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Fibroblast growth factor 9 prevents MPP^+-induced death of dopaminergic neurons and is involved in melatonin neuroprotection *in vivo* and *in vitro*

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Abstract

Oxidative stress and down-regulated trophic factors are involved in the pathogenesis of nigrostriatal dopamine(DA)rgic neurodegeneration in Parkinson's disease. Fibroblast growth factor 9 (FGF9) is a survival factor for various cell types; however, the effect of FGF9 on DA neurons has not been studied. The antioxidant melatonin protects DA neurons against neurotoxicity. We used MPP^+ to induce neuron death *in vivo* and *in vitro* and investigated the involvement of FGF9 in MPP^+ intoxication and melatonin protection. We found that MPP^+ in a dose- and time-dependent manner inhibited FGF9 mRNA and protein expression, and caused death in primary cortical neurons. Treating neurons in the substantia nigra and mesencephalic cell cultures with FGF9 protein inhibited the MPP^+-induced cell death of DA neurons. Melatonin co-treatment attenuated MPP^+-induced FGF9 down-regulation and DA neuronal apoptosis *in vivo* and *in vitro*. Co-treating DA neurons with melatonin and FGF9-neutralizing antibody prevented the protective effect of melatonin. In the absence of MPP^+, the treatment of FGF9-neutralizing antibody-induced DA neuronal apoptosis whereas FGF9 protein reduced it indicating that endogenous FGF9 is a survival factor for DA neurons. We conclude that MPP^+ down-regulates FGF9 expression to cause DA neuron death and that the prevention of FGF9 down-regulation is involved in melatonin-provided neuroprotection.

Keywords: dopaminergic neurons, fibroblast growth factor, melatonin, MPP^+, neuroprotection, Parkinson's disease.

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Accumulated evidence (Dauer and Przedborski 2003; Dawson and Dawson 2003) suggests that mitochondrial dysfunction and oxidative stress are involved in the pathogenesis of the nigrostriatal loss of dopaminergic neurons in Parkinson's disease (PD). Mitochondrial inhibitors of MPP^+ and rotenone are extensively used to selectively kill nigrostriatal dopaminergic neurons and generate PD models in rodents (Dawson and Dawson 2003; Lin *et al.* 2008). MPP^+-treated animals and neurons lose endogenous antioxidant glutathione and overproduce reactive oxygen species (Chen *et al.* 2002; Chuang and Chen 2004; Chinta and Andersen 2008). In addition, the expression of brain-derived neurotrophic growth factor (BDNF) and glial cell line-derived neurotrophic factor (GDNF) was significantly lower in the substantia nigra of persons with PD and dopamine (DA) neurons in MPP^+-treated animals (Howells *et al.* 2000; Chauhan *et al.* 2001; Collier *et al.* 2005). Direct administration of GDNF or BDNF to the substantia nigra prevents the toxin-induced death of dopaminergic neurons (Kirik *et al.* 2004; Sun *et al.* 2005). In contrast, inhibiting BDNF by antisense oligonucleotide infusion (Porritt *et al.* 2005) and conditional GDNF knock out (Pascual *et al.* 2008) resulted in the death of nigral dopaminergic neurons. These findings suggest that the down-regulation of trophic factors is also involved in the pathogenesis of PD.

Fibroblast growth factors (FGFs) and their receptors constitute a complex signaling system that participates in cell proliferation, differentiation, and survival (Reuss and

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Abbreviations used: BDNF, brain-derived neurotrophic growth factor; BSA, bovine serum albumin; DA, dopamine; FGF, fibroblast growth factor; GDNF, glial cell line-derived neurotrophic factor; MAP2, microtubule associated protein 2; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PBS, phosphate-buffered saline; PD, Parkinson's disease; TH, tyrosine hydroxylase; TH-ir, TH-immunoreactive.

von Bohlen und Halbach 2003). Of the 23 FGF family members, seven have been identified in the substantia nigra (Todo *et al.* 1998; Ohmachi *et al.* 2000; Dono 2003). FGF2 which is widely expressed in the brain, and FGF20 which is especially expressed in the substantia nigra, are important for the survival of dopaminergic neurons (Ohmachi *et al.* 2003; Hsuan *et al.* 2006; Murase and McKay 2006). Furthermore, FGF9 is expressed by neurons and glial cells to support the survival of neurons as well as to increase glial cell proliferation in an autocrine and/or paracrine manner, (Tagashira *et al.* 1995; Pataky *et al.* 2000). Astrocytes in the brains of persons with Alzheimer's disease expressed higher than normal levels of FGF9 (Nakamura *et al.* 1998). However, the involvement of FGF9 in PD has not yet been investigated.

Melatonin (5-methoxy-*N*-acetyltryptamine), a neurohormone, is produced in the pineal gland and nocturnally secreted. Reports have shown that melatonin acts as an antioxidant and scavenges radicals at the mitochondria level to protect cells from oxidative insults (Chen *et al.* 2002; Tan *et al.* 2002; Chuang and Chen 2004; Jou *et al.* 2007; Reiter *et al.* 2008). Moreover, melatonin may up-regulate the expression of GDNF and BDNF genes to promote neuronal survival and protect neurons from oxidative insults (Tang *et al.* 1998; Chen *et al.* 2003; Lee *et al.* 2006; Imbesi *et al.* 2008; Kong *et al.* 2008). However, whether melatonin modulates FGF9 expression to protect dopaminergic neurons from MPP^+ toxicity is still unknown.

To address this issue, we examined, *in vivo* and *in vitro*, the effect of MPP^+ on FGF9 expression and the role of FGF9 in MPP^+-induced neurotoxicity and melatonin-mediated neuroprotection. We showed that MPP^+ down-regulated FGF9 expression which caused apoptosis in neurons, and that FGF9 protected neurons against MPP^+ intoxication and was involved in the protection afforded by melatonin.

Materials and methods

Chemicals and antibodies

All reagents and chemicals were purchased from Sigma-Aldrich Co. (St Louis, MO, USA) unless otherwise stated. Mouse anti-microtubule associated protein 2 (MAP2) and rabbit anti-glial fibrillary acidic protein were purchased from Millipore (Billerica, MA, USA); mouse anti-tyrosine hydroxylase (TH) antibody was purchased from Sigma-Aldrich Co. Goat anti-FGF9 antibody was purchased from R & D Systems, (Minneapolis, MN, USA). All Alexa Fluor series conjugated secondary antibodies were purchased from Invitrogen Corp. (Carlsbad, CA, USA).

Experimental animals and treatments

Adult male Wistar rats (weight: 260–320 g; age: 8 weeks) from the Animal Resource center at the National Cheng Kung University Medical College were the test animals. The rats were housed separately at 24°C with a 12/12-h light/dark cycle; food and water were provided *ad libitum*. The protocols for this study were approved by the Animal Care and Use Committee of National Cheng Kung University and were in accord with national guidelines for the care and use of laboratory animals.

