed as mean ± SEM. The differences of rotarod test among different groups of mice were analyzed by two-way ANOVA, followed by Tukey's post hoc test. The data of all other experiments were analyzed by one-way ANOVA, followed by Tukey's post hoc test. Significance level was set at $P < 0.05$.

Results

DHF Ameliorates MPTP-induced Motor Deficits

It has been well documented that a core symptom of PD is motor abnormality [26,27], and a recent study shows that the intervention with DHF ameliorates MPTP-induced motor deficit in gait analysis [22]. To further determine whether DHF can rescue MPTP-induced motor deficits, we here introduced another 3 behavioral tasks: rotarod test, pole test and wire suspension test. In rotarod test, the mice treated with MPTP spent significantly less time on the rod during test compared with those treated with saline (Figure 2A), indicating an impairment of motor balance and coordination. Importantly, daily DHF treatment (5 mg/kg, i.p.) fully rescued the MPTP-induced motor deficit, as reflected by a dramatic increase in the latency to fall off (saline: n = 16; DHF: n = 16; MPTP + saline: n = 16; MPTP + DHF: n = 18; Figure 2A). In pole test, the mice treated with MPTP spent much more time descending the pole compared with those treated with saline, whereas DHF treatment significantly shortened the descending time during test (saline: n = 16, 6.1 ± 0.5 s; DHF: n = 16, 5.8 ± 0.4 s, $P > 0.05$ vs. saline; MPTP + saline: n = 16, 7.6 ± 0.3 s, $P < 0.05$ vs. saline, $P < 0.01$ vs. DHF; MPTP + DHF: n = 18, 5.9 ± 0.2 s, $P > 0.05$ vs. saline, $P < 0.01$ vs. MPTP + saline; Figure 2B). Similarly, the mice treated with MPTP spent much more time reaching the platform during the wire suspension test, and DHF treatment ameliorated the motor capacity to control levels, as reflected by a similar time in reaching the platform (saline: n = 16, 10.5 ± 0.7 s; DHF: n = 16, 10.4 ± 0.9 s, $P > 0.05$ vs. saline; MPTP + saline: n = 16, 14.9 ± 1.0 s, $P < 0.01$ vs. saline, $P < 0.01$ vs. DHF; MPTP + DHF: n = 18, 11.3 ± 0.8 s, $P > 0.05$ vs. saline, $P < 0.05$ vs. MPTP + saline; Figure 2C).

DHF Reduces MPTP-induced Loss of Dopaminergic Neurons

To confirm the protective effects of DHF on dopaminergic neurons after MPTP insult, we examined the level of TH-positive neurons in the SN and neuronal terminals in the striatum. The results showed that the MPTP administration markedly decreased the number of TH-positive neurons in the SN compared with saline control, and DHF treatment significantly blocked the loss of TH-positive neurons (saline: n = 7; DHF: n = 7, 104.7 ± 2.5%

