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Mitoapocynin Treatment Protects Against Neuroinflammation and Dopaminergic Neurodegeneration in a Preclinical Animal Model of Parkinson's Disease

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Abstract

Mitochondrial dysfunction, oxidative stress and neuroinflammation have been implicated as key mediators contributing to the progressive degeneration of dopaminergic neurons in Parkinson's disease (PD). Currently, we lack a pharmacological agent that can intervene in all key pathological mechanisms, which would offer better neuroprotective efficacy than a compound that targets a single degenerative mechanism. Herein, we investigated whether mito-apocynin (Mito-Apo), a newly-synthesized and orally available derivative of apocynin that targets mitochondria, protects against oxidative damage, glial-mediated inflammation and nigrostriatal neurodegeneration in cellular and animal models of PD. Mito-Apo treatment in primary mesencephalic cultures significantly attenuated the 1-methyl-4-phenylpyridinium (MPP⁺)-induced loss of tyrosine hydroxylase (TH)-positive neuronal cells and neurites. Mito-Apo also diminished MPP⁺-induced increases in glial cell activation and inducible nitric oxide synthase (iNOS) expression. Additionally, Mito-Apo decreased nitrotyrosine (3-NT) and 4-hydroxynonenol (4-HNE) levels in primary mesencephalic cultures. Importantly, we assessed the neuroprotective property of Mito-Apo in the MPTP mouse model of PD, wherein it restored the behavioral performance of MPTP-treated mice. Immunohistological analysis of nigral dopaminergic neurons and monoamine measurement further confirmed the neuroprotective effect of Mito-Apo against MPTP-induced nigrostriatal dopaminergic neuronal loss. Mito-Apo showed excellent brain bioavailability and also markedly attenuated MPTP-induced oxidative markers in the substantia nigra (SN). Furthermore, oral administration of Mito-Apo significantly suppressed MPTP-induced glial cell activation, upregulation of proinflammatory cytokines, iNOS and gp91phox in IBA1-positive cells of SN. Collectively, these results demonstrate that the novel mitochondria-targeted compound Mito-Apo

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Conflict of Interest

A.G.K. and B.K. hold a patent entitled "Neuroprotective Compounds and Their Use" for the Mitoapocynin treatment of Parkinson's disease licensed to PK Biosciences Corporation (Ames, IA). The other authors have no conflicts of interest.

exhibits profound neuroprotective effects in cellular and pre-clinical animal models of PD by attenuating oxidative damage and neuroinflammatory processes.

Keywords

Mito-apocynin; microglia; Parkinson's disease; mitochondria; neuroinflammation; drug therapy

Introduction

Parkinson's disease (PD) is a common neurodegenerative movement disorder characterized by motor and non-motor symptoms (Dauer and Przedborski, 2003; Vila and Przedborski, 2004). The pathological hallmarks of PD are the loss of dopaminergic neurons and their terminals in the nigrostriatal axis and the appearance of Lewy bodies in the surviving neurons in the substantia nigra pars compacta (SNpc). The underlying cellular and molecular mechanisms that cause the disease are not yet known, but studies suggest that mitochondrial dysfunction, oxidative stress, and glia-mediated inflammation increase the risk of developing PD (Dauer and Przedborski, 2003; Goldman, 2014; Blesa et al., 2015; Klingelhoefer and Reichmann, 2015; Mullin and Schapira, 2015). Sustained microglial and astroglial activation near dying dopaminergic neurons in the SNpc is evident in PD patients and animal models of the disease (McGeer et al., 1988; Hirsch and Hunot, 2009). Several *in vitro* cell culture and *in vivo* animal studies have demonstrated the elevation of key enzymes involved in the production of reactive oxygen species (ROS) due to mitochondrial impairment and reactive nitrogen species (RNS) in dopaminergic neurons of the SN, including microglial gp91phox (Nox2) and inducible nitric oxide synthase (iNOS) (Gao et al., 2003b; Wu et al., 2003; Hunter et al., 2007; Hirsch et al., 2012; Murakami and Hirano, 2012; Strowig et al., 2012; Yan et al., 2014; Rocha et al., 2015). In addition, different pro-inflammatory cytokines, including tumor necrosis factor alpha (TNF- α), interleukins IL-1 β and IL-6, interferon gamma (IFN- γ), as well as other immune neurotoxins, are found either in the CSF or affected regions of PD brains (Nagatsu et al., 2000). However, the mechanism of glial cell activation in the diseased state is not completely known. Collectively, these findings suggest that targeting multiple pathogenic mechanisms, including mitochondrial impairment, oxidative stress and inflammatory processes, could provide an advantage over targeting a single disease process, which is the strategy often tested by others for PD.

Several inhibitors of NADPH oxidase have been tested for their anti-inflammatory and antioxidant effects in dopaminergic cells, including apocynin (4-hydroxy-3-methoxyacetophenone), a non-toxic plant-derived molecule that is well-tolerated in cell culture and animals models of PD (Gao et al., 2003a; Anantharam et al., 2007; Cristovao et al., 2009). We recently demonstrated diapocynin to be neuroprotective and anti-neuroinflammatory in the MPTP animal model as well as in the progressively degenerative LRRK2^{R1441G} transgenic mouse model (Ghosh et al., 2012b; Dranka et al., 2013). In these studies, both apocynin and diapocynin were orally administered at 300 mg/kg body weight. Although these high doses were tolerated in animals, more efficacious apocynin analogs are needed. To this effect, we designed a new class of apocynins (mito-apocynins) designed to

enhance their cellular and mitochondrial uptake. Mito-apocynins were synthesized by the hybrid technique of attaching a mitochondria-targeting group to apocynin (Jin et al., 2014).

In this study, we synthesized Mito-Apo (apocynin conjugated to the mitochondria-targeting triphenylphosphonium cation moiety TPP⁺) (Fig. 1.) and investigated its preclinical efficacy in both cell culture and animal models of PD by determining its antioxidant and anti-inflammatory properties. The presence of a highly lipophilic and delocalized cationic moiety in Mito-Apo makes it more cell-permeable and enhances its availability to mitochondria. Here, we demonstrate that Mito-Apo attenuates MPP⁺-induced dopaminergic neurodegeneration in primary mesencephalic neuronal cultures by attenuating glia-mediated inflammatory reactions and subsequent oxidative damage to dopaminergic neurons. Notably, oral administration of Mito-Apo reaches to the nigrostriatal region in mice where it protects dopaminergic neurons and terminals in an MPTP-mouse model of PD by mitigating glial activation and glia-mediated inflammatory reactions and oxidative stress.

Materials and Methods

Chemicals and biological reagents

1-Methyl-4-phenylpyridinium (MPP⁺ iodide) and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP-HCl) were purchased from Sigma (St. Louis, MO, USA). RPMI, neurobasal medium, B27 supplement, fetal bovine serum (FBS), L-glutamine, IR-dye-tagged secondary antibodies, penicillin, and streptomycin and other cell culture reagents were purchased from Invitrogen (Gaithersburg, MD).

