MANF improves the MPP+/MPTP-induced Parkinson’s disease via improvement of mitochondrial function and inhibition of oxidative stress

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Abstract: Objective: This study aimed to investigate the therapeutic effect of mesencephalic astrocyte-derived neurotrophic factor (MANF) on the MPTP/MPP+-induced model of Parkinson’s disease (PD) and the potential mechanism. Methods: Male C57BL/6 mice PD model with MPTP-induced were randomly injected bilaterally with MANF or PBS into the striatum. Two weeks later, Rotarod test, immunohistochemistry, and detection of dopamine (DA) and its metabolites, superoxide dismutase (SOD), glutathione (GSH), and malondialdehyde (MDA) were performed. A cell model of PD was established by incubating SH-SY5Y cells with MPP+, cells were pretreated for 2 h with different concentrations of MANF before 24 h incubation with MPP+. Cell viability, expression of Bax, and Bcl-2, gene expression levels of Heme oxygenase 1 (HMOX1) and Superoxide dismutase 2 (SOD2), and mitochondrial transmembrane potential were detected. Results: The latency reduction in PD mice was partially restored after MANF treatment (P<0.05); MANF significantly reduced the loss of tyrosine hydroxylase (TH)-positive dopaminergic neurons in the substantia nigra pars compacta (SNpc) (P<0.01); MANF significantly increased the striatal DA level in PD mice (P<0.05) and markedly increased the SOD activity (P<0.01) and GSH production (P<0.01). MANF pre-treatment significantly decreased the MPP+-induced reduction of cell viability (P<0.01), inhibited the ratio of Bax/Bcl-2 expression (P<0.01), activated gene expression levels of HMOX1 (P<0.01) and SOD2 (P<0.05), and reversed MPP+-induced loss of mitochondrial membrane potential (P<0.01). Conclusion: MANF can attenuate the neuronal lesion in MPTP/MPP+-induced PD mice, which may be related to the improvement of mitochondrial function and inhibition of oxidative stress.

Keywords: MANF, MPTP/MPP+, neurorestorative effect, Parkinson’s disease, mitochondrial function

Introduction

Parkinson’s disease (PD) is a disabling neurodegenerative disorder characterized clinically by tremor, rigidity, bradykinesia and postural instability [1] and pathologically by the extensive loss of dopaminergic neurons in the substantia nigra pars compacta (SNpc) [2]. Currently, the most widely used treatment for PD is dopamine replacement therapy, but long-term treatment can produce debilitating adverse effects and may not affect the progression of PD.

At the cellular level, PD is involved in the alteration of mitochondrial electron transport chain function, the change in dopamine metabolism, and the excess production of reactive oxygen species (ROS) due to oxidative stress. Thus, it has been proposed that to improve the mitochondrial function is effective to delay the progression of dopaminergic neurons loss.

Neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and its metabolite 1-methyl-4-phenylpyridinium ion (MPP+) are widely used to establish model of PD since it shares tremendous similarities in neural biochemical, pathology and behaviors with human PD [3].

Mesencephalic astrocyte-derived neurotrophic factor (MANF) is a novel neurotrophic factor and can selectively protect nigral dopaminergic neurons. It has been proven that MANF has neuro-
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Tyrosine hydroxylase immunohistochemistry

Two weeks after MANF treatment, 6–7 mice from each group were intraperitoneally anesthetized with 10% chloral hydrate and the brain was perfusion-fixed with 4% paraformaldehyde following a normal saline flush, and then transferred to a 30% sucrose solution. Serial coronal sections through the substantia nigra were cut frozen at a thickness of 20 μm. Immunohistochemistry was performed for tyrosine hydroxylase (TH) as described [9]. After inhibition of endogenous peroxidase activity, sections were incubated in 5% bovine serum albumin (BSA) in PBS for 30 min at room temperature. Sections were then incubated with rabbit anti-TH primary antibody (1:1000, Millipore) overnight at 4°C. After rinsing with PBS, sections were incubated in a biotinylated secondary antibody (1:50) with horseradish peroxidase (HRP) for 2 h and then visualized with diaminobenzidine (DAB). At last, the sections were dehydrated through an alcohol series and mounted.

The brain sections were photographed at a magnification of 200× (Nikon) and TH positive dopaminergic neurons were manually counted in the SNpc of right hemisphere by an investigator blind to the experiment. At least three sections were used for each animal and a mean was calculated.