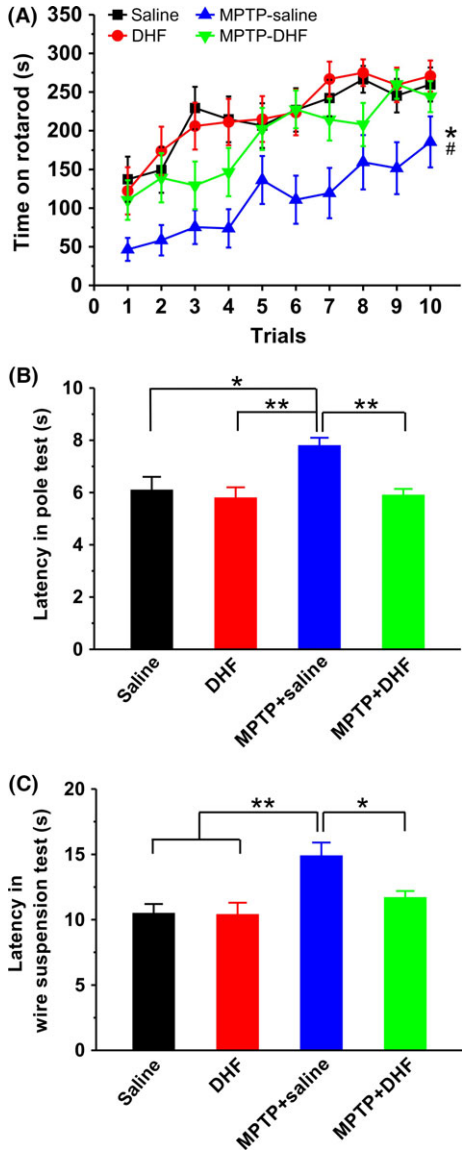

Figure 2 Behavioral analysis of motor functions. (**A**) Latency to fall off the rod in the rotarod test was significantly reduced by MPTP administration, and DHF treatment restored the latency to control level. Two-way ANOVA was used in this experiment (group: $F_{3,\ 620} = 70.111$, $P < 0.001$; trial: $F_{9,\ 620} = 22.352$, $P < 0.001$; group × trial, $F_{27,\ 620} = 0.491$, $P = 0.987$). (**B**) Latency to descend in the pole test was markedly increased by MPTP administration, and DHF treatment restored the latency to control level. One-way ANOVA was used in this experiment ($F_{3,\ 62} = 6.366$, $P = 0.001$). (**C**) Latency to reach the platform in the wire suspension test was significantly increased by MPTP administration, and DHF treatment restored the latency to control level. One-way ANOVA was used in this experiment ($F_{3,\ 62} = 5.702$, $P = 0.002$). Data are expressed as mean ± SEM, *$P < 0.05$, **$P < 0.01$.

saline, $P > 0.05$ vs. saline; MPTP + saline: n = 7, 61.2 ± 2.8% saline, $P < 0.01$ vs. saline; MPTP + DHF: n = 7, 90.9 ± 4.0% saline, $P < 0.05$ vs. saline, $P < 0.01$ vs. MPTP + saline; Figure 3A). Similarly, MPTP administration significantly decreased the neuronal terminals in the striatum, as reflected by a dramatic decrease in the optical density of TH, whereas DHF treatment significantly ameliorated it although the optical density of TH is still lower than control level (saline: n = 7; DHF: n = 7, 106.4 ± 10.8% saline, $P > 0.05$ vs. saline; MPTP + saline: n = 7, 33.0 ± 6.2% saline, $P < 0.01$ vs. saline; MPTP + DHF: n = 7, 70.9 ± 7.9% saline, $P < 0.05$ vs. saline, $P < 0.01$ vs. MPTP + saline; Figure 3B).

DHF Rescues the Decrease in MPTP-induced TH Expression

To further determine the protective effects of DHF on dopaminergic neurons, we next examine the TH expression in the SN and striatum using Western immunoblotting. The results showed that the MPTP administration significantly decreased TH expression in the SN (saline: n = 10; MPTP + saline: n = 10, 62.9 ± 7.3% saline, $P < 0.01$ vs. saline; Figure 4A) and striatum (saline: n = 10; MPTP + saline: n = 10, 56.8 ± 5.5% saline, $P < 0.01$ vs. saline; Figure 4B) compared with saline control. Consistent with recent reports [21,22], DHF treatment fully rescued the decrease in TH expression in both the SN (DHF: n = 10, 114.6 ± 7.7% saline, $P > 0.05$ vs. saline; MPTP + DHF: n = 10, 91.0 ± 8.4% saline, $P > 0.05$ vs. saline, $P < 0.05$ vs. MPTP + saline; Figure 4A) and striatum (DHF: n = 10, 113.1 ± 14.4% saline, $P > 0.05$ vs. saline; MPTP + DHF: n = 10, 94.7 ± 17.5% saline, $P > 0.05$ vs. saline, $P < 0.05$ vs. MPTP + saline; Figure 4B).