Mito-apocynin synthesis

The novel compound Mito-apocynin (Mito-Apo) was synthesized by modifying the previously described protocol for Mito-Q and long chain Mito-Apo-C₁₁ synthesis (Ghosh et al., 2010; Dranka et al., 2014). Figure 1 shows the key steps involved in the synthesis. Briefly, acetylvannillic acid chloride was synthesized from first mixing acetylvannillic acid with thionyl chloride, and then the mixture was dissolved in dichloromethane and aminoethyltriphenylphosphonium bromide and pyridine. The acetylated Mito-Apo was purified on a silica gel column followed by removal of the acetyl protective group. The final product was purified and characterized by HPLC and LC-MS as described previously (Ghosh et al., 2010; Dranka et al., 2014).

Primary neuronal cultures and treatments

Primary mesencephalic and primary striatal cultures were prepared from the ventral mesencephalon and striatum regions respectively from gestational 14-day-old mouse embryos as described previously (Ghosh et al., 2013; Ngwa et al., 2014). Briefly, mesencephalic tissues from E14 mouse embryos were dissected and maintained in ice-cold calcium-free Hank's balanced salt solution and then dissociated in Hank's balanced salt solution containing trypsin-0.25% EDTA for 20 min at 37°C. The dissociated cells were then plated at an equal density of 0.6 million cells per well on 12-mm coverslips precoated with 0.1 mg/ml poly-D-lysine. Cultures were maintained in neurobasal media fortified with B-27 supplement, 500 mM L-glutamine, 100 IU/ml penicillin, and 100 µg/ml streptomycin.

The cells were maintained in a humidified CO₂ incubator (5% CO₂ and 37°C) for 24 h. One-half of the culture media was replaced every other day. Approximately 6- to 7-day-old cultures were used for experiments. Both primary mesencephalic and striatal neuronal cells were exposed to 10 μM MPP⁺ in the presence or absence of Mito-Apo (10 μM) for 24 h.

Primary microglial and astroglial cultures and treatments

Primary microglial and astroglial cultures were prepared from C57BL/6 postnatal day 1 (P1) mouse pups. Mouse brains were harvested, meninges removed, and then placed in Dulbecco's modified Eagle's medium/F-12 nutrient media (DMEM-F12, GIBCO Cat # 11320) supplemented with 10% heat-inactivated FBS, 50 U/mL penicillin, 50 μg/mL streptomycin, 2 mM L-glutamine, 100 μM non-essential amino acids, and 2 mM sodium pyruvate (Invitrogen). The brain tissue was then incubated in 0.25% trypsin for 30 min with gentle agitation. The trypsin reaction was stopped by adding an equal volume of DMEM/F12 complete media, and the brain tissue was washed three times. The tissue was then triturated gently to prepare a single cell suspension and then passed through a 70-μm nylon mesh cell strainer to remove tissue debris and aggregates. Next, the cell suspension was made up in DMEM/F12 complete media and seeded into T-75 flasks. Cell cultures were placed in a humidified CO₂ incubator at 37°C where the mixed glial cells were grown to confluence in media that was changed after 4 to 5 days. Using differential adherence and magnetic separation, microglial cells were separated from confluent mixed glial cultures to >97% purity, as described in our recent publication (Gordon et al., 2011). Both microglia and astrocytes were allowed to recover for at least 48 h after plating. Primary microglia and astrocytes were treated in DMEM/F12 complete media containing 2% FBS.

Immunocytochemistry

The primary mesencephalic and glial cultures were fixed with 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS) for 20 min and processed for immunocytochemical staining, as described previously (Ghosh et al., 2010). First, nonspecific sites were blocked with 2% bovine serum albumin, 0.5% Triton X-100 and 0.05% Tween-20 in PBS for 45 min at RT. Cells were then incubated overnight at 4°C with different primary antibodies such as TH (1:1600, mouse monoclonal; EMD Millipore, Billerica, MA) and GFAP (1:1000, mouse monoclonal; EMD Millipore). Appropriate secondary antibodies (Alexa Fluor 488 or 594 or 555; Invitrogen) were used followed by incubation with 10 μg/ml Hoechst 33342 (Invitrogen) for 5 min at RT to stain the nucleus. Coverslips containing stained cells were washed twice with PBS and mounted on poly-D-lysine-coated slides (Sigma, St Louis). Images of cells were captured with a SPOT digital camera (Diagnostic Instruments, Inc., Sterling Heights, MI) attached to an inverted fluorescence microscope (model TE-2000U; Nikon, Tokyo, Japan).

Quantification of TH-positive cell numbers and neuronal processes

From each cover-slipped primary neuronal culture, the number of TH-positive dopaminergic neurons and the length of their neuronal processes were quantified using MetaMorph software, Version 5.0 (Molecular Devices) as described previously (Ghosh et al., 2013). For counting TH cells, pictures were first taken at 20x magnification, then thresholded. After that, neurons were counted using the Integrated Morphometry Analysis function. For

measurement of neuronal processes, pictures were taken using a 60x oil immersion lens, and the lengths of the processes were marked by applying the region and length measurement function in the Integrated Morphometry analysis.

High-affinity [³H]dopamine uptake assays

Cells in each well were washed twice with 1 ml of Krebs-Ringer buffer (16 mM NaH₂PO₄, 16 mM Na₂HPO₄, 119 mM NaCl, 4.7 mM KCl, 1.8 mM CaCl₂, 1.2 mM MgSO₄, 1.3 mM EDTA, and 5.6 mM glucose, pH 7.4). The cells were then incubated with 25 nM [³H]dopamine in Krebs-Ringer buffer (0.4 ml/well) for 20 min at 37°C. Nonspecific uptake of dopamine was determined in parallel wells incubated with tritiated dopamine and 10 μM mazindol, an inhibitor of neuronal dopamine uptake. Briefly, the cells were washed 3 times with ice-cold Krebs-Ringer buffer (1 ml/well) and lysed with 1 N NaOH (0.5 ml/well). After washing, lysates were mixed with 5 ml of scintillation fluid (Scinti-verse BD), and radioactivity was determined with a liquid scintillation counter (Tri-Carb 1600 TR; Packard, Meriden, CT). Specific dopamine uptake was calculated by subtracting the amount of radioactivity observed in the presence of mazindol from that observed in its absence.

Animals and treatments

Eight- to ten-week-old male C57BL/6 mice weighing 24 to 28 g were housed in standard conditions: constant temperature (22 ± 1°C), relative humidity (30%), and a 12-h light cycle. Animal experiments were approved and supervised by the Institutional Animal Care and Use Committee (IACUC) at Iowa State University (Ames, IA).

Sub-acute treatment paradigm—Mice received Mito-Apo (3 mg/kg/day) in 10% ethanol by oral gavage beginning 1 day before the MPTP treatment, 5 days of co-treatment with MPTP (25 mg/kg/day, intraperitoneal injection), Mito-Apo treatment continued daily for another 6 days and mice were sacrificed 24 h later. Control mice received 10% ethanol in saline.

Acute Paradigm—Mice received Mito-Apo (3 mg/kg/day) in 10% ethanol by oral gavage beginning 1 day before the MPTP treatment, 3 days of co-treatment with MPTP (25 mg/kg/day, intraperitoneal injection). Mice were sacrificed 24 h after the last dose of MPTP. Control mice received 10% ethanol in saline.

