

Original Article

Nicotinamide mononucleotide adenylyltransferase 1 ameliorates dopaminergic neuron death via attenuating apoptosis and oxidation in MPTP-treated mice

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Abstract: Background: Axonal degeneration is a primary or contributing component of Parkinson's disease (PD). Studies have found that the full sequence of nicotinamide mononucleotide adenylyltransferase 1 (NMNAT1) is the chief component of Wallerian degeneration slow (Wld^s) protein, which could delay axonal degeneration. In addition, Nmnat1 is required and sufficient for the protective effects of Wld^s. However, the role of NMNAT1 in PD has not been clearly established. Methods: In the present study, we established a PD model using the neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). Mice were divided into the following groups: vehicle group, MPTP-treated vehicle group and MPTP-treated NMNAT1-overexpression group. We evaluated motor coordination ability with the rotarod test. The nigrostriatal pathway was tested by TH immunofluorescence staining and catecholamine measurements. In addition, we assessed the possible molecular mechanisms of NMNAT1-mediated neuroprotection via Western blot analysis in the PD model. Results: We found that overexpression of NMNAT1 significantly improved motor coordination ability in MPTP-treated mice. Further studies showed that NMNAT1 could attenuate dopaminergic neural death and increase striatal levels of DA as well as the metabolites DOPAC and HVA in MPTP-treated mice. In addition, we found that overexpression of NMNAT1 abrogated the MPTP-induced decrease in the Bcl-2/Bax ratio and SOD1 expression. Conclusions: Our study demonstrated that overexpression of NMNAT1 could improve motor coordination ability and ameliorate degeneration of the nigrostriatal pathway in MPTP-treated mice. The mechanisms of this NMNAT1-mediated effect might rely on the inhibition of apoptosis and oxidation. Together, these findings reveal novel roles for NMNAT1 in PD and provide new therapeutic avenues for the treatment of the disease.

Keywords: NMNAT1, axonal degeneration, Parkinson's disease

Introduction

Parkinson's disease (PD), affecting as much as 1% of the worldwide population over 60 years of age, is a progressive, neurodegenerative disorder characterized by selective loss of dopaminergic neurons in the substantia nigra (SN), leading to dopamine (DA) depletion in the striatum and the presence of Lewy bodies (LB) in the remaining dopaminergic neurons [1, 2]. Although genetic and environmental factors have been implicated in the etiology of PD, the precise pathogenic mechanism remains unknown. Axonal degeneration is a common component of many neurodegenerative diseases including PD, and growing evidence suggests that axon degeneration precedes neuronal cell death [3, 4]. These findings indicate a promising target for neuroprotective therapy of PD.

In the past, axonal degeneration was thought to be due to the passive death of the distal portion of severed axons caused by nutrient deprivation from the neuronal cell body. However, the first indication that Wallerian degeneration is an active process came from studies of Wallerian degeneration slow (Wld^s) mice in which axonal degeneration was delayed after injury [5]. The Wld^s mutation is a translocation, resulting in the overexpression of Wld^s, which is composed of the N-terminal 70 amino acids of the ubiquitination factor E4b (Ube4b) linked to full-length NMNAT1 by an 18 amino acid linker region [6].

The full sequence of NMNAT1, the chief component of Wld^s protein, is a nicotinamide adenine dinucleotide (NAD⁺) synthesizing enzyme [7]. Though the axonal protection of NMNAT1 or its

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product NAD⁺ has been reproduced in a number of studies, the effect of NMNAT1 on Parkinson's disease has not been clearly established [8, 9]. In the present study, we demonstrated that overexpression of NMNAT1 can improve motor coordination ability and ameliorate degeneration of the nigrostriatal pathway in MPTP-treated mice. The mechanisms of this NMNAT1-mediated effect might rely on inhibition of apoptosis and oxidation.

Materials and methods

Chemicals and drugs

Unless otherwise stated, all chemicals were purchased from Sigma Chemical Co. (St. Louis, MO, USA). A primary antibody against NMNAT1 was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). A primary antibody against tyrosine hydroxylase (TH) was from Millipore (Billerica, MA, USA). All other chemicals and reagents were of the highest grade available from local commercial sources.