Brain tissue preparation and DA detection

Mice were sacrificed by cervical dislocation 14 days after MANF treatment and the dopamine and its metabolites 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) were detected by HPLC. The brains were rapidly removed fresh and the striatum was separated on an ice-cold plate. For each animal, the striatum of both hemispheres was transferred into a 1.5-ml flask, weighed and homogenized in ice-cold solution A (0.2 mM HClO₄), and then the homogenate was centrifuged at 10,000 rpm for 15 min at 4°C. The supernatant was filtered through a nylon syringe filter (0.2 μm in pore size) and 20 μL of the filtrate was injected for HPLC. The mobile phase, containing 80 mM sodium dihydrogenphosphate monohydrate, 2.0 mM 1-octanesulfonic acid sodium salt, 100 μl/l triethylamine, 5 nM EDTA, and 10% acetonitrile, pH 3.0, was perfused at 0.25 ml/min. Data were calibrated with an external standard. The contents of dopamine and its metabolites
were calculated and expressed as ng/mg tissue weight.

**Detection of anti-oxidative activity**

Mice were euthanized and the substantia nigra was separated, homogenized in PBS and centrifuged at 10,000 g for 10 min at 4°C. The protein concentration was quantified with BCA protein assay kit. Superoxide dismutase (SOD) activity, glutathione (GSH) content, and malondialdehyde (MDA) content were determined with commercially available kits according to the manufacturer’s instructions.

**Cell culture and treatments**

Human neuroblastoma SH-SY5Y cells were cultured in DMEM supplemented with 10% (v/v) FBS, and 100 U/mL penicillin/streptomycin at 37°C in an environment with 5% CO₂ and 95% humidified air. SH-SY5Y cells were pretreated for 2 h with different concentrations of MANF (50, 200, 400 ng/mL) before 24-h incubation with 2 mM MPP⁺.

**MTT assay of cell viability**

MTT assay was used to evaluate the viability of SH-SY5Y cells. Cells were seeded in 96-well plates with five wells in each group. After 24-h treatment, 20 μL of MTT solution (5 mg/ml) was added to each well, followed by incubation at 37°C for 4 h. Then, the supernatant was removed and 150 μL of DMSO was added into each well. The absorbance of each well was measured at 570 nm with an automated microplate reader. The cell viability was averaged and normalized to that of control cells.

**Western blot analysis**

Cells were homogenized in RIPA lysis buffer and the protein concentration in the supernatant was quantified by BCA kit, proteins (30 μg) was loaded onto 10% SDS-PAGE. After electrophoresis, proteins were transferred to PVDF membranes. Membranes were blocked with 5% BSA in TBST for 1 h at room temperature and then were incubated with Bax (1:1000), Bcl-1 (1:1000) overnight at 4°C followed by incubation for 1 h with HRP secondary antibodies (1:5000). Bands of proteins were visualized using an ECL detection system and digitized data were quantified using Image J software and the relative intensities were normalized to that of GAPDH.

**Quantitative real-time PCR analysis**

Total RNA from SH-SY5Y cells was isolated using TRIzol reagent. mRNA was enriched from the total RNA using oligo dT magnetic beads. Then, the mRNA was fragmented into short fragments using an RNA fragmentation kit. Double stranded cDNA was purified using a QIAquick PCR extraction kit and used for end repair and base A addition. Then, the following RT-PCR primer pairs were used: TBP, 5’-AGCCAAGAGTGAAGAAGACATCC-3’ (forward) and 5’-CACAGCTCTCTCAACCATATC-3’ (reverse); Heme oxygenase 1 (HMOX1), 5’-AACATTGAGAAGCCGCT-3’ (forward) and 5’-GACGACAGGGCGAGACTG-3’ (reverse); Superoxide dismutase 2 (SOD2), 5’-TCAATAAGGAACGGGGAGCG-3’ (forward) and 5’-ACACATCAATCCCCAGCATG-3’ (reverse); Primers for TBP mRNA were used to normalize mRNA expression.

**Measurement of mitochondrial membrane potential**

JC-1, a lipophilic cationic dye, was used to detect mitochondrial transmembrane potential change. SH-SY5Y cells were seeded in 6-well plates and JC-1 kit was used according to manufacturer’s instructions. After the indicated treatments, cells were incubated with JC-1 solution for 20 min at 37°C, then rinsed twice with JC-1 buffer. Fluorescent signal was measured by flow cytometry. The mitochondrial membrane potential was calculated as the ratio of JC-1 red to green fluorescence intensity.