DHF Inhibits the MPTP-Induced α-synuclein Overexpression

Previous studies have reported that the loss of dopaminergic neurons in PD is associated with the overexpression of α-synuclein in the cytoplasm of these neurons [4–10]. We next wanted to determine the effects of DHF treatment on the expression of α-synuclein. As shown in Figure 5, MPTP administration significantly increased the expression of α-synuclein in both the SN (saline: n = 6; MPTP + saline: n = 6, 149.2 ± 18.2% saline, $P < 0.05$ vs. saline; Figure 5A) and striatum (saline: n = 6; MPTP + saline: n = 6, 144.3 ± 16.9% saline, $P < 0.05$ vs. saline; Figure 5B) compared with saline control. More importantly, DHF treatment reduced the expression of α-synuclein to control level in both the SN (DHF: n = 6, 85.5 ± 7.2% saline, $P > 0.05$ vs. saline; MPTP + DHF: n = 6, 112.5 ± 9.7% saline, $P > 0.05$ vs. saline, $P < 0.05$ vs. MPTP + saline; Figure 5A) and striatum (DHF: n = 6, 100.8 ± 2.5% saline, $P > 0.05$ vs. saline; MPTP + DHF: n = 6, 95.5 ± 7.7% saline, $P > 0.05$ vs. saline, $P < 0.01$ vs. MPTP + saline; Figure 5B).

DHF Reverses MPTP-induced Decrease in p-TrkB

Previous study has shown that DHF exerts neuroprotective effects by acting as a selective TrkB agonist [21]. There are two forms of TrkB exist, full-length isoform (TrkB-FL) and a truncated protein lacking the tyrosine-kinase domain, which is strikingly similar to the inactive TrkB-T1 isoform (TrkB-T1). Thus, we next examined the effects of DHF on all TrkB isoforms (panTrkB, including TrkB-FL and TrkB-T1) and p-TrkB expression. The results showed that both MPTP and DHF had no effect on the expression of panTrkB in

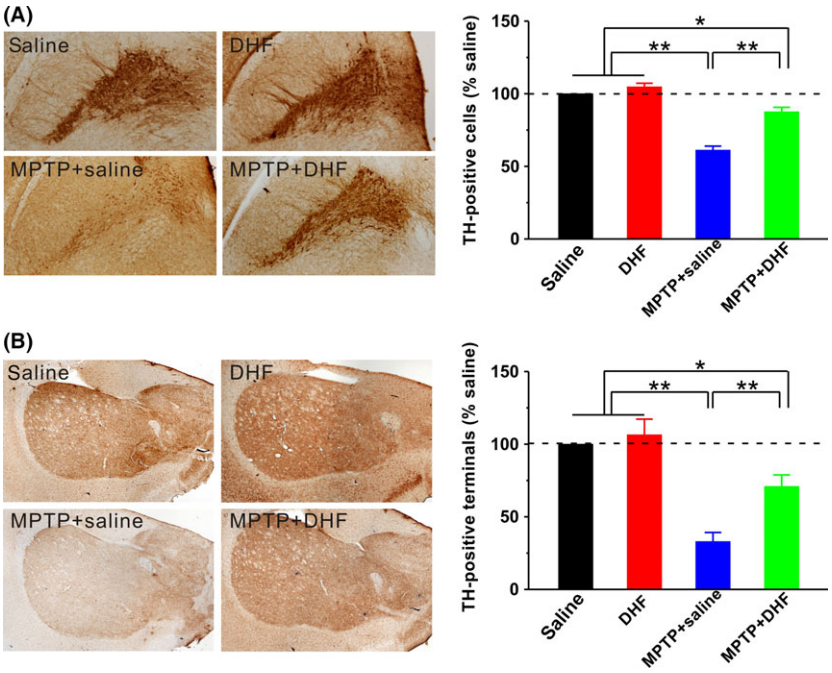

Figure 3 Immunohistochemical analysis of TH-positive neurons. Representative photomicrographs on the left panels, along with bar graph summarizing group data on the right panel. The number of dopaminergic neurons in the SN (**A**) and neuronal terminals in the striatum (**B**) was significantly decreased by MPTP administration, and DHF treatment significantly restored it although the number is still less than control level. One-way ANOVA was used in this experiment (SN: $F_{3, 24} = 87.347$, $P < 0.001$; striatum: $F_{3, 24} = 20.614$, $P < 0.001$). Data are expressed as mean ± SEM, *$P < 0.05$, **$P < 0.01$.

