Obligatory Role for Complex I Inhibition in the Dopaminergic Neurotoxicity of 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)

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Administration of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) to mice and nonhuman primates causes a parkinsonian disorder characterized by a loss of dopamine-producing neurons in the substantia nigra and corresponding motor deficits. MPTP has been proposed to exert its neurotoxic effects through a variety of mechanisms, including inhibition of complex I of the mitochondrial respiratory chain, displacement of dopamine from vesicular stores, and formation of reactive oxygen species from mitochondrial or cytosolic sources. However, the mechanism of MPTP-induced neurotoxicity is still a matter of debate. Recently, we reported that the yeast single-subunit nicotinamide adenine dinucleotide (reduced) dehydrogenase (NDI1) is resistant to rotenone, a complex I inhibitor that produces a parkinsonian syndrome in rats, and that overexpression of NDI1 in SK-N-MC cells prevents the toxicity of rotenone. In this study, we used viral-mediated overexpression of NDI1 in SK-N-MC cells and animals to determine the relative contribution of complex I inhibition in the toxicity of MPTP. In cell culture, NDI1 overexpression abolished the toxicity of 1-methyl-4-phenylpyridinium, the active metabolite of MPTP. Overexpression of NDI1 through stereotactic administration of a viral vector harboring the NDI1 gene into the substantia nigra protected mice from both the neurochemical and behavioral deficits elicited by MPTP. These data identify inhibition of complex I as a requirement for dopaminergic neurodegeneration and subsequent behavioral deficits produced by MPTP. Furthermore, combined with reports of a complex I defect in Parkinson’s disease (PD) patients, the present study affirms the utility of MPTP in understanding the molecular mechanisms underlying dopaminergic neurodegeneration in PD.

Key Words: Mitochondria; complex I; MPTP; Parkinson’s disease; dopamine transporter; viral expression.

Parkinson’s disease (PD) is a progressive neurodegenerative disorder estimated to affect greater than 1% of all adults over the age of 65 worldwide (Olanow and Tatton, 1999). Although genetic mutations can cause PD, the vast majority of cases are idiopathic (Vila and Przedborski, 2004) and there is increasing evidence that exposure to environmental toxicants may play a significant role in PD (Sherer et al., 2002). A key indication that environmental factors may play a role in PD came with the discovery of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), which caused an acute and permanent parkinsonian syndrome in intravenous drug users (Langston et al., 1983). Since this discovery, MPTP has been used to develop animal models of PD in several species, as a tool to understand the pathogenesis of PD and to test a variety of therapeutic interventions designed to prevent or reduce dopaminergic neurodegeneration (Dauer and Przedborski, 2003).

Despite intensive investigation, the molecular mechanism of MPTP has not been definitively demonstrated. MPP+, the active metabolite of MPTP, has been shown to inhibit complex I of the mitochondrial electron transport chain, which was thought to be the mechanism by which MPTP induced parkinsonism (Nicklas et al., 1985). However, the weak inhibitory ability of MPP+ at complex I (Ramsay et al., 1991; Richardson et al., 2005) has led some to question the role of complex I inhibition in MPTP toxicity (Lotharius and O’Malley, 2000; Nakamura et al., 2000). Unfortunately, complex I activity was not determined in these studies and the contribution of complex I in the toxicity of MPP+ could not directly be determined. Others have hypothesized alternate mechanisms of action for MPTP including release of vesicular...
stored dopamine and subsequent oxidative damage, release of stored iron deposits, increased cytoplasmic calcium and intraneuronal calcium release, and redox cycling of MPP⁺ (Chen et al., 1995; Koouncmchoo et al., 2006; Lotharius and O’Malley, 2000; Obata, 2002, 2006; Trevor et al., 1987). Thus, the molecular mechanism of MPTP remains to be established.