**Statistical analysis**

Data are expressed as mean ± standard error (S.E.M). Statistical analysis was performed using SPSS version 20.0. One-way analysis of variance (ANOVA) followed by Post-hoc LSD test was used to compare data among groups. A value of P<0.05 was considered statistically significant.

**Results**

**MANF improves motor behavior in MPTP-treated mice**

One week after the last MPTP administration, the latency of Rotarod test (206.7±7.2, n=26) reduced significantly as compared to control group (274.1±14.1, n=9) (P<0.01), which indi-
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cates that the MPTP-induced mouse PD model is established successfully (Figure 1A). Then, one week after MANF or PBS treatment, no significant difference was observed between PBS-treatment group (180.7±16.1, n=13) and MANF-treatment group (174.9±13.6, n=13) (P>0.05); however, after 2-week MANF treatment, the latency reduction after MPTP injection was significantly attenuated in MANF-treatment group (203.4±13.9, n=13) as compared to PBS-treatment group (182.5±15.9, n=13) (P<0.05) (Figure 1B).

**Loss of TH-positive neurons in the SNpc**

TH is a rate-limiting enzyme in the dopamine biosynthesis and a marker of dopaminergic neuron. MPTP mainly damages the dopaminergic neurons which could cause a prominent TH-positive cell loss in the SNpc. [10]. In addition, results showed MANF significantly inhibited the loss of TH-positive dopaminergic neurons (37.1±4.4, n=6) as compared to PBS-treatment group (20.9±3.5, n=6) (P<0.01) (Figure 2). These demonstrate that MANF can rescue dopaminergic neurons from MPTP neurotoxicity in mice.

**Levels of dopamine and its metabolites**

MPTP markedly reduced in the levels of dopamine (DA) and its metabolites (DOPAC and HVA) in the striatum. However, after MANF treatment, the striatal DA, DOPAC and HVA levels increased significantly as compared to PBS treated mice (DA: 13.86±0.9 ng/mg, n=7, vs 11.0±2.0 ng/mg, n=7, P<0.05; DOPAC: 5.5±0.8 ng/mg, n=7, vs 3.8±1.1 ng/mg, n=7, P<0.05; HVA: 1.15±0.08 ng/mg, n=7, vs 0.84±0.13 ng/mg, n=7, P<0.01, Figure 3).

**Anti-oxidative indexes**

SOD is an important anti-oxidase in nearly all living cells. GSH is one of the most important non-enzymatic antioxidants in cells and possesses the capabilities of free radical scavenging, detoxification and cellular immunity. GSH can scavenge the O₂⁻ and H₂O₂, and thus the amount of GSH is an important factor related to anti-oxidative capability. MDA was a stable product of lipid peroxidation and may indirectly reflect the extent of cell damage. As mentioned previously, MPTP may induce the free radical production and lipid peroxidation in dopaminergic neurons. These results indicated that, compared with PBS-treatment group, MANF significantly increased the SOD activity (SOD: 375.0±15.0, n=7, vs 276.4±32.0, n=7, P<0.01) and GSH production (GSH: 290.2±34.6, n=7, 219.7±11.92, n=7, P<0.01; Figure 4A, 4B), and decreased the MDA production (0.88±0.067, n=7, vs 0.89±0.044, n=7, P>0.05; Figure 4C). These indicate that MANF may improve the anti-oxidative capacity in MPTP-treated mice.

**Effects of MANF on cell viability**

The dose-cytotoxicity relationship of MPP⁺ was determined in SH-SY5Y cells which were treat-
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Figure 2. Effect of MANF on the loss of TH-positive neurons in the SNpc. Photographs were captured at a magnification of 100×. A. Control group. B. MANF+MPTP-treated group. C. PBS+MPTP-treated group. D. Quantitative analysis of TH-positive neurons in the substantia nigra pars compacta. MANF significantly inhibited the loss of TH-positive neurons as compared to PBS-treatment group. Data are presented as mean ± S.D. *P<0.05, **P<0.01 vs PBS-treatment group.

As illustrated in Figure 5A, MTT assay showed MPP+ (2 mM for 24 h) decreased cell viability to 50.49%, however, pre-treatment with 50, 200 or 400 ng/mL MANF for 2 h before 2 mM MPP+ incubation for 24 h prevented SH-SY5Y cells from MPP+ induced injury, and could restore the cell viability to 52.33%, 66.93% and 75.58%, respectively (P>0.05, P<0.01, and P<0.01). Moreover, various concentrations of MANF (50, 200 and 400 ng/mL) alone failed to significantly affect the viability of SH-SY5Y cells after 24-h treatment (Figure 5B). It suggests that MANF significantly decreases MPP+-induced reduction of cell viability in a dose dependent manner.