Animal and drug treatment

All experiments were conducted in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. This study was approved by the Committee on the Ethics of Animal Experiments of Qingdao University. All efforts were made to minimize animal suffering. Ten-week-old C57BL/6 mice were housed at room temperature under a 12-h light/dark cycle, and all mice were male. The mice were provided access to food and water ad libitum. For the chronic MPTP regimen, mice were intraperitoneal injected with MPTP hydrochloride (30 mg/kg) in phosphate-buffered saline (PBS) or PBS alone twice a week for 5 weeks. The mice were sacrificed by decapitation 5 weeks after MPTP or saline injection, and the brains were removed for analysis.

Intracerebral administration of viral vectors

Mus musculus green fluorescent protein (GFP) and NMNAT1 overexpressing lentiviruses (LV-NMNAT1) were produced and titrated as described by Shanghai Shengbo Biomedical Technologies Inc [10]. Lentiviral vectors coding for GFP (LV-GFP) were used as a control. In addition, NMNAT1 was connected to GFP in

LV-NMNAT1. A week before the induction of the PD model, viral preparations (0.4 μ L, LV-NMNAT1 3×10^8 TU/ml, LV-GFP 5×10^8 TU/ml) in a 2 μ L volume were injected bilaterally into the SN of mice according to the stereotaxic atlas of the mouse brain using the following coordinates: ± 1.3 mm medial/lateral, -3.0 mm anterior/posterior, and -4.7 mm dorsal/ventral from the bregma. The preparation was injected with a speed of 0.2 μ L/min over a period of 2 minutes by a 28 G needle [11]. Before waking, the mice were allowed to recover in a heated chamber.

All mice were divided into three groups as follows: Vehicle group (N = 12): mice were given LV-GFP injections in the SN and intraperitoneal injections of PBS; MPTP group (N = 12): mice were given LV-GFP injections in the SN and intraperitoneal injections of MPTP; NMNAT1 group (N = 12): mice were given LV-NMNAT1 injections in the SN and intraperitoneal injections of MPTP.

Western blot analysis

The right part of the SN was exactly dislodged from the mouse brains, washed with ice-cold PBS and lysed with lysis buffer and protease inhibitors. The lysates were centrifuged for 20 min at 12,000 rpm at 4°C, and the supernatants were used for protein concentration determination by a Bradford assay kit. Total proteins were separated by 10% SDS-polyacrylamide gel electrophoresis and then transferred to polyvinylidene fluoride (PVDF) membranes. After being blocked for 2 hours in 10% non-fat milk at room temperature, the membranes were incubated overnight with rabbit NMNAT1 (1:500), rabbit TH (1:5000), rabbit SOD1 (1:500), rabbit Bcl-2 (1:1000) and rabbit Bax (1:1000) primary antibodies at 4°C. A secondary antibody conjugated to horseradish peroxidase was used at 1:10000. Blots were also probed with an anti- β -actin monoclonal antibody (1:10000) as a loading control. Cross-reactivity was visualized using ECL Western blotting detection reagents and analyzed by scanning densitometry using a UVP Image System (Upland, CA, USA).

Rotarod test

Motor coordination and balance were assessed using a rotarod apparatus (Med Associates, USA). After the chronic PD model was produced,

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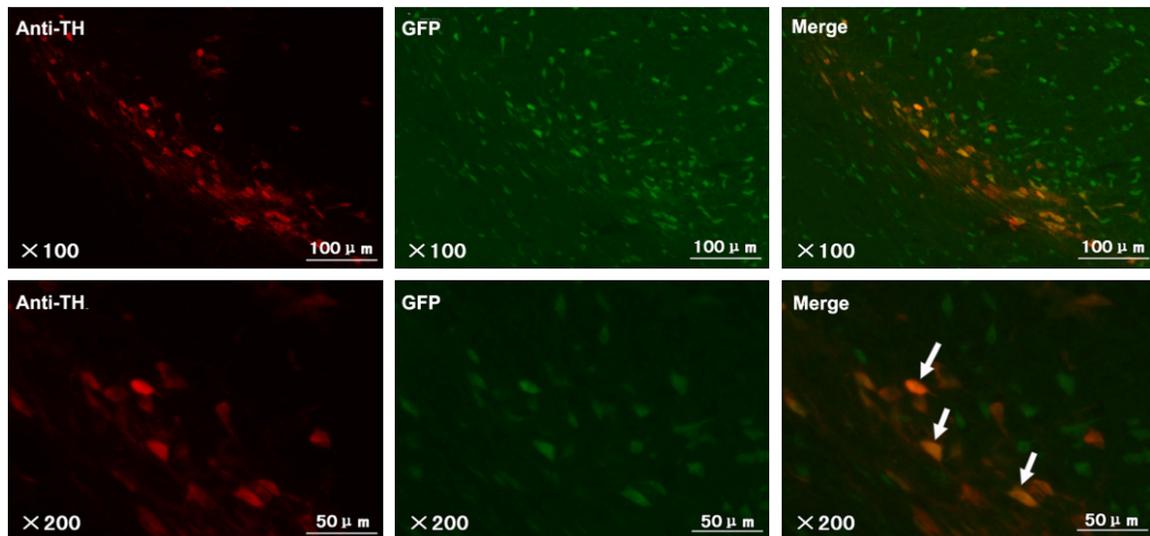