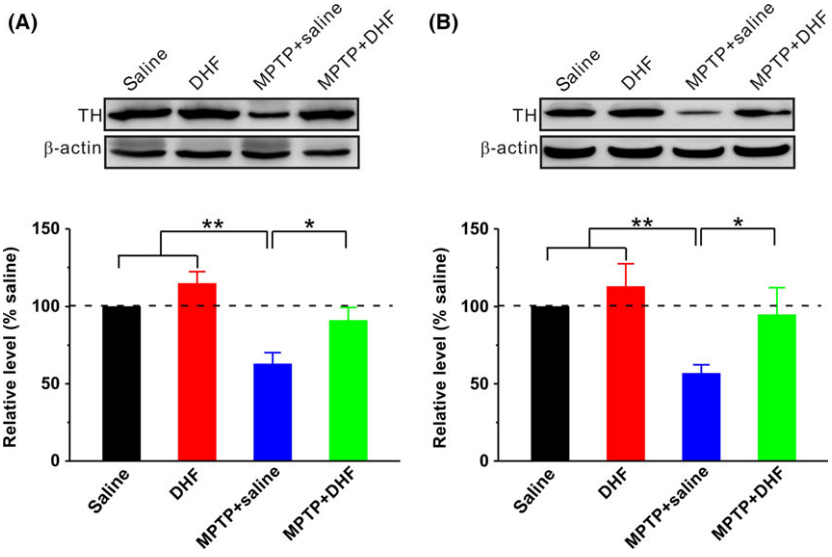

Figure 4 Western blot analysis of TH. Representative blots on the upper panels, along with bar graph summarizing group data on the bottom panel. MPTP administration significantly decreased TH expression in the SN (**A**) and striatum (**B**), and DHF treatment restored the expression of TH to control level. One-way ANOVA was used in this experiment (SN: $F_{3, 36} = 10.347$, $P < 0.001$; striatum: $F_{3, 36} = 4.294$, $P = 0.011$). Data are expressed as mean ± SEM, *$P < 0.05$, **$P < 0.01$.

the SN (saline: n = 8; DHF: n = 8, 102.4 ± 9.5% saline, $P > 0.05$ vs. saline; MPTP + saline: n = 8, 99.7 ± 6.3% saline, $P > 0.05$ vs. saline; MPTP + DHF: n = 8, 104.0 ± 8.7% saline, $P > 0.05$ vs. saline, $P > 0.05$ vs. MPTP + saline; Figure 6A and C) and striatum (saline: n = 8; DHF: n = 8, 104.7 ± 7.7% saline, $P > 0.05$ vs. saline; MPTP + saline: n = 8, 95.6 ± 8.2% saline, $P > 0.05$ vs. saline; MPTP + DHF: n = 8, 96.2 ± 7.3% saline, $P > 0.05$ vs. saline, $P > 0.05$ vs. MPTP + saline; Figure 6B and D). However, the expression of p-TrkB dramatically decreased after MPTP treatment in the SN (saline: n = 8; MPTP + saline: n = 8, 66.5 ± 3.2% saline, $P < 0.05$ vs. saline; Figure 6A and E) and striatum (saline: n = 8; MPTP + saline: n = 8, 68.9 ± 10.1% saline, $P < 0.05$ vs. saline; Figure 6B and F). As expected, DHF treatment reversed the expression of p-TrkB in the SN (DHF: n = 8, 169.2 ± 16.1% saline, $P < 0.05$ vs. saline; MPTP + DHF: n = 8, 97.1 ± 5.7% saline, $P > 0.05$ vs. saline, $P < 0.05$ vs. MPTP + saline; Figure 6A and E) and striatum (DHF: n = 8, 131.92 ± 10.8% saline, $P < 0.05$ vs. saline; MPTP + DHF: n = 8, 107.2 ± 7.6% saline, $P > 0.05$ vs. saline, $P < 0.01$ vs. MPTP + saline; Figure 6B and F). These results suggest that DHF induces the TrkB activation in MPTP-treated mice.