The rats were intraperitoneally (i.p.) anesthetized with sodium pentobarbital (43 mg/kg) and mounted on a stereotaxic apparatus (David Kopf Instruments, Tujunga, CA, USA). An intrastriatal microinjection was made through a 28-gauge stainless steel cannula connected to a 10-μL microsyringe (Hamilton Co., Reno, NV, USA). The coordinates for the striatum were: anterior-posterior, 1 mm from the bregma; mesolateral, −3.5 mm from the midline; and dorsoventral, −5.5 mm below the skull surface. One microliter of 50 mM MPP^+ or vehicle solution [phosphate-buffered saline (PBS)] was infused into the right striatum, and then melatonin (10 mg/mL, i.p.) was injected 0, 1, 2, 3, 4, 24, 48, and 71 h later. Recombinant FGF9 protein was infused using a cannula guide and needle (C313 and C313I; Plastics One, Roanoke, VA, USA) implanted into two sites above the sbstantia nigra. The stereotaxic coordinates of the substantia nigra were: AP, −0.53 mm from the bregma; ML, ±0.22 mm from the midline; and DV, −0.62 mm below the skull surface. FGF9 (100 ng/μL/day) was infused using an infusion pump (Harvard Apparatus, Holliston, MA, USA) at a constant flow rate (0.1 μL/min) for 10 min on three consecutive days. The vehicle solution for FGF9 was 1% bovine serum albumin (BSA) in PBS. One hour after the final melatonin or FGF9 injection, the rats were decapitated and their striatums and substantia nigras were collected for the assays of FGF9 mRNA and protein expression. Part of rats were perfused and fixed for histological examination using immunohistochemistry.

Primary cortical and mesencephalic neuron culture

We removed the cerebral cortex from 16.5-day-old and mesencephalon from 14.5-day-old Sprague–Dawley rat embryos and seeded the cells ($8 \times 10^5/cm^2$) on culture dishes pre-coated with poly-D-lysine (50 μg/mL), and maintained in Dulbecco's modified Eagle's medium/Ham's F12 1 : 1 (Dulbecco's modified Eagle's medium/F12) supplemented with 2% B27 supplement (Invitrogen), 200 U/mL of penicillin, and 100 μg/mL of streptomycin. The cultures were maintained at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. Half of the medium was replaced 3 days after the cells were plated. Six-day-old cultures underwent B27 withdrawal for 22–24 h and then were used for various treatments. Neurons and dopaminergic neurons were identified by immunostaining with anti-MAP2 and anti-TH antibody, respectively.

Immunohistochemistry

The Wistar rats were perfused transcardially with 0.9% saline solution and then 4% paraformaldehyde. Their brains were dissected and coronal sections were collected using a cryostat (Thermo Shandon Ltd., Runcorn, Cheshire, UK). Ten-micrometer midbrain sections were reacted with anti-TH antibody or anti-FGF9 antibody. Avidin-biotin-peroxidase (ABC; Vector Laboratories, Burlingame, CA, USA) and glucose oxidase–nickel–diaminobenzidine methods were used to detect antibody. TH-positive (TH^+) dopaminergic neurons were counted as previously described (Lin *et al.* 2008). The percentage of TH^+ neuron loss was calculated using the following formula: $TH_{ip}/TH_c \times 100\%$, where TH_c is the number of TH^+

neurons in the contralateral substantia nigra and TH_{ip} is the number of TH^+ neurons in the ipsilateral substantia nigra.

Neuron viability assays

To determine cortical neuron viability, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was performed or the number of $MAP2^+$ cells in each of five randomly selected fields in each experiment was directly counted. MTT was converted to formazan in living cells. After treatments, cells were incubated with 0.25 mg/mL MTT for 4 h at 37°C and lysed in 5% (wt/vol) sodium dodecyl sulphate at 37°C for overnight. The optical density was determined at the absorbance of 540 nm. To detect mesencephalic dopaminergic neuron death, apoptotic TH^+ neurons with fragmented nuclei (visualized using Hoechst 33342 staining [Invitrogen]) were counted. Cells from primary cortical and mesencephalic cultures were fixed with 4% paraformaldehyde for 15 min and permeabilized with 0.025% (vol/vol) Triton X-100 in PBS. After blocking with 1% BSA in PBS, the cells were incubated with anti-MAP2 or TH antibody (1 : 1000) and then reacted with Alexa Fluor conjugated secondary antibody (1 : 1000) and Hoechst 33342 (0.5 μg/mL) for 1 h at 24°C. Images were captured using Olympus microscope BX61 and digital camera DP70 (Olympus, Tokyo, Japan).

Total RNA extraction and RT-PCR and real-time PCR

Total RNA was extracted from harvested cells using a reagent (Trizol; Invitrogen) and converted to cDNA using Moloney murine leukemia virus reverse transcriptase (Promega, Madison, WI, USA). The cDNA was reverse transcribed at 42°C for 90 min, heated to 95°C for 2 min, reacted with PCR mix solution [1 × PCR buffer, 1.5 mM of $MgCl_2$, 0.2 mM of dNTPs, 0.4 μM of primer, and 0.5 U of *Taq* polymerase (ABgene, Epsom, Surrey, UK)], and then amplified in a thermocycler (PX2; Thermo Scientific, Milford, MA, USA). FGF9 primers: 5′-AGGTGAAGTTGGGAGCTATT-3′; 5′-CCGTCCTTATTTAATGCAAC-3′ and glyceraldehyde-3-phosphate dehydrogenase primers: 5′-AGGTCGGTGTCAACGGATTT-3′; 5′-CAGCATCAAAGGTGGAGGAA-3′ were used. The PCR products were separated on a 5% acrylamide gel using 1× TBE buffer [0.09 M Tris, 0.09 M boric acid, and 0.001 M EDTA (pH 8.0)] and then stained with ethidium bromide. The gel image was analyzed using AlphaImager software (Alpha Innotech Corp., San Leandro, CA, USA).

For the real-time PCR reaction, cDNA was reacted with PCR mix solution [1× master mix (ABgene) and 0.4 μM of primer] (LightCycler Real-time PCR; Roche Diagnostics, Mannheim, Germany). FGF9 primers: 5′-CCAGGGAACCAGGAAAGAC-3′; 5′-CACTGTCCACACCACGAATG-3′ and 18S primers: 5′-GTTGGTTTTCGGAACTGAGGC-3′; 5′-GTCGGCATCGTTATG-GTCG-3′ were used. The cycling conditions were 95°C for 10 min, 50 cycles at 95°C for 15 s, and 50 cycles at 60°C for 1 min. The number-of-cycles threshold was converted to the copy number of the amplicon according to the standard curve.

Detecting FGF9 protein levels

For total protein extraction, cells and brain tissue were lysed and homogenized with modified RIPA buffer [50 mM of Tris-base (pH 7.4), 50 mM of NaCl, 1% Triton X-100, 0.1% sodium dodecyl sulphate, 1 mM of EDTA, 1% Na-deoxycholate, 1 mM of phenylmethylsulfonyl fluoride, 1 μg/mL of leupeptin, 1 μg/mL of aprotinin, 1 mM of Na_3VO_4, and 1 mM of NaF]. The supernatant solution was collected using centrifugation at 12 000 *g* for 15 min at 4°C, and then protein concentration was measured using a Bradford assay (Bio-Rad, Hercules, CA, USA). FGF9 protein concentration was detected using an ELISA analysis according to the manufacturer's instructions (R & D Systems).

Statistical analysis

The data were analyzed in GraphPad Prism 3.0 (GraphPad Software, San Diego, CA, USA). We used the unpaired Student's *t*-test to compare statistical significance between two independent groups. For multiple comparisons, we used one-way ANOVA and then the Newman–Keuls test. Statistical significance was set at $p < 0.5$.