Figure 1. Lentivirus-mediated expression of NMNAT1 in the SN. TH immunofluorescence staining on the SN sections demonstrates the localization of the NMNAT1 protein within TH-positive dopaminergic neurons (as indicated by the arrow).

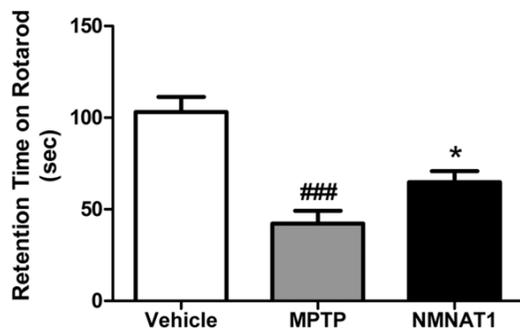


Figure 2. NMNAT1 improved the motor coordination ability of MPTP-intoxicated mice. Motor coordination ability was assessed by the rotarod test. The residence time of mice on rotarod treadmills in NMNAT1 group is longer than that in the MPTP group (* $P<0.05$). In addition, the residence time markedly decreased in mice following MPTP treatment (### $P<0.001$). $N = 10$. Error bars equal SEM.

the mice were placed on a rolling rod with an initial speed of 4 rpm. Then, two trials with an interval trial time of one hour were performed using an accelerating speed level (4 to 40 rpm) mode of the apparatus. The mean latency to fall off the rotarod was recorded.

TH immunofluorescence staining

The animals were anesthetized with 8% chloral hydrate (400 mg/kg, i.p.). The mice were sacrificed by decapitation. The brains were removed, and the left part of the brains was post fixed in

4% paraformaldehyde for 12 hours and transferred to 30% sucrose until sectioning. Sections (20 μm) were cut on a freezing microtome (Leica, Germany). According to the stereotaxic atlas of the mouse brain, alternating SN sections (30 slices) were used for TH immunostaining. After three washes in 0.1 mol/L PBS plus 0.1% Triton X-100, the sections were incubated overnight with a rabbit TH (1:2000) primary antibody at 4°C. Then, the sections were washed three times with PBS and incubated in an Alexa Fluor 555 donkey anti-rabbit IgG secondary antibody for 2 h at room temperature. Next, the sections were rinsed with PBS three times and subsequently dehydrated in 80% ethanol for 5 min. The slides were rinsed with distilled water, mounted in 70% glycerin, and examined using a fluorescence microscope (Carl Zeiss, Germany).

DA measurements

The right striatum was homogenized in lysis buffer for the analysis of dopamine (DA) and its metabolites using high-pressure liquid chromatography (HPLC). Samples were diluted 1:20 in HPLC buffer, separated on a catecholamine ESA HR-80 column (ESA, Chelmsford, MA) using the same HPLC buffer for the mobile phase, and analyzed by electrochemical detection (ESA) against prerun standards (DA; 3,4-dihydroxyphenylacetic acid, DOPAC; and homovanillic acid, HVA).