DHF Reverses MPTP-induced Decrease in GSH and SOD

In addition, it has been well documented that MPTP destroys the defense of antioxidant enzymes in mice brain [28], whereas DHF

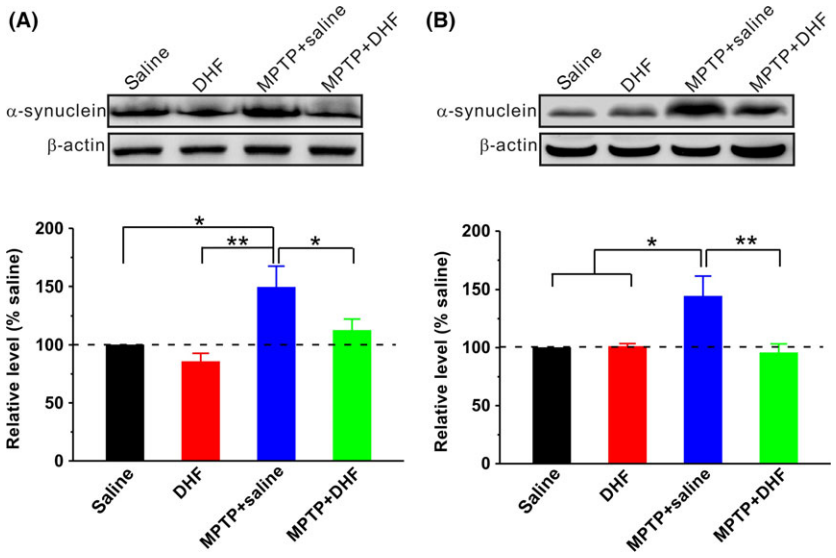

Figure 5 Western blot analysis of α-synuclein. Representative blots on the upper panels, along with bar graph summarizing group data on the bottom panel. MPTP administration significantly increased α-synuclein expression in the SN (**A**) and striatum (**B**), and DHF treatment restored the expression of α-synuclein to control level. One-way ANOVA was used in this experiment (SN: $F_{3, 20} = 6.258$, $P = 0.004$; striatum: $F_{3, 20} = 5.944$, $P = 0.005$). Data are expressed as mean ± SEM, $^{*}P < 0.05$, $^{**}P < 0.01$.