Results

MPP^+ in a dose- and time-dependent manner down-regulated FGF9 mRNA and protein expression and reduced cell viability in primary cortical neurons

To examine the effect of MPP^+ on FGF9 expression, we treated primary cortical neurons with 10^{-3}–10^{-6} M of MPP^+ and measured the cell viability, FGF9 mRNA, and protein expression at different time points after treatment. MPP^+ in a dose-dependent manner induced significant cell death 24 and 48 h after treatment (Fig. 1a). A 10^{-4} M dose of MPP^+ induced a 50% decrease in cell viability 48 h post-treatment, as did a 5×10^{-4} M dose 24 h post-treatment (Fig. 1a). MPP^+ also in a dose-dependent manner decreased FGF9 mRNA expression 24 h after treatment (Fig. 1b): MPP^+ (10^{-4} M) treatment for 24 h significantly down-regulated FGF9 mRNA expression compared with the untreated control group (Con); however, this dose of MPP^+ did not cause as much cell death as the 5×10^{-4} M dose did (Fig. 1a). We further found that significant down-regulation of FGF9 mRNA expression had occurred as early as 4 h after treatment (Fig. 1c), whereas significant down-regulation of FGF9 protein expression occurred 8 h after MPP^+ treatment and persisted for an additional 16 h (Fig. 1d). Because the down-regulation of FGF9 mRNA and protein expression occurred much earlier than MPP^+-induced neuron death, these results implied that FGF9 down-regulation was a cause but not a consequence of neuron death.

Melatonin prevented MPP^+-induced FGF9 down-regulation and death in primary cortical neurons

To examine the effect of melatonin on MPP^+-induced neuron death, we directly counted the number of MAP2-ir cells (indicated neurons) 48 h after treatment in primary cortical neurons (Fig. 2a). MPP^+ and melatonin co-treatment in a dose-dependent manner (10^{-6}–10^{-4} M) inhibited the MPP^+-induced loss of cortical neurons 48 h after treatment (Fig. 2b). After determining an optimal dose of melatonin

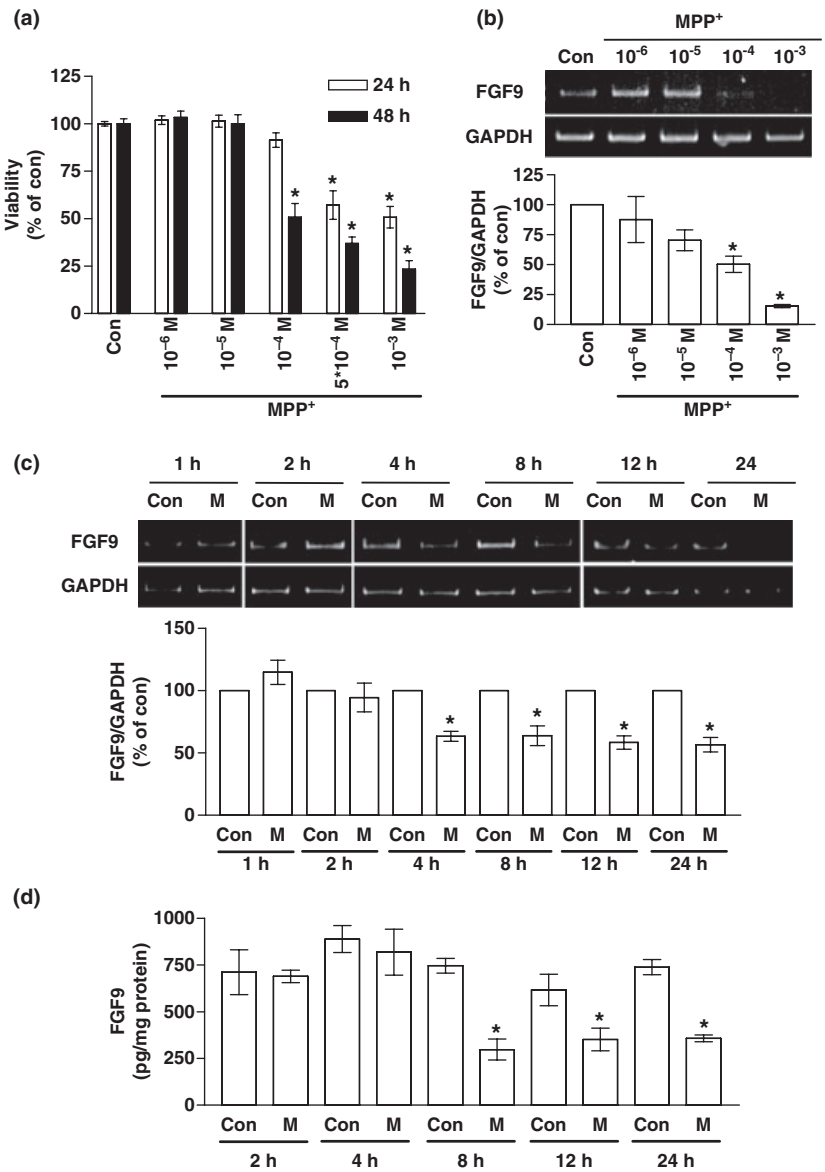

Fig. 1 MPP^+ in a dose- and time-dependent manner down-regulated FGF9 expression and induced death in primary cortical neurons. MTT assays were used to measure cell viability 24 and 48 h post-treatment with the indicated dose of MPP^+ (a). RT-PCR was used to measure FGF9 mRNA expression 24 h post-treatment (b). Primary cortical neurons treated with 10^{-4} M MPP^+ (M) were harvested at the indicated time points and FGF9 mRNA expression was measured using RT-PCR (c); protein concentration was measured using ELISA (d). Densitometric analysis was used to determine the quantitative data of b and c (lower panel). Values are means ± SEM. ($n \geq 3$) $^*p < 0.05$, compared with the untreated group (Con) at each corresponding time point.

(10^{-4} M), we investigated its effect on MPP^+-induced FGF9 down-regulation. We found that 8 h after co-treatment with melatonin (10^{-4} M) and MPP^+ (10^{-4} M), melatonin had significantly inhibited the reduction of FGF9 mRNA and protein expression compared with the MPP^+-only group (Fig. 2c and d). However, treatment with melatonin alone did not change FGF9 mRNA or protein expression. These results indicated that melatonin prevented FGF9 down-regulation to protect cortical neurons from MPP^+ intoxication.

Melatonin protection of dopaminergic neurons was FGF9-dependent

Because the uptake of MPP^+ into cells can be mediated by DA transporter, DA neurons are more vulnerable to MPP^+ (Gainetdinov *et al.* 1997). We further examined the role of FGF9 on MPP^+ toxicity and melatonin protection in dopaminergic neurons using a primary mesencephalic neuron culture. Dopaminergic neurons were identified in cells double-stained with anti-MAP2 (neuron marker) and anti-TH (dopaminergic neuron marker) antibody. The cells were also stained with Hoechst 33342 to identify apoptotic neurons with fragmented nuclei (Fig. 3a). We found that exposure to MPP^+ (10^{-6} M) for 48 h selectively induced apoptosis in dopaminergic neurons [TH-immunoreactive (TH-ir) and MAP2-ir cell)], but not in other neurons (MAP2-ir only cells) (Fig. 3a). We counted the number of apoptotic and dopaminergic neurons and found that MPP^+ treatment for 48 h resulted in dose-dependent apoptosis in dopaminergic neurons (Fig. 3b). MPP^+ at 10^{-7} M induced significant apoptosis in dopaminergic neurons, all of which