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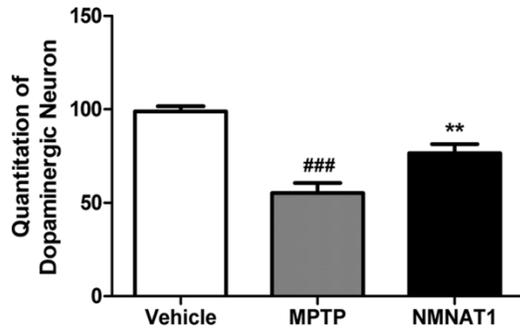


Figure 3. NMNAT1 attenuated dopaminergic neural death in MPTP-treated mice. Loss of TH-positive cells in the SN is observed in mice following MPTP treatment. Counts of TH-positive neurons demonstrate significant MPTP-induced dopaminergic neuron loss (### $P < 0.001$). Dopaminergic neurons of the mice from the NMNAT1 group are protected against MPTP toxicity compared with those from the MPTP group (** $P < 0.01$). $N = 6$. Error bars equal SEM.

Statistical analysis

SPSS 19.0 was used to analyze the data. All data are expressed as the mean values \pm SEM. Differences between means in two groups were compared using unpaired two-tailed t-test. One-way analysis of variance (ANOVA) followed by Student-Newman-Keuls test were used to compare the differences between means in >2 groups. $P < 0.05$ was considered statistically significant.

Results

Lentivirus-mediated expression of NMNAT1 in the SN

To investigate the efficiency and stability of our lentivirus-mediated transfer, we analyzed the pattern of SN GFP fluorescence in mice after LV-GFP or LV-NMNAT1 delivery. In the LV-NMNAT1 mice, the GFP fluorescence was prominently localized to the injection site (**Figure 1**). In addition, NMNAT1 was connected to GFP in LV-NMNAT1. **Figure 1** shows high viral transfection efficiency. In addition, brain sections from some noninjected animals and lentiviral transduction animals were stained with TH, and immunoreactivity in the SN was quantitatively assessed. We found no evidence of neuronal injury in the SN, indicating that cytotoxicity caused by the lentiviral vector or the injection itself was negligible.

We next investigated the protein expression levels of NMNAT1 in the SN of mice. The results showed that the expression level of NMNAT1 protein was nearly 2.4 times higher in the NMNAT1 group than that in the MPTP group (**Figure 5B**). This result further demonstrated the success of LV-NMNAT1 transfection.

NMNAT1 improved the motor coordination ability of MPTP-intoxicated mice

Compared with the mice from the vehicle group, the mice from the MPTP group manifested PD symptoms such as decreased body weight and reduced autonomic activity. The rotarod test was used to evaluate the motor coordination of the mice. As shown in **Figure 2**, the residence time obviously decreased in mice following MPTP treatment ($P < 0.001$), which indicated that the chronic PD model was successfully produced. Next, we found that the residence time of mice on rotarod treadmills in the NMNAT1 group was longer than that in the MPTP group ($P < 0.05$). These results showed that NMNAT1 could improve the injured motor coordination ability in MPTP-treated mice.

NMNAT1 attenuated dopaminergic neural death in MPTP-treated mice

TH-positive neurons in alternating SN sections (30 slices) of every mouse were counted, and the average count of TH-positive neurons per slice was calculated. Consistent with previous studies, dopaminergic neurons (TH-positive) were significantly decreased (**Figure 3**). However, this effect was reversed by NMNAT1 over-expression (**Figure 3**), indicating that NMNAT1 could protect dopaminergic neurons against cell death in mice treated with MPTP.

NMNAT1 ameliorated degeneration of the nigrostriatal pathway in MPTP-treated mice

Dopaminergic function was gravely damaged in mice following treatment with MPTP, as demonstrated by quantitation of DA (**Figure 4A**) and the metabolites DOPAC and HVA (**Figure 4B**) ($P < 0.001$). Further study showed that striatal levels of DA (**Figure 4A**) in the NMNAT1 group were over 2-fold greater than those in the MPTP group ($P < 0.001$). In addition, the metabolites DOPAC and HVA (**Figure 4B**) were also significantly increased.

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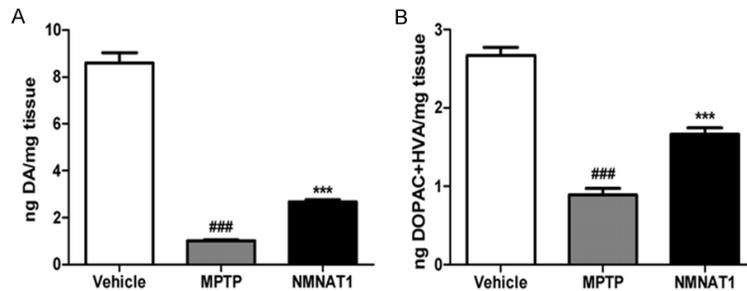


Figure 4. NMNAT1 ameliorated degeneration of the nigrostriatal pathway in MPTP-treated mice. Dopaminergic function is gravely damaged in mice following treatment with MPTP, as demonstrated by quantitation of DA (A) and the metabolites DOPAC and HVA (B) (### $P < 0.001$). Striatal levels of DA (A) and the metabolites DOPAC and HVA (B) in the mice of the NMNAT1 group are over 2-fold greater than those of the MPTP group (** $P < 0.001$). $N = 6$. Error bars equal SEM.