HPLC analysis of striatal dopamine and its metabolites

Samples were prepared and quantified as described previously (Ghosh et al., 2010). In brief, 7 days after MPTP injection, mice were sacrificed and striata were collected and stored at -80°C. On the day of analysis, neurotransmitters from striatal tissues were extracted using an antioxidant extraction solution (0.1 M perchloric acid containing 0.05% Na₂EDTA and 0.1% Na₂S₂O₅) and isoproterenol (internal standard). Dopamine (DA), 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) were separated isocratically using a reversed-phase column with a flow rate of 0.6 mL/min. An HPLC system (ESA Inc., Bedford, MA) with a thermostatted automatic sampler (model 542; ESA Inc.) was used for these experiments. The electrochemical detection system consisted of a Coulochem model 5100A with a microanalysis cell (model 5014A, ESA Inc.) and a guard cell (model 5020, ESA

Inc.). Data acquisition and analysis were performed using EZStart HPLC Software (ESA Inc.).

HPLC detection of Mito-Apo in the brain

Mice were administered 3 mg/kg Mito-Apo by oral gavage and then sacrificed at 1, 2, 4, or 24 h (n=4 per time-point). Samples of various brain regions were dissected out and stored at -80°C. Tissue homogenates were then made using an antioxidant buffer containing 0.2 M perchloric acid, 0.1% Na₂S₂O₅, and 0.05% Na₂EDTA. The standards prepared and used for quantification were 0.1 µg, 0.3 µg, 1.0 µg, 10 µg, and 30 µg of Mito-Apo. Samples were centrifuged and passed through a 0.2-µm filter before analysis on an Agilent Technologies 1200 Series HPLC system. Samples were injected at a volume of 20 µL. Separation was performed with a Kinetex C18 column, using a gradient elution (solvent A = 90% water, 10% acetonitrile and solvent B = 100% acetonitrile) operated over seven minutes at a flow rate of 1.5 mL/min and a temperature of 40°C. Mito-Apo was quantified by UV absorbance (collected at wavelengths of 262 nm and 292 nm) with an average retention time of 3.8 min. Standards were fit by linear regression and data were analyzed using GraphPad Prism 4.0 software (GraphPad Software Inc., San Diego, CA).

qRT-PCR

After treatment, total RNA was extracted from the SN of mouse brains using Absolutely RNA Miniprep Kit (Agilent Technologies, Santa Clara, CA) following the manufacturer's protocol. Total RNA was treated with DNase I to remove DNA contamination and then reversibly transcribed into first-strand cDNA using the SuperScript III first-strand synthesis system (Invitrogen, Carlsbad, CA) as described in the kit instructions. SYBR-green quantitative PCR was performed with validated primers of TNF-α, IL-1β, iNOS, and IL-6 as well as control 18S primers (Qiagen, Valencia, CA) using RT² SYBR[®] Green qPCR Master Mix (SABiosciences, Frederick, MD).

Western blotting

Mice were sacrificed three days after MPTP treatment and the SN was dissected out. SN lysates containing equal amounts of protein were loaded in each lane and separated in a 10-15% SDS-polyacrylamide gel, as described previously (Jin et al., 2011). Proteins were transferred to a nitrocellulose membrane and nonspecific binding sites were blocked with Licor Odyssey blocking buffer. The membranes were then incubated with different primary antibodies such as anti-IBA-1 (Abcam), anti-GFAP (EMD Millipore), anti-iNOS (Santa Cruz), anti-gp91phox (Abcam), anti-3NT (EMD Millipore) and anti-4HNE (R & D Systems). Next, membranes were incubated with one of the following secondary antibodies: Alexa Fluor 680 goat anti-mouse, Alexa Fluor 680 donkey anti-goat (Invitrogen) or IR dye 800 donkey anti-rabbit (Rockland). To confirm equal protein loading, blots were reprobbed with a β-actin antibody (Sigma; 1:10000 dilution). Western blot images were captured with a Licor Odyssey machine (Licor, NE), and the bands were quantified using NIH ImageJ software.

Immunohistochemistry (IHC)

Three days after the last MPTP treatment, mice were perfused with 4% PFA and post-fixed with PFA and 30% sucrose. Next, fixed brains were cut into 30- μ m sections and were incubated overnight at 4°C with different primary antibodies such as anti-IBA-1, anti-GFAP, anti-iNOS, anti-3NT, anti-gp91phox and anti-4HNE. Appropriate secondary antibodies (Alexa Fluor 488, 594 or 555 from Invitrogen) were used, followed by incubation with 10 μ g/ml Hoechst 33342 for 5 min at RT to stain the nucleus. Images of sections were captured with a SPOT digital camera (Diagnostic Instruments) attached to an inverted fluorescence microscope (Nikon TE-2000U).

DAB immunostaining and stereological counting

DAB immunostaining was performed in striatal and SN sections, as described previously (Ghosh et al., 2009; Dranka et al., 2013). In brief, 30- μ m sections were incubated overnight at 4°C with one of the following primary antibodies: anti-TH antibody (EMD Millipore, rabbit anti-mouse, 1:1800), anti-IBA-1 (Abcam, goat anti-mouse, 1:1000) or anti-GFAP (EMD Millipore, mouse anti-mouse, 1:1000). Next, sections were incubated in biotinylated anti-rabbit, goat or mouse secondary antibody followed by incubation with avidin peroxidase (Vectastain ABC Elite kit, Vector laboratories). Immunolabeling was observed using diaminobenzidine (DAB), which yielded a brown stain. Total numbers of TH-positive neurons in SN were counted stereologically with Stereo Investigator software (MBF Bioscience, Williston, VT, USA) using an optical fractionator (Ghosh et al., 2010).

Behavioral measurements

Four days after the last MPTP injection, open-field and rotarod experiments were conducted as described previously (Ghosh et al., 2010). Briefly, a computer-controlled open-field test (Versamax monitor, model RXYZCM-16, AccuScan, Columbus, OH) was used to measure the spontaneous activity of mice. The activity chamber was 40×40×30.5 cm, made of clear Plexiglas and covered with a ventilated Plexiglas lid. Data were collected and analyzed by a VersaMax Analyzer (AccuScan, model CDA-8, Columbus, OH). Before any treatment, mice were placed inside the infrared monitor for 5 min daily for 3 consecutive days to acclimatize them. Locomotor activities were monitored during 10 min test sessions. Locomotor coordination was assessed using the rotarod (AccuScan) preset to a constant 20-rpm speed. Mice were given a 5-7 min rest interval to eliminate stress and fatigue.

Data analysis

Data analysis was performed using Prism 4.0 software. Raw data were first analyzed using one-way analysis of variance and then Tukey's post-test was performed to compare all treatment groups. Differences with $p < 0.05$ were considered significant.