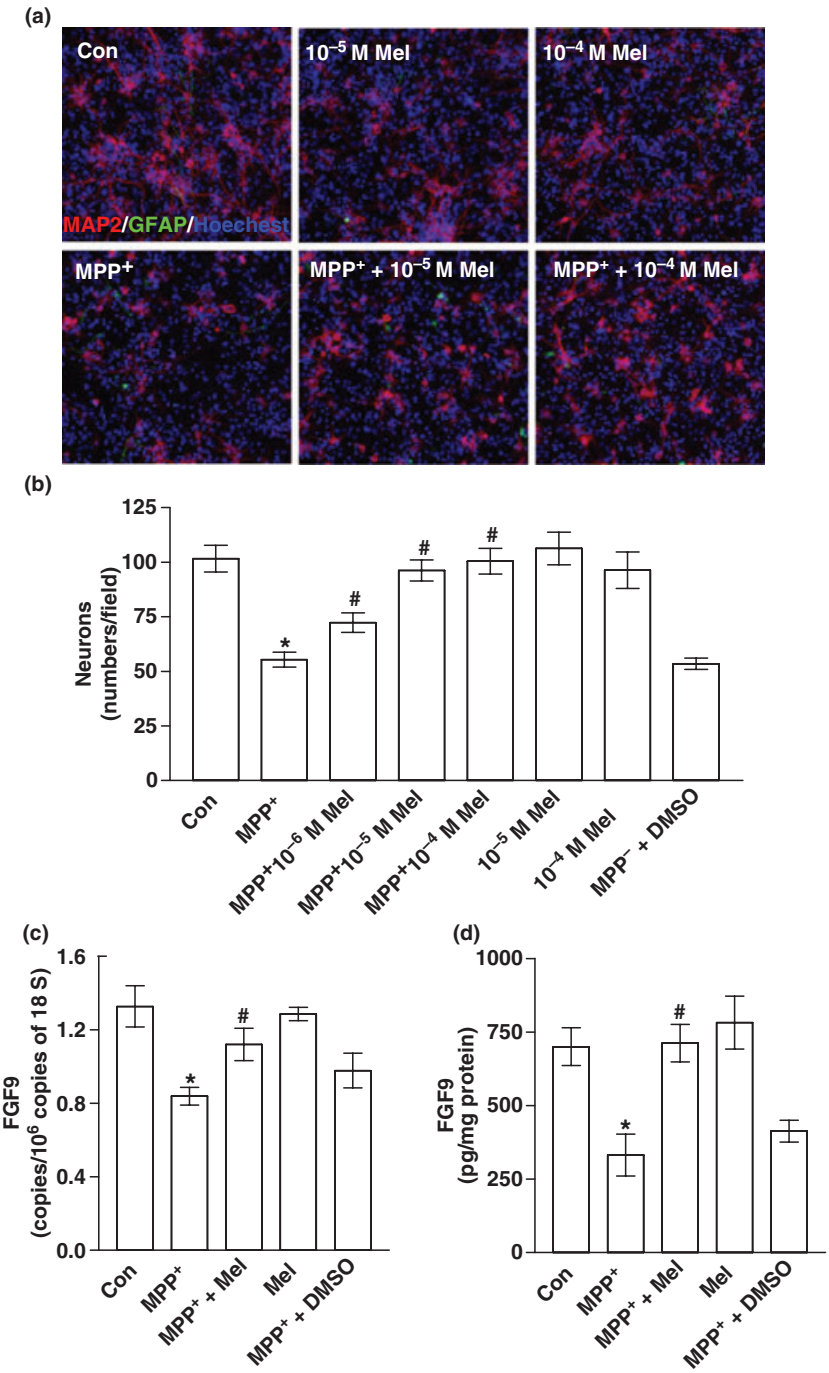

Fig. 2 Melatonin (Mel) prevented MPP^+-induced neuron death and FGF9 down-regulation in primary cortical neurons. (a) Forty eight hours after 10^{-4} M MPP^+ treatment, primary cortical cells were fixed and immunostained with anti-MAP2 antibody (neuronal marker), anti-GFAP antibody (astroglial marker), and Hoechst 33342 (nuclei). The number of MAP2-positive cells per field for each treatment group is shown in (b). Real-time PCR and ELISA were respectively used to measure FGF9 mRNA (c) and protein (d) expression 8 h after the indicated treatments [doses: MPP^+, [10^{-4} M]; Mel, [10^{-4} M]; vehicle (dimethylsulfoxide, DMSO) [0.02%]]. Values are means ± SEM. ($n \geq 4$) $^*p < 0.05$, compared with the untreated (Con) group; #$p < 0.05$, compared with the MPP^+-treated group.

died when exposed to 10^{-4} M MPP^+ for 48 h (Fig. 3b); the same dose of MPP^+ caused an approximately 50% loss of cortical neurons (Fig. 1a). These results indicated that mesencephalic dopaminergic neurons were selectively vulnerable to MPP^+. Furthermore, melatonin (5×10^{-5}–10^{-4} M) and 10^{-6} M MPP^+ co-treatment in a dose-dependent manner reduced the MPP^+-induced apoptosis in dopaminergic neurons (Fig. 3c). To determine whether FGF9 was essential for melatonin's protective effect, we blocked FGF9 by treating the neurons with FGF9 neutralizing antibody. FGF9 neutralizing antibody (10 μg/mL) induced apoptosis in dopaminergic neurons and inhibited the neuroprotective effect of melatonin (Fig. 3c). Treatment with FGF9 neutralizing antibody doubled the level of apoptosis compared with the Con group; however, FGF9 neutralizing antibody had no effect on MPP^+-induced neurotoxicity (Fig. 3c). Co-treatment with FGF9 neutralizing antibody (Fig. 3c) significantly blocked the reduction of MPP^+-increased apoptosis in dopaminergic neurons in the melatonin + MPP^+ co-treated group (Fig. 3c).