Because the administration of MPTP to experimental animals is the most widely used model of PD, determination of the mechanism by which MPTP causes dopaminergic neurodegeneration may provide critical information on pathogenic processes involved in PD. Previously, we have demonstrated that the single-subunit nicotinamide adenine dinucleotide (reduced) dehydrogenase of Saccharomyces cerevisiae (NDI1) can serve as a replacement for complex I in mammalian cells and restore electron transfer in cell devoid of mitochondrial DNA (Bai et al., 2001; Seo et al., 1998, 2000) and that NDI1 expression in neuroblastoma cells abolishes the toxicity of rotenone, a complex I inhibitor we have demonstrated to produce features of PD in rats (Betarbet et al., 2000; Sherer et al., 2003). More recently, we have reported that NDI1 can be expressed in vivo using viral-mediated techniques and that unilateral expression of NDI1 partially protected against reduced tyrosine hydroxylase (TH) immunoreactivity resulting from an acute high-dose regimen of MPTP (4 × 15 mg/kg over 1 day; Seo et al., 2006). These findings suggested that NDI1 could provide some protection from MPTP neurotoxicity. However, the in vivo nature of our experiments prevented unequivocal identification of complex I as the target of MPTP. Additionally, the unilateral nature of our experiments did not allow us to determine whether NDI1-mediated neuroprotection would ameliorate the behavioral deficits observed following MPTP administration (Tillerson et al., 2002). Here, we sought to definitively determine the role of complex I inhibition in the neurotoxicity produced by MPTP. Using an in vitro system, we demonstrate that overexpression of NDI1 in cells totally abolishes the toxicity of MPP⁺, the active metabolite of MPTP. Furthermore, viral-mediated overexpression of NDI1 in vivo resulted in significant reduction of MPTP dopaminergic neurotoxicity and the total recovery of behavioral deficits caused by MPTP.

**MATERIALS AND METHODS**

**Cell culture.** SK-N-MC neuroblastoma cells stably expressing NDI1 protein have been described previously (Sherer et al., 2003). To demonstrate proper localization of NDI1 to the mitochondria, immunofluorescence microscopy was performed as described previously (Seo et al., 2000). Briefly, cells were fixed and immunostained with a previously characterized affinity-purified rabbit antibody to NDI1 and an antibody to the mitochondrial F1-adenosine triphosphatase (ATPase) (Molecular Probes, Eugene, OR). Secondary antibodies were FITC-conjugated goat anti-rabbit for NDI1 and rhodamine red goat anti-mouse for F1-ATPase (Molecular Probes). For studies on MPP⁺ toxicity, parental SK-N-MC or SK-N-MC cells stably expressing NDI1 (Sherer et al., 2003) were transfected with 4 μg of the dopamine transporter (DAT) in pcDNA 3.1 using Lipofectamine as described previously (Ramachandiran et al., 2006). Twenty-four hours after transfection, cells were split into 24-well plates for determination of 1-methyl-4-phenylpyridinium (1H-MPP⁺) uptake (Pfit et al., 1993) on the following day (48 h after transfection). Briefly, cells were washed with 1 ml uptake buffer (4mM Tris, 6.25mM N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid, 120mM NaCl, 5mM KCl, 1.2mM CaCl₂, 1.2mM MgSO₄, 5.6mM glucose, and 0.5mM ascorbic acid, pH 7.2) and incubated in 0.5 ml uptake buffer containing various concentrations (0.5–40μM) of cold MPP⁺ and 0.25 μCi of ‘¹H-MPP⁺ for 10 min at 37°C. Nonspecific uptake was determined in the presence of 10μM nonifensine. Uptake was terminated by aspirating the uptake buffer and washing each well twice with 1 ml of ice-cold uptake buffer. Cells were lysed by 1% sodium dodecyl sulfate and transferred into vials containing 5 ml of scintillation cocktail and radioactivity measured by liquid scintillation counting. Uptake values were corrected for the amount of protein to account for variation in plating density and kinetic (Kₘ and Vₘₐₓ) values were calculated by nonlinear regression using GraphPad Prism 4.0. For studies on cell death, SK-N-MC cells stably expressing NDI1 were transfected with the DAT as described above. Cells were then incubated for 48 h with 100μM MPP⁺ and cell death determined using Sytox green (Molecular Probes, Eugene, OR) with fluorescent detection as described previously (Sherer et al., 2003). Following the last measurement, cells were incubated for 1 h with 1% Triton, and total fluorescence was determined to normalize for variation in plating density.

**Animal studies.** Eight-week-old male C57BL/6j mice were obtained from an in-house breeding colony at the Scripps Research Institute. Mice received stereotoxic injections (anterior-posterior –3.3 mm, medial-lateral +1.4 mm, and dorso-ventral –3.9 mm) of phosphate-buffered saline vehicle (n = 14) or rAAV-NDI1 (n = 17), a recombinant adeno-associated serotype 2 virus carrying the NDI1 gene (1 × 10¹¹ IU/ml in phosphate-buffered saline), bilaterally into the substantia nigra (Seo et al., 2004, 2006). A total of 31 mice were shipped to Emory University at 4–5 months of age and quarantined for 3 months. Therefore, mice were 8 months of age at the time of experimentation, an age at which MPTP produces a sustained loss of dopaminergic terminals and cell bodies (Ali et al., 1991; Tillerson et al., 2002). Mice were then assigned to one of four treatment groups: (1) vehicle-saline (n = 3); (2) vehicle-MPTP (2 × 15 mg/kg; n = 6); (3) NDI1-saline (n = 5); and (4) NDI1-MPTP (n = 12). The use of a higher number of animals in the NDI1-MPTP group was based on previous observations that the expression of NDI1 varies from animal to animal (Seo et al., 2006). Animals underwent behavioral testing 7 days following MPTP treatment and were then sacrificed (Tillerson and Miller, 2003). The brain was removed and divided at the midline, with one half of the brain placed in freshly prepared 4% parafomaldehyde and the striatum dissected from the other side and frozen for neurochemical determinations. All animal procedures were conducted in accordance with the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals and approved by both the Institutional Animal Care and Use Committee at the Scripps Research Institute and the Institutional Animal Care and Use Committee at Emory University.