Results

Mito-Apo protects against MPP⁺-induced neurotoxicity in primary mesencephalic cultures

Primary mesencephalic and primary striatal cultures were isolated from the E14 embryos of C57BL/6 mice and processed as described in "Materials and Methods". After six days in

culture, primary mesencephalic neurons were exposed to 10 μ M MPP⁺ in the presence or absence of 10 μ M Mito-Apo. After a 24-h treatment, tyrosine hydroxylase (TH)-immunostaining assays for cell morphology, TH⁺ neuron counting and neurite length and [3H] dopamine uptake assays for dopamine neuronal functions were then performed. For TH ICC studies, cultures were fixed and stained for TH, and images (Fig. 2A) were captured with a Nikon TE 2000U microscope. Compared to control cultures, MPP⁺-treated TH-immunoreactive neurons underwent extensive degenerative changes, as evidenced by reduced neurites and shrunken cell bodies (Fig. 2A). However, these changes were largely reversed by Mito-Apo co-treatment. Quantification of TH cell counts from multiple images from the neuronal cultures revealed that MPP⁺ reduced the TH⁺ cell count by approximately 75% (Fig. 2B) compared to untreated controls. Treating with 10 μ M Mito-Apo significantly protected against the MPP⁺-induced loss of TH⁺ cells. Similarly, MPP⁺ induced an ~85% loss in TH neurite length (Fig. 2C) compared to untreated controls ($p < 0.01$), whereas neurite length in Mito-Apo-co-treated cultures was significantly longer relative to MPP⁺-alone, remaining comparable to that of untreated controls. The functional integrity of dopaminergic neurons was also examined by dopamine reuptake capacity, which serves as a neurite functional viability indicator (Ghosh et al., 2013). Treating with MPP⁺ for 24 h dramatically diminished dopamine uptake compared to untreated controls, while Mito-Apo significantly improved dopamine uptake in MPP⁺-co-treated cultures (Fig. 2D). Together, these data suggest that Mito-Apo elicits neuroprotective effects in the MPP⁺-induced dopaminergic degenerative model.

Mito-Apo attenuates MPP⁺-induced increases in the expression of 3-nitrotyrosine (NT) and 4-hydroxynonenal (HNE) in primary mesencephalic cultures

Next, we investigated whether Mito-Apo could also attenuate MPP⁺-induced oxidative and nitrative stress in primary mesencephalic cultures. We used 3-NT staining as a marker of nitric oxide-dependent oxidative stress and 4-HNE as a marker of membrane lipid peroxidation induced by hydroxyl radicals ($\cdot$ OH) (Stadler et al., 2008). Double-immunolabeling with antibodies directed against TH and 3-NT revealed that MPP⁺ significantly increased the immunoreactivity of 3-NT-modified proteins in primary dopaminergic neurons, while adding Mito-Apo significantly inhibited 3-NT immunoreactivity levels in MPP⁺-treated cultures (Fig. 3A). Similarly, we observed an increased 4-HNE immunoreactivity in dopaminergic neurons in the presence of MPP⁺ as evidenced by 4-HNE and TH double-immunolabeling, and co-treating with Mito-Apo effectively attenuated 4-HNE immunoreactivity, demonstrating the efficacy of Mito-Apo in inhibiting oxidative damage in dopaminergic neurons (Fig. 3B). Together, these results suggest that Mito-Apo mitigates MPP⁺-induced increases in the levels of 3-NT and 4-HNE in TH⁺ neurons, further supporting a neuroprotective role for Mito-Apo.

Mito-Apo mitigates MPP⁺-induced glial activation and inducible nitric oxide synthase (iNOS) upregulation in primary microglia and astrocyte cultures

Activation of microglia and astrocytes plays an important role in the pathogenesis of PD, as well as in other neurodegenerative disorders (Dauer and Przedborski, 2003; Gao et al., 2003b; Ghosh et al., 2007). It has been well-established that MPP⁺ causes glial cell proinflammatory activation, which is believed to exacerbate dopaminergic degeneration by

augmenting oxidative stress (Block and Hong, 2007). Ionized calcium-binding adaptor molecule 1 (IBA1) and glial fibrillary acidic protein (GFAP) are markers of microglia and astrocyte activation, respectively (Ghosh et al., 2012b). The key proinflammatory enzyme iNOS is typically elevated in disease conditions (Kavanagh et al., 2014) and thereby serves as a marker for glial pro-inflammatory activation. To determine if Mito-Apo protects against MPP⁺-induced microglia activation and astrogliosis, we treated mouse primary microglia and astrocytes with 10 μ M MPP⁺ in the presence or absence of 10 μ M Mito-Apo for 24 h. In immunocytochemistry experiments, the expression of IBA-1 and GFAP were significantly increased in MPP⁺-treated primary microglia (Fig. 4A) and astrocytes (Fig. 4B), respectively. In contrast, these effects were greatly inhibited by co-treating with Mito-Apo. In colocalization studies, MPP⁺ significantly increased iNOS expression in both IBA-1- and GFAP-expressing cells, whereas Mito-Apo significantly restored iNOS expression back to control levels in MPP⁺-co-treated cultures (Fig. 4A-B). Together, these results suggest that Mito-Apo co-treatment mitigates MPP⁺-induced glial activation and iNOS upregulation in primary microglia and astrocytes, suggesting an anti-inflammatory effect for Mito-Apo.

Mito-Apo improves motor behavior in MPTP-treated mice

After establishing the efficacy of Mito-Apo in mitigating inflammatory reactions and protecting dopaminergic neurons against MPP⁺ toxicity in primary cultures of neurons, microglia and astrocytes, we investigated the neuroprotective effect of Mito-Apo in a preclinical animal model of PD. The MPTP mouse model is a reliable and effective rodent model which has been extensively used to test putative neuroprotective agents (Grunblatt et al., 2000; Meissner et al., 2004; Zhang et al., 2007; Prediger et al., 2011; Seidl and Potashkin, 2011; Ghosh et al., 2012a). We first evaluated whether Mito-Apo improves MPTP-induced motor deficits by testing the behavioral performance in the open-field test and on the rotarod, as described recently (Ghosh et al., 2010). Animals receiving Mito-Apo (3 mg/kg/day) orally were evaluated four days after MPTP treatment. Representative activity maps (Fig. 5A) of open-field movements of saline-treated control, MPTP and MPTP/Mito-Apo-co-treated mice revealed decreased movements of MPTP-treated mice in contrast to improved locomotion in MPTP/Mito-Apo-co-treated mice. MPTP decreased horizontal activity (Fig. 5B), vertical activity (Fig. 5C), stereotypy count (Fig. 5D), total movement time (Fig. 5E), rearing count (Fig. 5F) and the time spent on the 20-rpm rotarod (Fig. 5G), which are all consistent with our previous observations (Ghosh et al., 2010). Importantly, most of the key motor deficits observed in the MPTP mouse model of PD were restored significantly by Mito-Apo treatment (Fig. 5B-G), suggesting potential neurobehavioral improvement with Mito-Apo treatment for PD.

Mito-Apo inhibits microglia and astrocyte activation in the SN *in vivo*

Increasing evidences suggest that activated glial cells in the SN pars compacta (SNpc) play an important role in dopaminergic neurodegeneration in human PD patients and in the MPTP mouse model (Dauer and Przedborski, 2003; Block and Hong, 2007; Ghosh et al., 2007; Panicker et al., 2015). Thus, we evaluated whether the neuroprotective effect of Mito-Apo is due to attenuated gliosis in the SN. For determining neuroinflammatory responses, a three day MPTP model was used (Przedborski et al., 2004; Jackson-Lewis and Przedborski, 2007; Ghosh et al., 2012b). Three days post-MPTP treatment, mice were sacrificed and

nigral tissue sections were processed for DAB immunostaining of IBA-1 and GFAP. Increased numbers of activated microglia (characterized by amoeboid shape with thickened processes) and astrocytes (characterized by bigger cell bodies with thickened processes) were noted in the SN of MPTP-treated mice compared with those in saline-treated control mice (Fig. 6A-B). However, Mito-Apo greatly decreased the number of IBA1-positive microglial cells (Fig. 6A) and GFAP-positive astroglial cells (Fig. 6B) in the MPTP-treated SN. To further corroborate these findings, Western blotting revealed significantly increased expression of IBA-1 and GFAP in MPTP-treated SN tissues compared with saline-treated control samples, whereas orally administered Mito-Apo attenuated the expression of IBA-1 (Fig. 6C-D) and GFAP (Fig. 6E-F) in MPTP-treated mice. These results, therefore, support an anti-inflammatory property for Mito-Apo *in vivo*.