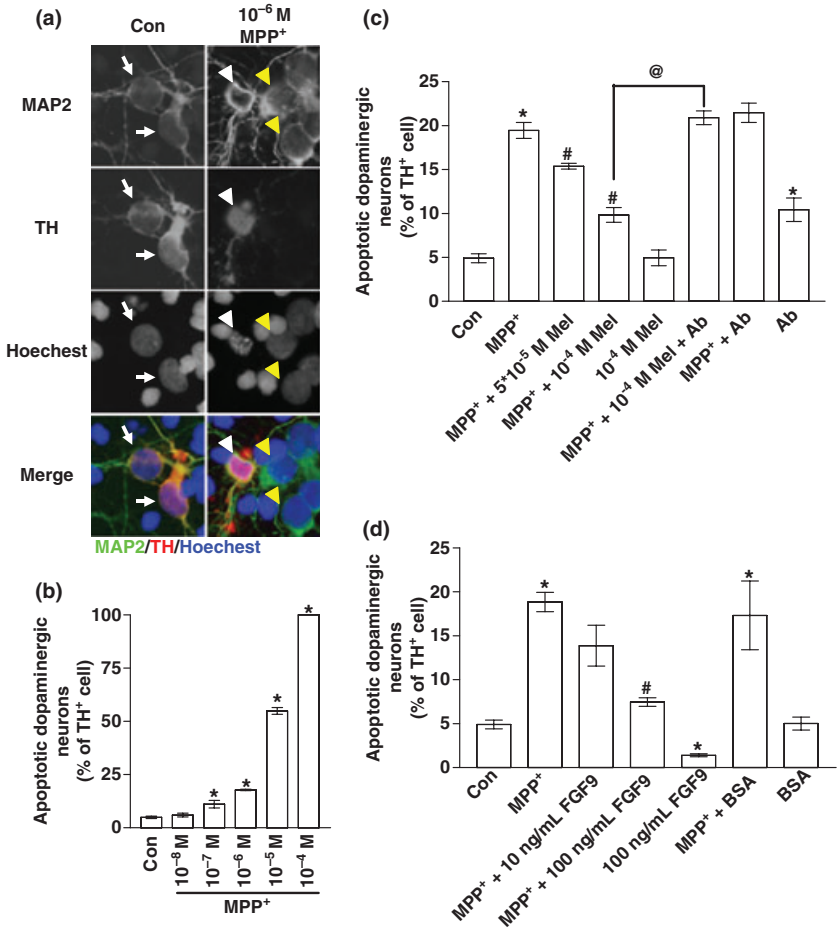

Fig. 3 Anti-FGF9 neutralizing antibody blocked the protective effect of melatonin (Mel) in a culture of primary mesencephalic neurons. (a) Primary mesencephalic neurons were fixed and immunostained with anti-MAP2 antibody, anti-tyrosine hydroxylase [TH, dopaminergic neuron marker] antibody, and Hoechst 33342, as indicated. Compared with healthy TH-positive dopaminergic neurons (arrows) in the untreated (Con) group, fragmented nuclei (apoptotic characteristic; white arrowheads) were found selectively in dopaminergic neurons but not in non-dopaminergic neurons (yellow arrowheads) 48 h after MPP^+ (10^{-6} M) treatment. The percentages of apoptotic dopaminergic neurons 48 h after the indicated doses of MPP^+ are shown in b. (c) The percentages of apoptotic dopaminergic neurons were also counted 48 h after treatment with MPP^+ (10^{-6} M), Mel (10^{-4} M), and anti-FGF9 neutralizing antibody (Ab, 10 μg/mL), and (d) co-treatment with MPP^+ (10^{-6} M) and recombinant human FGF9 protein (at the indicated dose). BSA was the vehicle for FGF9. Values are means ± SEM. ($n \geq 4$). $^*p < 0.05$ compared with untreated controls (Con); $\#p < 0.05$ compared with the MPP^+-treated group; $@p < 0.05$ compared with the MPP^+ + Mel co-treated group.

To further confirm that the prevention of MPP^+-induced FGF9 down-regulation was involved in melatonin's neuroprotection, we evaluated the effect of FGF9 on MPP^+ toxicity in dopaminergic neurons. We found that in the absence of MPP^+, FGF9 (100 ng/mL) treatment significantly reduced the number of apoptotic dopaminergic neurons compared with the Con group (Fig. 3d). FGF9 treatment was neuroprotective: co-treatment with FGF9 (10–100 ng/mL) and MPP^+ in a dose-dependent manner decreased the MPP^+-induced apoptosis in dopaminergic neurons (Fig. 3d). Moreover, that FGF9 neutralizing antibody selectively blocked the protective effect of FGF9 but not of FGF2 (Fig. S1) confirmed the specificity and effective dose (10 μg/mL) of FGF9 neutralizing antibody. These results suggested that the survival of dopaminergic neurons and the neuroprotection of melatonin were FGF9-dependent.

Melatonin inhibited the MPP^+-induced down-regulation of FGF9 expression in the rat nigrostriatal dopaminergic system

To test whether FGF9 is also involved in MPP^+-induced neurotoxicity and melatonin-mediated neuroprotection *in vivo*, we measured FGF9 protein and mRNA expression in the striatum and substantia nigra of MPP^+-only and MPP^+ + melatonin co-treated rats. Intrastriatal infusion of MPP^+ (100 nmole in 1 μL) significantly down-regulated FGF9 mRNA and protein expression not only in the lesioned striatum (Fig. 4) but also in the ipsilateral substantia nigra (Fig. 5) compared with the corresponding contralateral side 72 h after treatment. To rule out the possibility that the FGF9 down-regulation observed at 72 h after treatment was because of the MPP^+-induced cell death, we further analyzed FGF9 protein expression in the striatum 8 h after it had been treated with a low dose of MPP^+ (50 nmole in 1 μL): the treatment had not damaged the neurons. We found that exposure to low dose of MPP^+ for 8 h had caused a significant down-regulation of FGF9 protein expression (Fig. 4c). The FGF9 protein level in the ipsilateral side of the striatum was 6.33 ± 1.1 ng/mg, 8 h after low-dose MPP^+ treatment (Fig. 4c), but only 2.14 ± 0.61 ng/mg protein 72 h after high-dose (100 nmole in 1 μL) MPP^+ treatment (Fig. 4b) compared with 9.45 ± 0.21 and 7.28 ± 0.18 ng/mg protein in the ipsilateral striatum of each corresponding sham group. Co-treatment with melatonin significantly inhibited MPP^+-down-regulated

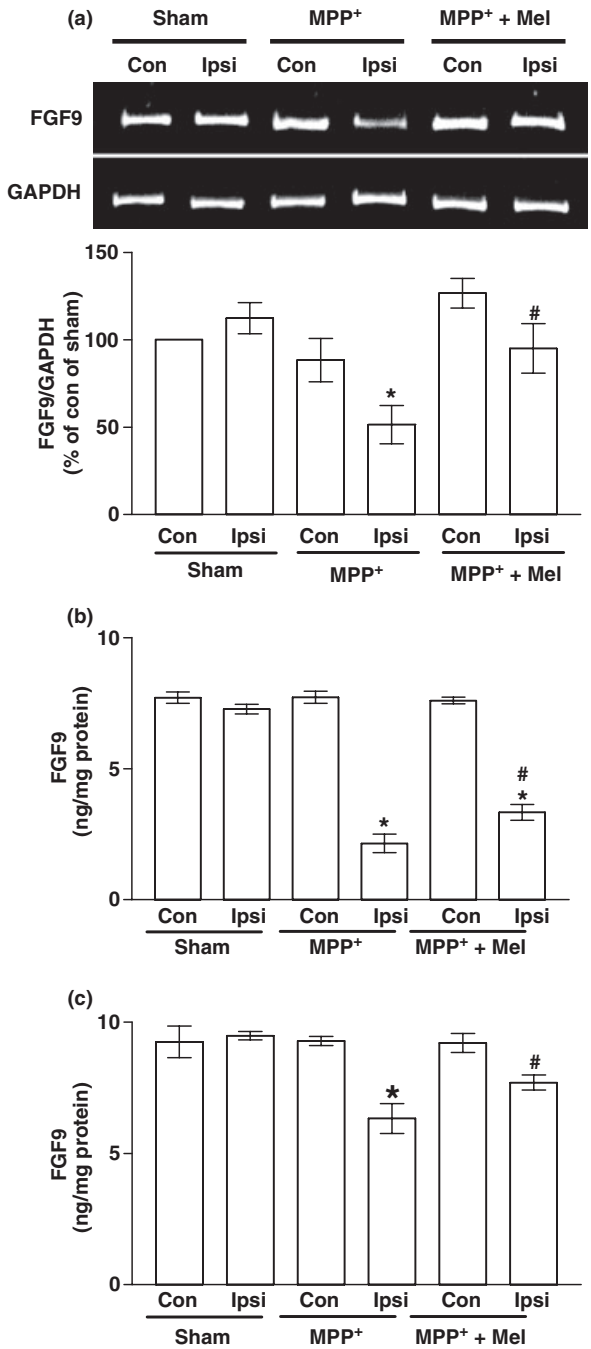

Fig. 4 Melatonin (Mel) co-treatment inhibited the down-regulation of FGF9 mRNA and protein expression in the striatum of MPP^+-treated rats. RT-PCR and ELISA were used to measure FGF9 mRNA (a) and protein expression (b) in the striatum of rats 72 h after MPP^+ treatment or MPP^+ + Mel co-treatment. MPP^+ (100 nmole/1 μL) was injected into the right side of the striatum (ipsilateral; Ipsi). Densitometric analysis was used to determine the quantitative data of upper panel and shown in lower panel of (a). c shows FGF9 protein expression in the striatum 8 h after treatment with a low dose of MPP^+ (50 nmole/1 μL). Values are means ± SEM ($n \geq 6$). *$p < 0.05$ compared with the contralateral side (Con) of each corresponding group. #$p < 0.05$ compared with the Ipsi of the MPP^+-treated group.