Expression of Bax and Bcl-2 in SH-SY5Y cells

The Bcl-2 family proteins play a pivotal role in the cellular apoptotic machinery caused by MPP+ [11, 12]. In this study, Bax protein expression significantly increased in the MPP+-treated group compared with control group, while pre-treatment with MANF inhibited Bax expression. In contrast, the levels of Bcl-2 decreased in the MPP+ group and increased after MANF treatment. There was a significant increase in the
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**Figure 3.** Effects of MANF treatment on the levels of striatal dopamine (DA) and its metabolites 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) in the brain. MANF significantly increased the levels of striatal DA, DOPAC and HVA as compared to PBS-treated mice. Data are presented as mean ± S.D. *P<0.05, **P<0.01 vs PBS-treatment group.

**Figure 4.** Effects of MANF on the anti-oxidative activity of PD mice. SOD, GSH, and MDA were measured in the brain. MANF increased the SOD activity and the GSH production, and decreased the MDA production. Data are presented as mean ± S.D. *P<0.05, **P<0.01 vs PBS-treatment group.

**Figure 5.** Effects of MANF on the viability of MPP⁺-treated SH-SY5Y cells. A. SH-SY5Y cells were seeded in 96-well plates and exposed to different concentrations of MPP⁺ for 24 h, and cell viability was assessed by MTT assay. ▲: control group, ▼: treatment with different concentrations of MPP⁺ for 24 h. SH-SY5Y cells treated with 2 mM MPP⁺ were used to establish the cytotoxic model of PD. B. Cells were pretreated with different concentrations of MANF (50, 200 and 400 ng/mL) for 2 h before incubation with 2 mM MPP⁺ for 24 h, and cell viability was assessed by MTT assay or cells were incubated with various concentrations of MANF for 24 h. Data are presented as mean ± S.E.M. *P<0.05, **P<0.01 vs MPP⁺-treated group. #P<0.05, ###P<0.01 vs control group.
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Figure 6. Expression of Bax and Bcl-2 in SH-SY5Y cells. A. Cells were pretreated with MANF (400 ng/ml) for 2 h, then incubated with 2 mM MPP+ for 24 h. Immunoblot was applied to measure the proteins expression of Bax and Bcl-2. Values of densitometric analysis were calculated from three independent experiments. GAPDH was used as internal control. B. MPP+-induced increase of Bax/Bcl-2 was attenuated by MANF pretreatment. Data are presented as mean ± S.E.M. *P<0.05, **P<0.01 vs MPP+-treated group. #P<0.05, ##P<0.01 vs control group.

Figure 7. Expression of HMOX1 and SOD2 in SH-SY5Y cells. A. MPP+ up-regulated the expression level of HMOX1, and MANF further strengthened the reaction. B. MANF significantly increased the expression level of SOD2 which was decreased by MPP+ treatment. Data are presented as mean ± S.E.M. *P<0.05, **P<0.01 vs MPP+-treated group. #P<0.05, ##P<0.01 vs control group.

ratio of Bax/Bcl-2 expression in MPP+-treated group compared with the control group, while the ratio decreased in cells pre-treated with MANF (Figure 6), suggesting that MPP+-induced alteration of Bcl-2 and Bax expression are inhibited by MANF treatment.

Expression of HMOX1 and SOD2 in SH-SY5Y cells

Heme oxygenase 1 (HMOX1) and Superoxide dismutase 2 (SOD2) are two of oxidative stress responsive genes, which are involved in anti-oxidative pathway. In this study, MPP+ up-regulated the expression level of HMOX1, and MANF further strengthened the reaction (P<0.01). Meanwhile, MANF significantly increased the expression level of SOD2 (P<0.05) which was decreased by MPP+ treatment (Figure 7). It indicated that MANF might be involved in anti-oxidative pathway.

Mitochondrial membrane potential in SH-SY5Y cells

Results showed 2 mM MPP+ significantly decreased the ratio of red/green fluorescence to 38.4% (P<0.01), indicating the depolarization of mitochondrial membrane. In contrast, pre-treatment with MANF (400 ng/ml) reversed the MPP+-induced reduction of mitochondrial membrane potential (P<0.01) (Figure 8). In brief, MANF decreases the MPP+-induced mitochondrial membrane potential decline.