Mito-Apo attenuates activation of NADPH oxidase in activated microglia of MPTP-treated mice

It has been demonstrated that NADPH oxidase-induced oxidative stress plays a pivotal role in the neurodegeneration of dopaminergic neurons in the MPTP mouse model of PD (Vejrazka et al., 2005). Apocynin is known to suppress NADPH oxidase (Anantharam et al., 2007; Ghosh et al., 2012a; Dranka et al., 2013; Dranka et al., 2014). Thus, in the mouse SN, we examined the protein expression of gp91phox, a major component of NADPH oxidase, three days after MPTP treatment. Western blot analysis showed an increased gp91phox expression in MPTP-treated mice compared to only weak expression in saline-treated mice (Fig. 7A-B). However, Mito-Apo attenuated the MPTP-induced gp91phox expression in the SN ($p < 0.05$; Fig. 7A-B). Double-immunolabeling studies confirmed that gp91phox immunoreactivity colocalized with IBA-1-positive microglia (Fig. 7C). Additionally, robust gp91phox immunoreactivity was seen specifically in larger cells whose shorter ramifications were thickened in the SN of MPTP-treated mice (Fig. 7C insets). Consistently, Mito-Apo attenuated MPTP-induced gp91phox protein expression in IBA-1-positive microglia in the SN. These results suggest that Mito-Apo effectively dampens the MPTP-induced activation of microglial NADPH oxidase.

Mito-Apo suppresses iNOS expression in the SN of MPTP-treated mice

Recently, we reported increased levels of iNOS in the SN of MPTP-treated mice (Ghosh et al., 2009; Ghosh et al., 2012b). Therefore, we investigated whether Mito-Apo modulates dopamine neuronal survival by inhibiting MPTP-induced iNOS expression. Immunoblotting of SN tissues collected three days after MPTP treatment revealed a marked increase in the expression of iNOS in MPTP-treated mice relative to PBS-treated control mice, which was attenuated when co-treated with Mito-Apo ($p < 0.05$; Fig. 8A-B). Additionally, immunofluorescence of iNOS in SN sections shows that MPTP markedly increased nigral iNOS protein expression, and that iNOS colocalized with IBA-1-positive microglia and GFAP-positive astrocytes, respectively (Fig. 8C and D). However, consistent with its inhibitory effect on glial activation, Mito-Apo attenuated MPTP-induced expression of iNOS in IBA-1- and GFAP-expressing cells (Fig. 8C and D).

Mito-Apo attenuates nitrative modification and lipid peroxidation in the SN *in vivo*

Nitration of protein tyrosine residues is believed to be a well-known marker of oxidative stress in PD and its animal models (Dawson and Dawson, 2003; Sherer et al., 2003; Sanders et al., 2014; Schapira et al., 2014). Under oxidative stress conditions, iNOS-derived oxidants selectively nitrate tyrosine residues, generating 3-NT (Ara et al., 1998). To observe iNOS-mediated oxidative damage to tyrosine residues, we checked the expression of 3-NT in the SN three days after MPTP treatment in the presence or absence of Mito-Apo. Western blot analysis showed a robust increase in 3-NT expression compared with that in PBS-treated control mice (Fig. 9A-B). However, Mito-Apo greatly attenuated the MPTP-induced 3-NT formation ($p < 0.05$; Fig 9B). Consistent with this result, IHC analysis demonstrated increased 3-NT expression in TH-positive dopaminergic neurons in the SN of MPTP-treated mice (yellow-colored cells show colocalization of TH and 3-NT) compared with PBS-treated control mice (Fig. 9C). However, this robust increase of 3-NT levels in MPTP-treated mice was dramatically inhibited by Mito-Apo, as very few TH-positive cells expressed nitrated protein (containing 3-NT) in their cytosol (Fig. 9C).

Studies from our lab and others have shown increased levels of 4-HNE in MPTP-treated mouse brains (Selley, 1998; Fujita et al., 2009; Ghosh et al., 2012a). In the present study, we also observed a robust increase in 4-HNE expression in the SN of MPTP-treated mice three days after MPTP administration compared to saline-treated mice (Fig. 10A-B). However, Mito-Apo significantly reduced the MPTP-induced expression of 4-HNE ($p < 0.01$; Fig. 10B). Consistent with the Western blotting data, IHC data show increased 4-HNE expression in the SN of MPTP-treated mice, with 70-80% of TH-positive dopaminergic neurons expressing 4-HNE in their cytosol (Fig. 10C). In contrast, MPTP mice co-treated with Mito-Apo showed dramatically reduced 4-HNE expression in dopaminergic neurons of the SN (Fig. 10C). Together, these results demonstrate that Mito-Apo attenuates oxidative damage in nigral neurons in the MPTP mouse model of PD.

Mito-Apo inhibits the expression of pro-inflammatory molecules *in vivo* in the midbrain of MPTP-treated mice

Activated glial cells release pro-inflammatory cytokines along with iNOS, thereby damaging dopaminergic neurons. Our qRT-PCR analysis revealed a marked increase in mRNA expression of iNOS, TNF- α , IL-1 β and IL-6 in the ventral midbrain three days after MPTP administration (Fig. 10A-D). However, Mito-Apo (3 mg/kg/day) strongly inhibited the MPTP-induced expression of iNOS, TNF- α and IL-1 β in the ventral midbrain (Fig. 11A-C). Although Mito-Apo appeared to similarly attenuate the expression of IL-6, the effect was not significant (Fig. 11D). These results suggest that Mito-Apo suppresses the expression of major pro-inflammatory molecules in the ventral midbrain of MPTP-treated mice.

Mito-Apo improves nigrostriatal dopaminergic neuron survival and striatal neurotransmitter levels in MPTP-treated mice

To demonstrate that Mito-Apo protects against MPTP-induced dopaminergic neuronal degeneration in the SN, we performed TH-immunohistochemical studies (Ghosh et al., 2010; Ghosh et al., 2012b; Ghosh et al., 2013). Sectioned brains collected seven days post-MPTP were immunostained with TH to detect dopaminergic neurons and fibers. DAB

immunostaining revealed a significant loss of TH-positive cell bodies and terminals in the SN and striatum, respectively, in subacutely MPTP-treated mice as compared to saline-treated mice (Fig. 12A and B). Higher magnified (10X) pictures (Fig. 12B, *lower panels*) clearly demonstrate neuron loss in the SNpc, lateralis (SNl) and reticularis (SNr) of MPTP-treated mice. Oral administration of Mito-Apo (3 mg/kg/day) significantly mitigated MPTP-induced dopaminergic neuronal cell death in the SN and terminal loss in the striatum as determined by TH-immunostaining (Fig. 12A-B). Additionally, stereological counting of TH⁺ neurons in the SN further confirmed the neuroprotective property of Mito-Apo (Fig. 12C).