FGF9 mRNA (Fig. 4a) and protein expression (Fig. 4b and c).

Moreover, we found a significant down-regulation of FGF9 mRNA expression (Fig. 5a) and loss of TH- (Fig. 5b and d) and FGF9-immunoreactive neurons (Fig. 5f and h) on the ipsilateral side of the substantia nigra compared with its contralateral side 72 h after the intrastriatal infusion of MPP^+. The co-treatment of melatonin and MPP^+ significantly attenuated MPP^+-induced FGF9 mRNA down-regulation (Fig. 5a). In the immunohistochemistry study, we found that melatonin + MPP^+ co-treatment prevented the MPP^+-induced loss of dopaminergic neurons and of FGF9-immunoreactivity: the number of TH-immunoreactive cells (TH-ir; indicated dopaminergic neurons) (Fig. 5c and e) and FGF9-immunoreactive cells (Fig. 5g and i) in the ipsilateral side was almost the same as on the contralateral side of the melatonin + MPP^+ co-treated substantia nigra and higher than the number on the ipsilateral side of the MPP^+-only group (Fig. 5c and e versus Fig. 5b and d; Fig 5g and i versus Fig 5f and h). Therefore, our *in vivo* results further supported the hypothesis that melatonin attenuated FGF9 down-regulation, thereby preventing MPP^+-induced nigrostriatal dopaminergic neurodegeneration.

MPP^+ treatment down-regulated FGF20 expression, but not FGF2 mRNA expression, in the substantia nigra

Because other FGF family members may compensate for the down-regulation of FGF9, we measured the mRNA expression of FGF20 and FGF2 in the substantia nigra 72 h after intrastriatal MPP^+ infusion with or without melatonin co-treatment. FGF20 mRNA expression in the ipsilateral substantia nigra was significantly down-regulated compared with the contralateral side of MPP^+-treated group; however, FGF2 expression was not significantly different between the ipsilateral and contralateral sides of the substantia nigra after MPP^+ treatment (Fig. 6b). Moreover, co-treatment with melatonin and MPP^+ did not alter MPP^+-induced FGF20 down-regulation or FGF2 expression in the substantia nigra (Fig. 6a).

Infusing FGF9 into the substantia nigra protected dopaminergic neurons from MPP^+-induced toxicity

To further examine the protective effect of FGF9 in dopaminergic neurons against MPP^+ toxicity *in vivo*, we daily injected human recombinant FGF9 (100 ng/μL/day) protein into the bilateral substantia nigra of rats and determined the number of TH-ir cells in the substantia nigra of MPP^+ + BSA (vehicle solution for FGF9) treated and MPP^+ + FGF9 co-treated rats. Intrastriatally infused MPP^+ caused a 50% loss of TH-ir neurons in the ipsilateral substantia nigra compared with the contralateral side, which was partially but significantly reduced by co-treatment with FGF9. The number of TH-ir neurons on the ipsilateral side of FGF9 + MPP^+ co-treated rats was significantly higher than

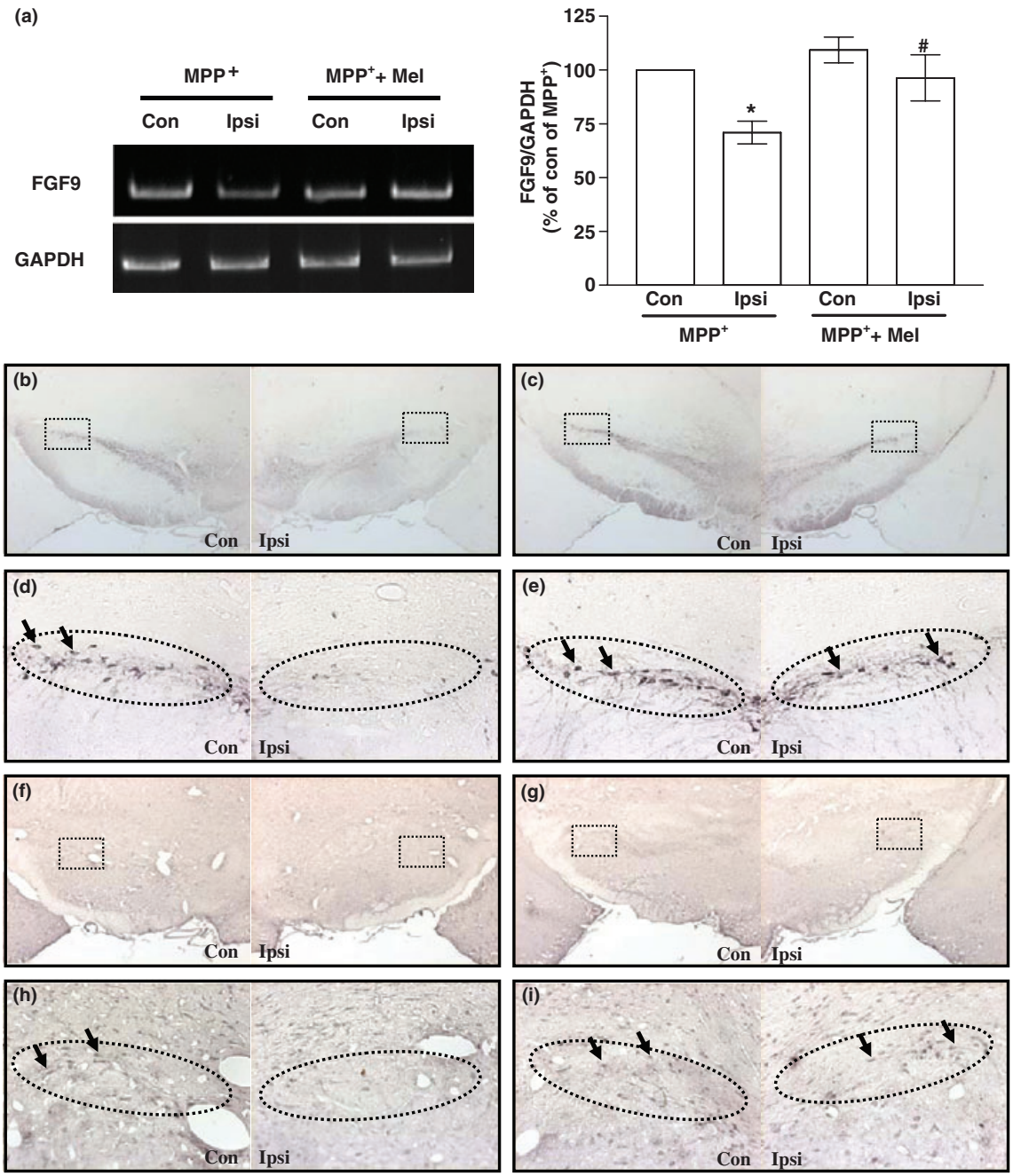

Fig. 5 Melatonin (Mel) + MPP^+ co-treatment reduced MPP^+-induced DA neuron loss and FGF9 down-regulation in the substantia nigra. (a) RT-PCR was used to measure FGF9 mRNA expression in the substantia nigra 72 h after treatment with MPP^+ and co-treatment with MPP^+ and melatonin (MPP^+ + Mel). Densitometric analysis was used to determine the quantitative data in the right panel (shown in the left panel) (a). Values are means ± SEM (n = 9). *p < 0.05 compared with the contralateral side (Con) of each corresponding group. The adjusted brain sections of MPP^+-treated (b, d, f, h) and MPP^+ + Mel co-treated (c, e, g, i) rats were immunostained with anti-TH (b–e) or anti-FGF9 antibody (f–i). The higher magnification of the square boxes in b, c, f, g are respectively shown in d, e, h, i. The dotted-line circles in d–e and h–i indicate the area of the substantia nigra. The TH-immunoreactive neurons are indicated by arrows in d and FGF9 immunoreactivity is shown by arrows in h.