These results suggested that NMNAT1 could ameliorate the decreased dopaminergic function induced by MPTP in mice.

Expression changes in TH, superoxide dismutase 1 (SOD1), B-cell lymphoma-2 (Bcl-2) and Bcl-2-associated X (Bax) of dopaminergic neurons in MPTP-treated mice

Since TH is a specific DA marker, we analyzed the protein levels of TH via Western blotting in the different groups. As shown in **Figure 5**, the protein levels of TH in the SN of mice from the NMNAT1 group were significantly higher than those in mice from the MPTP group ($P < 0.001$).

SOD1, a potentially protective enzyme that could suppress oxidative stress, was significantly upregulated in the NMNAT1 group relative to the MPTP group ($P < 0.001$). In addition, compared with the MPTP group, the NMNAT1 group exhibited significant upregulation of the anti-apoptotic molecule Bcl-2 ($P < 0.01$) and downregulation of the pro-apoptotic molecule Bax ($P < 0.001$). Meanwhile, the Bcl-2/Bax ratio was notably elevated ($P < 0.001$).

These results in part demonstrated that the beneficial roles of NMNAT1 in MPTP-treated mice might rely on regulating the expression of SOD1 and Bcl-2/Bax.

Discussion

The most important goal of research into neurodegenerative disorders such as PD is the identification and reversal of the earliest pathological changes in the affected neural systems.

Recent neuropathological and experimental studies suggest that the degeneration of dopaminergic terminals and axons precedes the demise of dopaminergic neurons in the SN, which ultimately results in the clinical symptoms of PD [12]. The first indication for Wallerian degeneration is an active process, which came from studies of Wld^s mice, in which axonal degeneration is delayed after injury [5]. Interestingly, besides axon protection, Wld^s can also prevent symptoms and rescue motor neurons when crossed with a

mouse model of progressive motor neuronopathy [13].

The full sequence of NMNAT1, the chief component of the Wld^s protein, is a NAD⁺ synthesizing enzyme [7]. At molecular level, although Wld^s is a fusion protein, $Nmnat1$ is required and sufficient for the protective effects of Wld^s . The axonal protection of NMNAT1 or its product NAD⁺ has been reproduced in a number of studies [8, 9]. Thus, we set out to investigate the role of NMNAT1 in PD. In this study, we used a well-described PD model to show that NMNAT1 markedly decreases neuronal loss and protects DA function as well as motor coordination ability against MPTP-induced injury.

What are the mechanisms underlying the protective effects of NMNAT1 in PD? To our current knowledge, several important but not mutually exclusive mechanisms may contribute to the progressive neurodegeneration in PD. These changes include oxidative stress, apoptosis, neuroinflammation and impairment of the ubiquitin-proteasome system [14]. For instance, oxidative damage to mitochondrial proteins has been observed in the brains of PD patients. This damage can be reproduced in isolated mitochondria treated with rotenone [15]. Therefore, antioxidants such as SOD1 can reduce reactive oxygen species (ROS) and protect dopaminergic neurons in the MPTP, 6-OHDA, rotenone and genetic models of PD both in vitro and in vivo [16, 17]. In the present study, SOD1 was significantly upregulated in the dopaminergic neurons of mice from the NMNAT1 group compared with that in mice from the MPTP