To further determine whether Mito-Apo protects against neurotransmitter depletion induced by MPTP, we measured striatal dopamine, DOPAC and HVA by HPLC (Fig. 12D-F). Our results revealed an ~80% loss of striatal dopamine in MPTP-treated mice relative to saline-treated mice. In contrast, Mito-Apo (3 mg/kg/day) increased dopamine levels by ~50% when compared to MPTP-alone ($p < 0.001$; Fig. 12D). MPTP also caused a ~76% and ~70% loss of DOPAC and HVA, respectively, in the striatum, and Mito-Apo treatment significantly attenuated MPTP-induced depletion of those metabolites (Fig. 12D-F). It is possible that neuroprotection afforded by Mito-Apo could be due to its interference with the toxic metabolic conversion of MPTP to MPP⁺ by inhibiting MAO-B enzyme activity. To address this possibility, we measured the level of MPP⁺ in striatum 3 h after the final MPTP injection, with or without Mito-Apo treatment. We found that Mito-Apo had no effect on striatal levels of MPP⁺ (MPTP-treated mice, 87.38 ± 13.59 ppb; MPTP plus Mito-Apo treated mice, 83.12 ± 9.86 ppb). Taken together, our histological and neurochemical data clearly demonstrated that Mito-Apo imparts a remarkable neuroprotective effect in the MPTP model of PD.

Brain bioavailability of Mito-Apo

The next logical step is to determine the amount of orally administered Mito-Apo that reached striatal and nigral tissues. Briefly, naïve C57BL/6 mice were orally gavaged 3 mg/kg Mito-Apo and then sacrificed at 1, 2, 4, or 24 h, and striatal and nigral regions were dissected and processed for HPLC detection as described in “Materials and Methods”. Striatal Mito-Apo levels were determined to be 72, 65, 43 and 19 ng/mg tissue at 1, 2, 4 and 24 h, respectively (Fig. 13). Similarly, Mito-Apo levels in the SN were determined to be 84, 48, 47 and 32 ng/mg tissue at 1, 2, 4 and 24 h, respectively (Fig. 13). These results suggest that Mito-Apo rapidly crosses the blood brain barrier to reach its target tissues, the striatum and SN, and persists in the CNS to produce its neuroprotective effect. We therefore can readily attribute the remarkable neuroprotective effect of Mito-Apo in the MPTP model of PD to its excellent bioavailability in the CNS.

Discussion

Neuroinflammation, oxidative and nitrative stress induced by NADPH oxidase and mitochondria-derived superoxide are key pathophysiological events contributing to the degeneration of dopaminergic neurons in PD (Wu et al., 2003; Mosley et al., 2006; Przedborski, 2007; Tansey and Goldberg, 2010; Cristovao et al., 2012; Zhang et al., 2014). In this study, we synthesized a novel anti-oxidant, mito-apocynin (Mito-Apo), by

conjugating triphenylphosphonium (TPP⁺) to apocynin to specifically target it to mitochondria. Mito-Apo attenuated the MPP⁺-induced loss of dopaminergic neurons and oxidative and nitrative stress in primary cultures. In animal studies, Mito-Apo blocked MPTP-induced glial activation, iNOS and gp91phox expression, and the formation of ONOO and 4-HNE, suggesting an anti-neuroinflammatory and antioxidant property. Importantly, Mito-Apo also protected against MPTP-induced motor deficits, striatal neurotransmitter depletion and nigrostriatal dopaminergic degeneration in animal studies, suggesting an *in vivo* neuroprotective property.

Many conventional antioxidant compounds (e.g., vitamin E, tempol, CoQ, creatine, minocycline and ubiquinone) have failed to attenuate ROS/RNS in preclinical models due to their inability to accumulate in the mitochondria (Murphy, 1997; Smith et al., 1999). Here we provide evidence that the mitochondria-targeted antioxidant Mito-Apo attenuates MPP⁺-induced increases in the expression of IBA-1 and GFAP in primary microglia and astrocyte cultures (Fig. 4), and rescued TH⁺ neurons in primary mesencephalic cultures (Fig. 2), suggesting that Mito-Apo exhibits both anti-neuroinflammatory and neuroprotective properties. Extension of these studies to the MPTP animal model revealed that Mito-Apo also effectively attenuated MPTP-induced IBA-1 and GFAP expression and TH⁺ neuronal loss in the mouse SNpc (Figs. 6 & 12).

Studies employing the MPTP animal model of PD have also shown NADPH oxidase activation, increased iNOS expression and elevated pro-inflammatory cytokines such as IL-1 β , IL-6 and TNF- α (Nagatsu et al., 2000; Wang et al., 2009; Lofrumento et al., 2011; Lim et al., 2015). Moreover, nitric oxide (*NO) generated by iNOS and derived from activated glial cells is believed to contribute to neurodegenerative diseases such as AD, PD, HIV-associated dementia, HD and ALS (Galea et al., 1992; Ghosh et al., 2009). Pro-inflammatory cytokines such as TNF- α , IL-6 and IL-1 β secreted from activated microglia facilitate intracellular death-related signaling pathways or activation of iNOS expression in the MPTP mouse model (Teismann et al., 2003). Furthermore, the promoter region of iNOS and pro-inflammatory cytokines contain the DNA binding sites for NF- κ B, a transcription factor that regulates glial cell-mediated inflammatory reactions (Hayden and Ghosh, 2004). *In vivo* studies have shown increased expression of iNOS and pro-inflammatory cytokines in ventral midbrain regions of MPTP-treated mice (Ghosh et al., 2007; Ghosh et al., 2009). Here we show that Mito-Apo attenuated MPP⁺-induced increases in iNOS expression in primary microglia and astrocyte cultures (Fig. 3). Furthermore, Mito-Apo treatment suppressed MPTP-induced expression of iNOS and the pro-inflammatory cytokines TNF α , IL-6 and IL-1 β in the mouse SNpc, suggesting an *in vivo* anti-inflammatory role (Fig 11).

NADPH oxidase and mitochondria-derived ROS are known to augment pro-inflammatory events in activated microglia, particularly in neurodegenerative disorders, such as PD, Alzheimer's disease, amyotrophic lateral sclerosis (ALS) and multiple sclerosis (Ansari and Scheff, 2011; Cristovao et al., 2012; Zhang et al., 2014). Recently, several studies have implicated Nox1-derived ROS in dopaminergic cell death induced by paraquat and 6-OHDA, and both of these toxins upregulated Nox1 (Choi et al., 2012; Cristovao et al., 2012; Hernandez et al., 2013). NADPH oxidase- and mitochondria-derived ROS both play equally important key roles in proinflammatory microglial activation (Dikalov, 2011; Mead et al.,

2012; Bordt and Polster, 2014). In the microglia, superoxide ($O_2^{\bullet-}$) is primarily produced by the three isoforms Nox1, Nox2 and Nox4, and among these, Nox2 is the most responsive and upregulated isoform in activated microglia (Fischer et al., 2012; Maghazal et al., 2012; Cooney et al., 2013).