the number on the ipsilateral side of MPP^+ + BSA treated rats, though the number of TH-ir neurons was lower on the ipsilateral side of FGF9 + MPP^+ co-treated rats compared with the contralateral side (Fig. 7). The *in vivo* results showed that FGF9 treatment partially protected nigral dopaminergic neurons from MPP^+ toxicity.

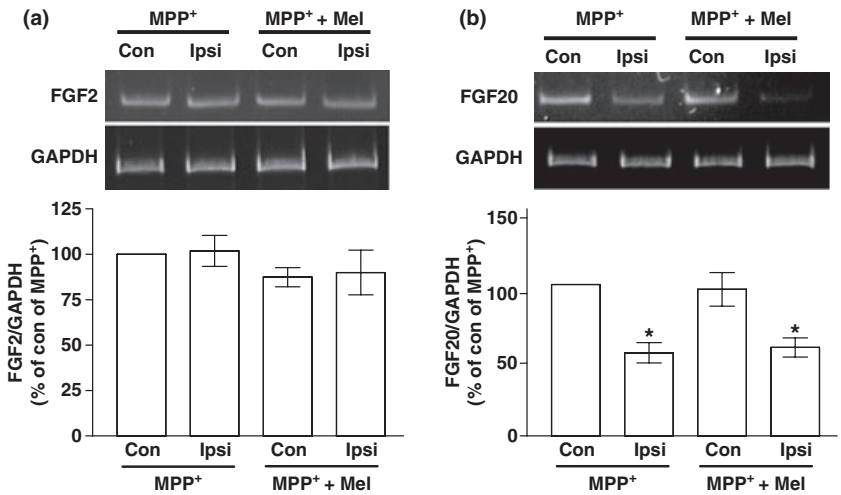

Fig. 6 MPP^+ treatment down-regulated FGF20, but not FGF2, mRNA in the substantia nigra. Both contralateral (Con) and ipsilateral (Ipsi) sides of the substantia nigra were collected 72 h after MPP^+ (100 nmole/1 μL) treatment and co-treatment with MPP^+ and melatonin (MPP^+ + Mel). RT-PCR was used to detect the mRNA expression of FGF2 (a), FGF20 (b), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (a, b) in the substantia nigra. Densitometric analysis was used to determine the quantitative data. FGF2 and FGF20 mRNA expression were normalized with GAPDH expression. Values are means ± SEM ($n \geq 6$) and presented as a percentage of their expression in the Con side of the MPP^+-treated group. *$p < 0.05$ compared with the Con of the MPP^+-treated group.

Discussion

Our novel finding *in vivo* and *in vitro* showed that FGF9 was down-regulated and melatonin co-treatment prevented this down-regulation in MPP^+-induced PD model. Because MPP^+ induced oxidative stress to cause cell death and melatonin had neuroprotective effect by preventing oxidative insult (Chuang and Chen 2004; Przedborski and Ischiropoulos 2005), these results suggested that MPP^+-down-regulated FGF9 expression may be because of the reactive oxygen species overproduction induced by MPP^+. Reports had shown that reactive oxygen species down-regulated the expression of trophic factors, for example, nitric oxide down-regulated BDNF secretion in cultured hippocampal neurons (Canossa *et al.* 2002) and oxidized low-density lipoprotein down-regulated FGF2 expression in cultured endothelial cells (Chang *et al.* 2001). Moreover, our unpublished data also showed that FGF9 mRNA expression was decreased after H_2O_2 treatment. Gene expression was controlled by the promoter activity and the mRNA stability. Recent evidence (Cantin *et al.* 2006; Rodriguez-Pascual *et al.* 2008) showed that oxidative stress may reduce mRNA stability to suppress gene expression. In particular, their results indicated that oxidative stress had no effect on gene transcription, but the mRNA stability was markedly reduced. On the other hand, other reports showed that oxidative stress repressed gene promoter activity by decreasing binding of transcription factors (Pugazhenthi *et al.* 2003; Kumar *et al.* 2008). Although our data indicated that MPP^+ treatment down-regulated FGF9 mRNA and protein expression *in vivo* and *in vitro*, the mechanisms involved in this down-regulation need to be further clarified.

Moreover, MPP^+ may cause the death of neurons by suppressing receptor expression instead of decreasing the level of FGF ligand, because FGF signaling is triggered by its four receptors, FGFR1–4 (Reuss and von Bohlen und Halbach 2003). Recent studies (Corso *et al.* 2005; Timmer *et al.* 2007) reported a significant loss of nigral dopaminergic neurons in FGFR3-deficient mice and in rats transfected with tyrosine-kinase-deleted FGFR1; another (Claus *et al.* 2004) showed that FGFR1-3 expression did not significantly change in a 6-hydroxydopamine-induced-PD model. Our finding that FGF9 co-treatment prevented MPP^+-induced toxicity indicated that the FGF receptors still functioned normally in the presence of MPP^+. However, the expression of FGF receptors and FGF9 in patients with PD needs to be detected to confirm this essential role of FGF9 in PD.

We also found that FGF9 was a survival and neuroprotective factor for dopaminergic neurons. First, we found that MPP^+-induced FGF9 down-regulation occurred much earlier than neuron death, a strong indication that FGF9 down-regulation was involved in MPP^+ toxicity. Second, FGF9 administration inhibited the MPP^+-induced death of dopaminergic neurons in mesencephalic culture and in the substantia nigra of rats. Third, in mesencephalic cultures, FGF9 alone reduced dopaminergic neuron apoptosis and FGF9 antibody increased it in an MPP^+-independent fashion, indicating that FGF9 was a trophic factor for dopaminergic neurons. Other reports (Garces *et al.* 2000; Kanda *et al.*