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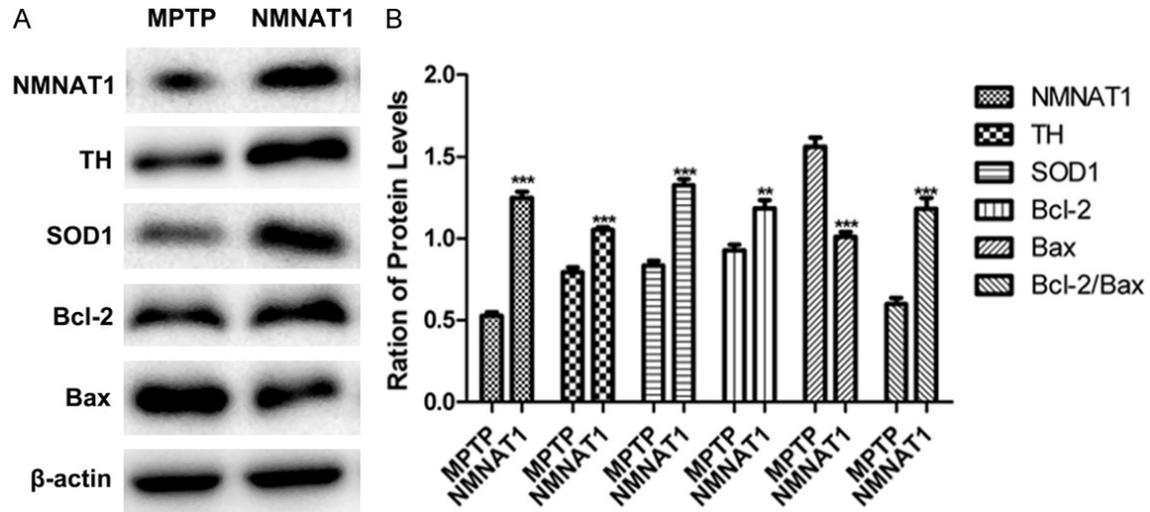


Figure 5. Determination of NMNAT1, TH, SOD1, Bcl-2, and Bax levels in the SN of C57BL/6 mice by Western blot analysis. A. Representative Western blots of SN lysates demonstrate significant increase in TH, SOD1, and Bcl-2, as well as a decrease in Bax in the NMNAT1 group compared with those in the MPTP group. B. Quantitation of band intensities is for statistical analysis (** $P < 0.01$, *** $P < 0.001$). $N = 6$. Error bars equal SEM.

group, which suggests that the beneficial roles of NMNAT1 in mice are possibly mediated by regulating the expression of SOD1, which could mitigate the effects of ROS.

Recent studies have suggested that the final death pathway of dopaminergic neurons in PD is connected with programmed necrosis or apoptosis, which may be linked to autophagy [18, 19]. Several key regulators of apoptosis, such as Bcl-2 and DAP (death-associated protein)-kinase, have also been shown to control some aspects of the autophagic process [20]. Using Western blot analysis, we showed that the upregulation of the anti-apoptotic molecule Bcl-2 and downregulation of the pro-apoptotic molecule Bax were prominently detected in mice of the NMNAT1 group. Meanwhile, the Bcl-2/Bax ratio was notably elevated. Therefore, the beneficial roles of NMNAT1 in mice are also possibly mediated by inhibition of apoptosis. There has been some controversy regarding whether NMNAT1 alone could exert protective effects. Antenor-Dorsey [21] suggested that Wid^S but not $Nmnat1$ protects dopaminergic neurons from MPP⁺ neurotoxicity. Others found that overexpressing $Nmnat1$ alone could mimic the protective effects of Wid^S [22, 23]. The most plausible explanation is that different expression levels of NMAT1 may lead to various results. For example, in *Drosophila*, the expression of mouse $Nmnat1$ in

ORNs using the Gal4/UAS binary expression system resulted in extremely strong protection of severed axons, which could be attributed to the fact that Gal4/UAS expresses extremely high levels of $Nmnat1$ in *Drosophila* ORNs [24]. Perhaps experiments aimed at observing the effects of different levels of NMNAT1 in PD models might provide new evidence.

In the present study, we demonstrated that overexpression of NMNAT1 could improve motor coordination ability and ameliorate degeneration of the nigrostriatal pathway in MPTP-treated mice. Moreover, our study sheds light on some molecular biological mechanisms of the neuroprotection of NMNAT1, which might be mediated by regulating the expression of SOD1 and Bcl-2/Bax. Although additional studies of models of neurodegenerative conditions may uncover the extent of involvement of these mechanisms in the etiology of PD, this study reveals a novel role for NMNAT1 in PD, suggesting new therapeutic avenues for the disease.

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Disclosure of conflict of interest

None.

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