Recent studies from our lab and others have shown that apocynin is a non-specific NADPH oxidase inhibitor (Hayashi et al., 2005; Sovari et al., 2008; Impellizzeri et al., 2011; Ghosh et al., 2012b; Dranka et al., 2014). Functional studies have revealed apocynin to attenuate superoxide formation and oxidative stress *in vivo* in the spinal cord (Bedard and Krause, 2007). Additionally, apocynin administered at a dose of 300 mg/kg/day protected against oxidative damage in an ALS model (Harraz et al., 2008), although this finding has been questioned by Trumbull et al. (2012). *In vitro* studies in dopaminergic neuronal cell lines and primary cultures also demonstrated the protective role of apocynin in the 1-methyl-4-phenyl-pyridinium ion (MPP⁺) model of PD (Anantharam et al., 2007). Recently, we have shown that diapocynin (apocynin dimer) reduced neuroinflammation and protected dopaminergic neurons in the MPTP mouse model of PD (Ghosh et al., 2012b) by preventing the assembly and activation of the NADPH oxidase complex. Diapocynin is 13-fold more lipophilic than the monomer apocynin (Luchtefeld et al., 2008). Later, we demonstrated that diapocynin successfully prevented motor deficits in the leucine-rich repeat kinase 2 (LRRK2_{R1441G}) transgenic mouse (Dranka et al., 2013). Very recently, we showed that treatment with a long-chain Mito-Apo analog, Mito-Apo-C₁₁, prevented hyposmia and reduced motor deficits in the progressive degenerative LRRK2_{R1441G} transgenic mouse model (Dranka et al., 2014). Here, we demonstrate that MPTP-induced increases in Nox2 enzyme (gp91phox) expression colocalized with IBA1-positive cells in the SNpc and that Mito-Apo effectively attenuated this increase in Nox2 expression, suggesting its antioxidant role *in vivo* (Fig. 7). Therefore, Mito-Apo may suppress MPTP-induced microglial Nox2 upregulation by inhibiting mitochondria generated superoxide.

Reactive glial cells induce $^{\bullet}NO$ and $O_2^{\bullet-}$ -generated by NADPH oxidase, and in mitochondria, react to form a potent oxidant, peroxynitrite ($ONOO^-$), which causes oxidative and nitrative modification of proteins through nitration of the proteins' tyrosine residues in dopaminergic neurons (Ara et al., 1998; Dawson and Dawson, 2003). Studies have shown iNOS expression and increased 3-NT levels in activated microglia in the SN of human PD brains (Hunot et al., 1996). Previous studies also have shown that iNOS facilitates dopaminergic neurodegeneration *via* MPTP-induced production of RNS in the MPTP mouse model of PD, resulting in enhanced 3-NT levels (Liberatore et al., 1999; Dehmer et al., 2000). In this study, the increased 3-NT expression we observed in primary mesencephalic culture and dopaminergic neurons in MPTP-treated mice was significantly reduced by Mito-Apo treatment in both models (Figs. 3 and 9). Besides peroxynitrite, 4-HNE, a well-known marker for oxidative insult caused mainly by the hydroxyl radical ($^{\bullet}OH$), was also formed during the course of disease. As an unsaturated aldehyde, more 4-HNE is generated due to decomposition of lipid peroxides in the SN of PD brains than in healthy controls (Yoritaka et al., 1996). Previous studies also demonstrated that 4-HNE blocked mitochondrial respiration and induced caspase-dependent apoptosis in neurons (Picklo et al., 1999). In our study, 4-HNE expression increased in both primary mesencephalic cultures and mice treated with MPP⁺ and MPTP, respectively (Figs. 3 and

10), but co-treatment with Mito-Apo significantly blocked the 4-HNE expression. These results further strengthen the anti-oxidant capability of Mito-Apo.

Therapy effective at halting the degeneration of dopaminergic neurons and their striatal terminals remains elusive. Administration of levodopa, the gold standard drug for PD does not affect disease progression. Other putative neuroprotective agents such as glial cell line-derived neurotrophic factor (GDNF), brain derived neurotrophic factor (BDNF) and other small compounds have been tested in different animal models of PD with no or little success in clinical trials (Chen and Le, 2006; Wu et al., 2011; Ybot-Gorin et al., 2011; Athauda and Foltynie, 2015). Additionally, these compounds also caused adverse side effects. Our novel compound, Mito-Apo, has several advantages compared to other experimental drugs: 1) orally administered Mito-Apo does not show any toxic effect in mice; 2) Mito-Apo, being a lipophilic molecule, diffuses through the blood-brain barrier and into mitochondria where it inhibits superoxide production; 3) Mito-Apo can be taken orally, which is the least painful route; 4) orally administered Mito-Apo protects the nigrostriatal axis in the MPTP model of PD; 5) Mito-Apo is at least 100-fold more efficacious than apocynin or diapocynin in preclinical animal PD model; and 6) low-dose Mito-Apo is CNS bioavailable and reaches its intended target tissues, the substantia nigra and striatum.

In summary, we have demonstrated that Mito-Apo protected dopaminergic neurons both in primary culture and an animal model in the presence of a potent dopaminergic toxin. Mito-Apo reduced oxidative and nitrative stress, glial activation and inflammatory reactions. Mito-Apo is CNS bioavailable and reversed the behavioral deficits caused by MPTP. Moreover, Mito-Apo restored dopamine and its metabolite levels and preserved TH⁺ neurons in the SNpc of MPTP-treated mice. These previously undiscovered properties of this novel mitochondria-targeted antioxidant strongly support continued research into potential clinical applications for Mito-Apo as a viable neuroprotective drug for treating Parkinson's disease.

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Fig.1. The synthetic pathway involved in the preparation of Mito-apocynin

Fig. 2. Mito-Apo protected dopaminergic neurons against MPP⁺-induced toxicity in primary mesencephalic cultures

Mesencephalic tissues from E14 mouse embryos were cultured and grown on poly-D-lysine-coated coverslips. The neuronal cultures were treated with 10 μM MPP⁺ for 24 h in the presence or absence of 10 μM Mito-Apo. After treatment, primary neurons were fixed with 4% PFA and incubated with anti-TH antibody and viewed under a Nikon TE2000 fluorescence microscope. **A.** TH immunolabeling in primary mesencephalic cultures from substantia nigra. **B.** Quantification of TH immunoreactive (i.r.) dopaminergic neurons. **C.** Quantification of TH i.r. dopaminergic axons. **D.** Dopamine uptake assay from primary striatal culture. Data are represented by the mean ± SEM of three independent experiments.

***, $p < 0.001$ vs. Control; @, $p < 0.05$ vs. MPP⁺; **, $p < 0.01$ vs MPP⁺.

Fig. 3. Mito-Apo attenuated MPP⁺-induced oxidative stress in primary mesencephalic cultures Mesencephalic tissues from E14 mouse embryos were cultured and grown on poly-D-lysine-coated coverslips. The neuronal cultures were treated with 10 μ M MPP⁺ for 24 h in the presence or absence of 10 μ M Mito-Apo. Cells were fixed with 4% PFA and incubated with different antibodies and viewed under a Nikon TE2000 fluorescence microscope. **A.** Double-labeling of TH and 3-NT. **B.** Double-labeling of TH and 4-HNE.