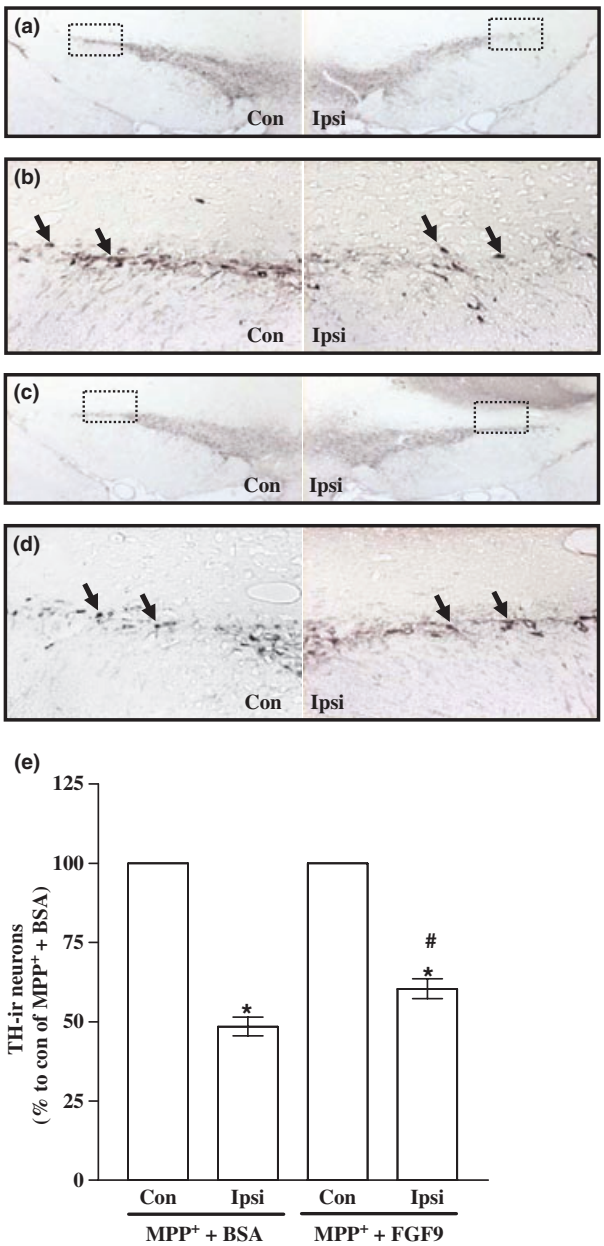

Fig. 7 Intranigral infusion of recombinant human FGF9 protein protected nigral dopaminergic neurons from MPP^+ toxicity. Human recombinant FGF9 (100 ng/μL/day) was daily infused into the bilateral substantia nigra for 3 days. The vehicle for FGF9 was 1% BSA in PBS. The brain sections from MPP^+ and BSA co-treated (MPP^+ + BSA; a, b) and MPP^+ and FGF9 co-treated (MPP^+ + FGF9; c, d) rats were immunostained with anti-TH antibody 72 h after the intrastriatal infusion of MPP^+ (100 nmole/1 μL). b and d are magnifications of the square boxes in a and c. The number of TH-immunoreactive (TH-ir) neurons (arrows) in b and d was counted and shown in e. Values are means ± SEM ($n \geq 4$) and presented as a percentage of the number of TH-ir neurons in the contralateral (Con) side of the MPP^+ + BSA co-treated group. *$p < 0.05$ compared with the Con side and #$p < 0.05$ compared with the ipsilateral side (Ipsi) of the MPP^+ + BSA co-treated group.

2000; Kinkl *et al.* 2003) showed that FGF9 promoted the survival of cholinergic neurons, motor neurons, and retinal ganglion cells.

In addition to FGF9 down-regulation, we found that FGF20, but not FGF2, mRNA expression was decreased in substantia nigra of MPP^+-treated rats. There are reports (Ohmachi *et al.* 2000, 2003) that FGF20 was selectively expressed in calbindin-negative dopaminergic neurons of substantia nigra that were preferentially lost in PD and promoted their survival *in vitro*, but there was no direct evidence of FGF20 expression in PD patients or animal models of PD. Our results clearly showed that in MPP^+-induced PD model, the nigral FGF20 was down-regulated and correlated with dopaminergic neuron death. This finding may explain why supra-nigral FGF9 administration significantly but partially rescued nigral dopaminergic neurons from MPP^+ intoxication compared with full protection in cortical and mesencephalic neuron cultures. On the other hand, we found that MPP^+ treatment did not suppress nigral FGF2 mRNA expression, although FGF2 co-treatment significantly prevented the MPP^+-induced death of cortical neurons (Fig. S1). Controversial findings showed increases or no change in FGF2 expression in MPTP- and 6-hydroxy dopamine-injured striatum and substantia nigra (Leonard *et al.* 1993; Zechel *et al.* 2006). Furthermore, reports (Chadi *et al.* 1993; Hsuan *et al.* 2006; Timmer *et al.* 2007) showed that FGF2 protected nigrostriatal and mesencephalic dopaminergic neurons from MPTP and rotenone toxicity. However, the protective effect of FGF2 may depend on astrocytes because the elevated FGF2 mRNA expression was in putative astroglial cells, but not in dopaminergic neurons, in response to 6-hydroxydopamine toxicity (Chadi *et al.* 2008). Our findings also indicated that FGF2 mRNA was chiefly expressed in primary cortical astrocytes, whereas FGF9 mRNA expression was higher in neurons (Fig. S2). These findings suggested that FGF2 protection against MPP^+-induced cortical neuron death and the unchanged expression of FGF2 mRNA in substantia nigra of MPP^+-treated rats may be attributed to an increased proliferation of astrocytes (astrogliosis) induced by neuron death after MPP^+ treatment (Sriram *et al.* 2004; McGeer and McGeer 2008).

Reports (Lee *et al.* 2006; Imbesi *et al.* 2008; Kong *et al.* 2008) had shown that melatonin treatment up-regulated BDNF and GDNF expression to promote the survival of neurons and the differentiation of neural stem cells. Reduced BDNF and GDNF expression was found in brains of persons with PD and MPP^+-treated animals (Howells *et al.* 2000; Chauhan *et al.* 2001; Collier *et al.* 2005). However, there is no evidence that melatonin prevents GDNF or BDNF down-regulation to protect neurons against neurotoxin-induced death. We have provided direct evidence that melatonin prevented MPP^+-induced FGF9 down-regulation to achieve

its neuroprotective effect *in vivo* and *in vitro*, although melatonin-only treatment did not up-regulate FGF9 expression. Because it had been reported that antioxidant of vitamin E and C prevented neurotoxin-induced down-regulation of trophic factors and cell death (Heaton *et al.* 2004; Grant *et al.* 2005), melatonin and its metabolites that are highly effective scavengers of radicals (Tan *et al.* 2007; Peyrot and Ducrocq 2008) may contribute to the prevention of MPP^+-induced FGF9 down-regulation and neuron death. On the other hand, treating the cells with an FGF9-specific neutralizing antibody blocked melatonin's protective effect, and co-treatment with melatonin and MPP^+ prevented FGF9, but not FGF20, down-regulation in the substantia nigra of MPP^+-treated rats. These findings showed the essential role of FGF9 in melatonin protection, in addition to its protective effect of reducing oxidative stress.

In conclusion, the present study indicates an important role of FGF9 for dopaminergic neurons in MPP^+-induced parkinsonism and melatonin protection. We clearly showed that MPP^+ down-regulated FGF9 expression to cause dopaminergic neuron death, and that preventing FGF9 down-regulation contributed to melatonin neuroprotection. However, the underlying mechanism of neuroprotective effect of FGF9 is worth further investigating. That FGF2 increased glutathione levels and the antioxidant enzymes of superoxide dismutase and glutathione reductase to protect neurons from oxidative insults (Mattson *et al.* 1995; Hou *et al.* 1997) supports our hypothesis that FGF9 treatment up-regulates endogenous antioxidant expression to protect neurons. Furthermore, in contrast to the poor penetration across the blood-brain-barrier and limited delivery of trophic factors into the brain, melatonin is lipid-soluble, freely permeates the brain, is safe and ready to use in clinical practice, and restores FGF9 content to normal; therefore, it is therapeutically efficacious for PD patients.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1 Anti-FGF9 neutralizing antibody specifically blocked FGF9-mediated neuroprotection.

Figure S2 The expression of FGF9 and FGF2 mRNA in neuron-enriched and astrocyte-enriched primary cortical cultures.

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