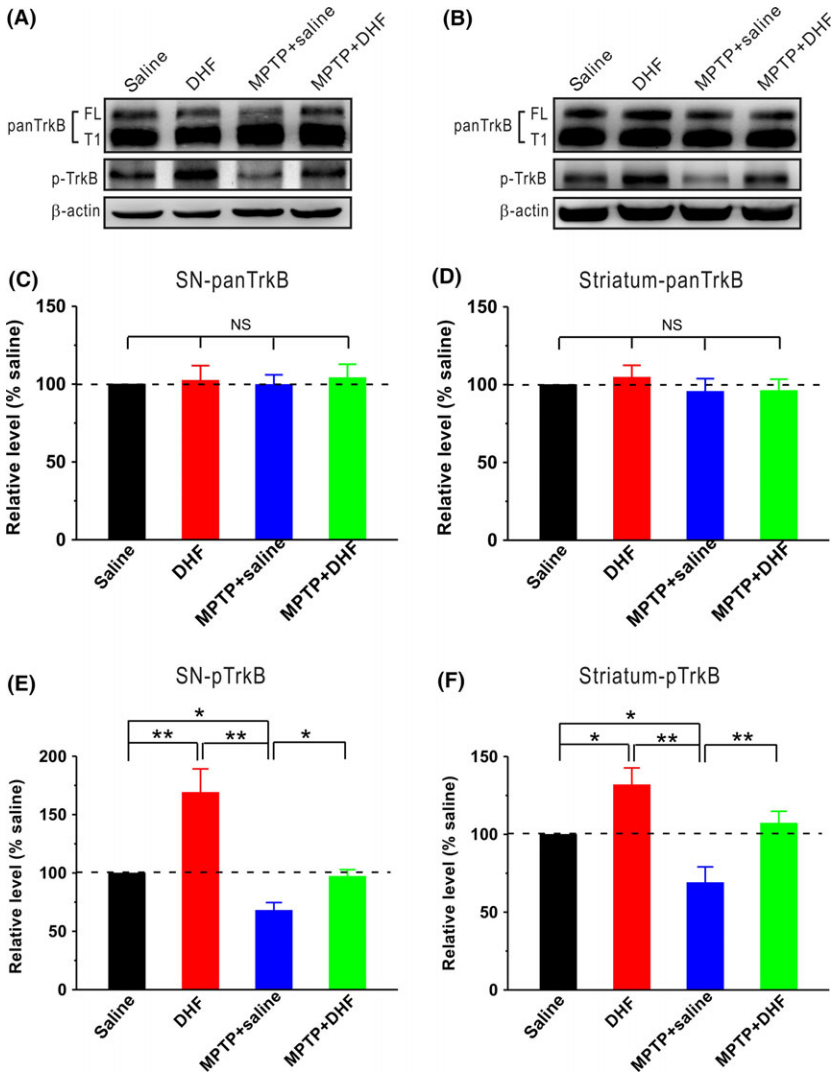

Figure 6 Western blot analysis of TrkB activation. (**A**–**B**) Representative blots showed the expressions of panTrkB and p-TrkB in the SN (**A**) and striatum (**B**). (**C**–**D**) MPTP and DHF had no effect on panTrkB expression in the SN (**C**) and striatum (**D**) compared with control. One-way ANOVA was used in this experiment (SN: $F_{3, 28} = 0.084$, $P = 0.968$; striatum: $F_{3, 28} = 0.387$, $P = 0.763$). (**E**–**F**) p-TrkB expression was dramatically reduced in the SN (**E**) and striatum (**F**) following MPTP treatment, and DHF treatment restored the expression of p-TrkB to control level (SN: $F_{3, 28} = 12.819$, $P < 0.001$; striatum: $F_{3, 28} = 9.764$, $P < 0.001$). Data are expressed as mean ± SEM, $^{*}P < 0.05$, $^{**}P < 0.01$.

Table 1 Effect of DHF on GSH level (n = 8 in each group) and SOD activity (n = 6 in each group) in the SN and striatum.

	GSH level (mg/g protein)		SOD activity (U/mg protein)	
Groups	SN	striatum	SN	striatum
Saline	14.98 ± 1.09	19.73 ± 0.97	36.17 ± 1.84	42.27 ± 1.58
DHF	19.60 ± 1.77	22.31 ± 0.84	40.25 ± 1.23	43.42 ± 1.68
MPTP + saline	6.05 ± 0.91[abc]	15.02 ± 0.43[abc]	27.41 ± 1.82 [abc]	29.51 ± 1.61 [abc]
MPTP + DHF	18.33 ± 2.03	19.96 ± 1.08	35.12 ± 1.89	37.81 ± 1.21

Data are expressed as mean ± SEM, [a]$P < 0.05$ vs. saline, [b]$P < 0.05$ vs. DHF, [c]$P < 0.05$ vs. MPTP + DHF.

has been shown to exert antioxidant action in *in vitro* model of PD [29]. We therefore examined whether DHF can suppress MPTP-induced oxidative stress *in vivo*. GSH was an important antioxidant, and SOD was a critical antioxidant enzyme in the body. As shown in Table 1, MPTP treatment significantly led to a decrease in GSH and SOD activity. Compared with saline treatment, DHF dramatically rescued the GSH and SOD activity following MPTP treatment in the SN and striatum (Table 1). These results indicated a powerful antioxidative capacity of DHF in MPTP-treated mice.