Fig. 4. Mito-Apo attenuated MPP⁺-induced glial activation and iNOS expression

Isolated primary microglia or astroglia from one-day-old pups were treated with 10 μ M MPP⁺ for 24 h in the presence or absence of 10 μ M Mito-Apo. Cells were fixed with 4% PFA and incubated with different antibodies and imaged through a Nikon TE2000 fluorescence microscope. **A.** Double-labeling of IBA-1 and iNOS in primary microglia cells. **B.** Double-labeling of GFAP and iNOS in primary astroglia cells.

Fig. 5. Mito-Apo rescued MPTP-induced behavioral and locomotor deficits

Sub-Acute MPTP-treatment paradigm as described in the methods section was used for this experiment. Control mice received saline. Mice were tested for locomotor activities four days after the last dose of MPTP. **A.** Movement track of mice collected over 10 min. Open-field data showing **B.** horizontal activity; **C.** vertical activity; **D.** stereotypy count; **E.** total movement time; **F.** rearing count and **G.** time spent on rotarod (sec) at 20-rpm speed. Data represented by the mean $\pm$ SEM of six to eight mice per group. ***, $p < 0.001$ vs. the control group; **, $p < 0.01$ vs. the MPTP group; *, $p < 0.05$ vs. the MPTP group; @, $p < 0.05$ vs. the Control group; #, $p < 0.01$ vs. the Control group.

Fig. 6. Mito-Apo blocked activation of microglia and astrocytes in the SN of MPTP-treated mice Acute MPTP-treatment paradigm as described in the methods section was used for this experiment. Control mice received 10% ethanol in saline. The substantia nigra (SN) tissue sections were immunostained with antibodies against IBA-1 (**A**) and GFAP (**B**). *Insets*, four-fold higher magnification of A-B. Dotted white lines indicate the SNpc. Representative Western blots illustrating the expression of IBA-1 (**C**) and GFAP (**E**) in SN. **D**. Bar graph showing mean IBA-1/ β -actin ratios $\pm$ SEM from Western blotting in SN of 6 mice per group. **F**. Bar graph showing mean GFAP/ β -actin ratios $\pm$ SEM from Western blotting in SN of 6 mice per group. ***, $p < 0.001$ vs. the Control group; *, $p < 0.05$ vs. the MPTP group.

Fig. 7. Mito-Apo attenuated activation of NADPH oxidase in the SN of MPTP-treated mice
 Acute MPTP-treatment paradigm as described in the methods section was used for this experiment. **A.** Representative Western blots illustrating the expression of gp91phox in SN. **B.** Bar graph showing mean gp91phox/β-actin ratios±SEM from Western blotting in SN of 6 mice per group. **C.** Substantia nigra tissue sections were double-labeled for gp91phox and IBA-1. Images were captured at 20X and 60X (*insets*) magnification. The SNpc zone is outlined in white dots. *Inset* pictures demonstrate colocalization of IBA-1 and gp91phox. **, $p < 0.01$ vs. the Control group; *, $p < 0.05$ vs. the MPTP group.

Fig.8. Mito-Apo decreased MPTP-induced nitric oxide damage in the SN of MPTP-treated mice
 Acute MPTP-treatment paradigm as described in the methods section was used for this experiment. **A.** Representative western blots illustrating the expression of iNOS in SN. **B.** Bar graph showing mean iNOS/β-actin ratios±SEM from Western blotting in SN of 6 mice per group. **C.** Double-labeling of GFAP and iNOS and **(D)** IBA-1 and iNOS in SN region of ventral midbrain. Images were captured at 20X and 60X (insets) magnification. *Inset* pictures demonstrate colocalization of iNOS and GFAP or IBA-1 ***, $p < 0.001$ vs. the Control group; *, $p < 0.05$ vs. the MPTP group.

Fig. 9. Mito-Apo decreased the formation of 3-nitrotyrosine in the SN of MPTP-treated mice
 Acute MPTP-treatment paradigm as described in the methods section was used for this experiment. **A.** Representative Western blots illustrating the expression of 3-NT in SN. **B.** Bar graph showing mean 3-NT/β-actin ratios±SEM from Western blotting in SN of 6 mice per group. **C.** Double-labeling of TH and 3-NT in the SN. Images were captured at 20X and 60X (*insets*) magnifications. The SNpc zone is outlined in white dots. Inset pictures demonstrate colocalization of TH and 3-NT. ***, $p < 0.001$ vs. the Control group; *, $p < 0.05$ vs. the MPTP group.

Fig. 10. Mito-Apo inhibited MPTP-induced formation of 4-hydroxynonenol in the SN of MPTP-treated mice

Acute MPTP-treatment paradigm as described in the methods section was used for this experiment. **A.** Representative Western blots illustrating the expression of 4-HNE in SN. **B.** Bar graph showing mean 4-HNE/β-actin ratios±SEM from Western blotting in SN of 6 mice per group. **C.** Double-labeling of TH and 4-HNE in the SN. Images were captured at 20X and 60X (*insets*) magnifications. The SNpc zone is outlined in white dots. *Inset* pictures demonstrate colocalization of TH and 4-HNE. ***, $p < 0.001$ vs. the Control group; **, $p < 0.01$ vs. the MPTP group.

Fig. 11. Mito-Apo attenuated the expression of proinflammatory molecules in the ventral midbrain of MPTP-treated mice

Acute MPTP-treatment paradigm as described in the methods section was used for this experiment. The mRNA expression of iNOS (**A**), TNF- α (**B**), IL-1 β (**C**) and IL-6 (**D**) was analyzed by quantitative real-time PCR. Data represented by the mean $\pm$ SEM of six mice per group. **, $p < 0.01$ vs. Control; *, $p < 0.05$ vs. Control; @, $p < 0.05$ vs MPTP; #, $p < 0.01$ vs MPTP; ns=non-significant.

Fig. 12. Mito-Apo prevented MPTP-induced neurotoxicity in the nigrostriatal axis

Sub-Acute MPTP-treatment paradigm as described in the methods section was used for this experiment. DAB-immunohistochemistry for TH in striatum (A) and SN (B), upper panel 2X magnification; lower panel 10X magnification. (C) Stereological counting of TH-positive neurons in the SN. Striatal dopamine (D), DOPAC (E) and HVA (F) were measured by HPLC. Data represented by mean $\pm$ SEM of six to eight mice per group. ***, $p < 0.001$ vs. Control; @, $p < 0.01$ vs. MPTP; #, $p < 0.05$ vs. MPTP.

Fig. 13. Mito-Apo bioavailability in the brain

Mice were administered Mito-Apo (3 mg/kg) once orally and sacrificed at 1, 2, 4, or 24 h post-gavage. Control mice received saline. Striatal and nigral regions were dissected out and processed for HPLC-UV detection as described in "Materials and Methods". **A**. Linear regression of Mito-Apo standards at 292 nm. Quantification of chromatographs for detection of Mito-Apo in SN tissue (**B**) and striatum (**C**). **D**, **E** Representative chromatographs of brain tissues analyzed for Mito-Apo detection at various time points in the SN and striatum, respectively. Data are represented as mean $\pm$ SEM of four mice per group. **, $p < 0.01$ vs. the control group.