Discussion

MANF is a novel evolutionarily conserved neurotrophic factor secreted by the endoplasmic
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In vitro and in vivo, MANF has been found to exert prominent neuroprotective effects on nigral dopaminergic neurons [13]. In this study, we further investigated the effects of MANF on the MPTP/MPP⁺-induced PD model and the potential mechanism. These results showed MANF was able to improve the mitochondrial function and inhibit the oxidative stress in both in vivo and in vitro PD model.

Mitochondrial dysfunction and cellular oxidative stress are the most prominent features in PD [14-17]. It has been confirmed that mitochondrial dysfunction may cause oxidative stress and neurodegeneration [3, 18], leading to neuronal loss. These findings indicate MANF can enhance the SOD activity and GSH production, scavenge lipid peroxidation product MDA, inhibit oxidative stress, and alleviate the ROS induced damage to dopaminergic neurons.

MPP⁺ accumulates in the mitochondria after being transported into the dopaminergic neurons, resulting in mitochondrial membrane potential decline [19-21]. In this study, MTT
assay confirmed that MPP+ treatment reduced the viability of SH-SY5Y cells in a dose-dependent manner, which was consistent with previous findings [22]. However, pretreatment with MANF partially reversed the reduction of cell viability caused by MPP+ treatment, which indicates that MANF protects SH-SY5Y cells against MPP+-induced cytotoxicity in a dose-dependent pattern. The mitochondrial depolarization is thought to mediate the neuronal apoptosis induced by MPP+ [23]. There is evidence showing that excess production of mitochondrial ROS is a main cause of mitochondrial depolarization [24]. These results revealed that MANF could partially reverse the decrease of MPP+-induced mitochondrial membrane potential, suggesting that MANF protected mitochondria from oxidative damage, and it might involve in the inhibition of MPP+-induced mitochondrial apoptosis via up-regulated HMOX1 and SOD2 expression which encode antioxidant.

Meanwhile, MPTP may also cause limbs incoordination and motor deficits in mouse PD model. In present study, MPTP significantly impaired the neurological function, but MANF treatment increased the latency in Rotarod test, suggesting that MANF can ameliorate MPTP-induced behavioral deficits.

Immunohistochemistry for TH demonstrated that the TH-positive cells in MANF-treated group remarkably increased as compared to PBS-treated group, suggesting that MANF alleviates the loss of dopaminergic neurons in the substantia nigra after MPTP injection, which is consistent with findings from rat PD model and cell model [5, 13]. These findings indicated the effect of MPTP on the dopaminergic neurons was more prominent than that on behavioral changes. Consistent with the features in human PD, the pathological changes often occur before the onset of clinical symptoms, which maybe related to the compensatory mechanism.

It has been reported that MPTP markedly reduces striatal dopamine, DOPAC and HVA [10]. These results further indicate that the depletion of dopamine, DOPAC and HVA in the striatum was partially inhibited by MANF-treatment, which was consistent with findings from immunohistochemistry for TH. This may be explained as that, in MPTP-induced PD model, MANF preserves more dopaminergic neurons and thus maintains dopamine at a relatively high level. Moreover, the amplitude of variation in dopamine level was not consistent with those in its metabolites, which was also reported in several previous studies [25, 26].

MANF belongs to secretory protein family, but the precise mechanisms and signaling pathway related to its protection on dopaminergic neurons are still poorly understood. There is evidence showing that the neuroprotective effects of MANF may be related to the inhibition of ER stress-induced cell death [27-29]. Studies on MANF structure suggests that the neurotrophic activity may associate to its N-terminal domains and ER stress response to its C-terminal domains [30]. These several previous researches had found the neuroprotective effect of MANF involved in ER stress and autophagy pathways [31-33], the present study demonstrated the neuroprotective effect of MANF may rely on promoting mitochondrial function, inhibiting oxidative stress, rectifying imbalance between oxidative stress and antioxidant system. MANF may exert its neurotrophic role in dopaminergic neurons through multiple methods besides ER stress and autophagy pathways.

However, there were some limitations in this study. The receptor for MANF has not been identified until now, more further studies are needed to elucidate the exact downstream signaling pathways that triggering the specific biological effects of MANF.

In summary, this study reveals MANF may be a promising agent for PD, or other neurodegenerative disorders, and its neuroprotective effects may be ascribed to the improvement of impaired mitochondrial function and inhibition of oxidative stress.

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Disclosure of conflict of interest

None.

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References


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