Discussion

In the present study, we confirm that DHF, a TrkB agonist, blocks the loss of dopaminergic neurons in the SN and neuronal terminals in the striatum and ameliorates motor deficits in different behavioral paradigms in the MPTP-induced mouse model of PD. We further demonstrate that the beneficial effects of DHF on PD are associated with the inhibition of α-synuclein expression and the activation of TrkB, as well as antioxidative activity in the SN and striatum. We have therefore provided evidence that DHF may suppress MPTP-induced toxic effects of α-synuclein overexpression and oxidative stress via activating TrkB and subsequently block the loss of dopaminergic neurons, thereby ameliorating behavioral decline.

The pathological hallmark of PD is a selective and progressive loss of dopaminergic neurons in the SN [1–3]. It has been reported that BDNF promotes neuronal survival and differentiation and modulates synaptic plasticity by activating the TrkB receptor tyrosine kinase [30]. In contrast, results obtained from genetic studies have shown that the deletion of either BDNF or TrkB in excitatory neurons causes dendritic degeneration and neuronal loss [31,32]. In addition, postmortem studies show that the level of BDNF is markedly reduced in the SN of PD patients [33–35], whereas the application of exogenous BDNF promotes the survival of dopaminergic neurons in PD animal models [19,36]. However, BDNF is difficult to use for PD treatment in clinic as mature BDNF does not cross the blood–brain barrier. Recent reports show that DHF as a bioactive high-affinity TrkB agonist exerts neuroprotective effects in animal model of PD [21,22]. In full agreement with these findings, we here reported that systemic treatment with DHF dramatically increased the TrkB activation following MPTP treatment (Figure 6) and subsequently reduced MPTP-induced loss of dopaminergic neurons in the SN and neuronal terminals in the striatum (Figures 3 and 4), and further behavioral experiments showed that DHF significantly ameliorated motor functions in different behavioral paradigms (Figure 2).

Besides the loss of dopaminergic neurons in the SN, another pathological change in PD is the overexpression of α-synuclein, especially main pathologic form of phosphorylated and insoluble α-synuclein [37,38]. We here reported that repeated MPTP application dramatically increased the expression of α-synuclein in the SN and striatum (Figure 5), which may contribute to the deficits of motor function. Indeed, this hypothesis is supported by some previous reports. For example, chronic MPTP treatment can induce α-synuclein oligomerization and such oligomers produce most pathological actions of α-synuclein, including mediating neuronal death and motor dysfunction [39,40]. In addition, MPTP application markedly increases α-synuclein expression in the animal model of PD [41], and the overexpression of α-synuclein causes oxidative stress and mitochondrial structural abnormalities [42]. Importantly, we here also found that chronic DHF treatment significantly inhibited MPTP-induced α-synuclein overexpression, which may reduce oxidative stress and mitochondrial dysfunction, thereby contributing to the amelioration of motor function. Indeed, we here found that DHF succeeded in reversing the decrease in the antioxidant GSH and antioxidant enzyme SOD in MPTP-treated mice (Table 1). These results were in agreement with previous reports that oxidative stress-mediated neuronal cell death may be associated with PD [43]. Although a large number of reports have shown that MPTP/MPP^+ administration causes cell death *in vitro* and *in vivo* [44–46], Aznavour and colleagues have recently reported that MPTP (5 mg/kg i.p. during 5 days) destroys TH protein rather than dopamine cells in a cat model of PD [47]. These contradictory results may be caused in part by different species and/or different doses of MPTP, which were used in these studies. For example, previous study has shown that the percentage cell death at 60 mg/kg of total MPTP used was lower than at 80 mg/kg in mice [46]. Although possible explanation for this controversy has been provided by previous research, further studies are required to determine the exact reason for the different results. Thus, future experiments examining mitochondrial function and neuronal cell death in MPTP-treated animals with or without DHF treatment will help determine whether DHF's therapeutic effect in the MPTP-induced mouse model of PD can be attributed to its amelioration of mitochondrial function and inhibition of cell death.

Conclusion

In summary, our study shows that DHF treatment suppresses MPTP-induced α-synuclein expression and oxidative stress, and then reduces the loss of dopaminergic neurons in the SN and striatum, thereby ameliorating motor deficits in a murine model of PD.

Conflict of Interest

The authors state no conflict of interest.

Acknowledgments

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