**Neurochemical determinations.** Western blots were performed as previously described (Richardson et al., 2006). Briefly, samples (20 μg) were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis on 10% precast NuPage gels (Invitrogen, Carlsbad, CA). Samples were electrophoretically transferred to a polyvinylidene difluoride membrane, and nonspecific sites were blocked in 7.5% nonfat dry milk in Tris-buffered saline (135mM NaCl, 2.5mM KCl, 50mM Tris, and 0.1% Tween 20, pH 7.4). Membranes were then incubated in a monoclonal antibody (Chemicon, Temecula, CA) to the N-terminus of DAT. Antibody binding was detected using a goat anti-rat horseradish peroxidase secondary antibody (ICN, Costa Mesa, CA) and enhanced chemiluminescence. The chemiluminescent signal was captured on an Alpha Innotech Fluorchem 8800 (San Leandro, CA) imaging system and stored as a digital image. Densitometric analysis was performed and calibrated to cobblotted dilutional standards of pooled cells from all control samples. Membranes were then stripped for 15 min at 25°C with Pierce (Rockford, IL) Stripping Buffer and subsequently reprobed with polyclonal anti-TH, polyclonal anti-vesicular monoamine transporter 2 (VMAT2), and monoclonal anti-[β-actin (Sigma-Aldrich, St. Louis, MO) antibody to ensure...
equal protein loading across samples. For assessment of astroglial and microglial activation, blots were probed with polyclonal antibodies to glial fibrillary acidic protein (GFAP) and glucos transporter-5 (Glut5).

High-performance liquid chromatography (HPLC) analysis of catecholamines by electrochemical detection was performed as previously described (Richardson and Miller, 2004). Briefly, dissected striata were sonicated in 0.1M perchloric acid containing 347 μM sodium bisulfite and 134 μM ethylenediaminetetraacetic acid. Homogenates were centrifuged at 12,000 × g for 10 min at 4°C, the supernatant was removed, and filtered through a 0.22-μm filter by centrifugation at 12,000 × g for 10 min. The supernatants were then analyzed for levels of dopamine, dihydroxyphenylacetic acid, and homovanillic acid. Levels were measured using HPLC with an eight-channel coulometric electrode array (ESA Coularray, Chelmsford, MA). Quantification was made by reference to calibration curves made with individual monoamine standards.

**Immunohistochemistry.** Immunohistochemical analysis of tissue was performed as previously described by Caudle et al. (2006). Brains were serially sectioned at 25 μm on a freezing microtome. Tissue was rinsed in 1× TBS and incubated in 3% H₂O₂ to quench endogenous peroxidases. Sections were blocked in 10% normal goat serum for 1 h at room temperature before being incubated overnight with monoclonal mouse anti-TH (1:2000) primary antibody. The following day tissue was rinsed in 1× TBS then incubated for 1 h in goat anti-mouse (1:200) secondary antibody conjugated to biotin. Tissue was rinsed in 1× TBS and incubated for 1 h in avidin-biotin complex solution. Tissue was rinsed in 1× TBS and the final product was visualized using 3,3′-diaminobenzidine tetrachloride. Free-floating slices were mounted onto slides, serially dehydrated in ethanol, and coverslipped. Immunostained sections were analyzed using bright-field microscopy, and images were captured on a Leitz microscope (Leica, Wetzlar, Germany). For final output, images were processed simultaneously and identically with Adobe Photoshop 7.0 software.

**Behavioral testing.** Behavioral testing to determine the behavioral impact of MPTP administration was performed via the grid performance test as described previously (Tillerson and Miller, 2003; Tillerson et al., 2002). Briefly, mice were lifted by their tail and slowly placed in the center of the horizontal grid and supported until they grabbed the grid with both their fore and hindpaws. The grid was then inverted and mice were videotaped while hanging upside down for 30 s. For analysis, videos were replayed using a recorder with slow motion and frame-by-frame option by a person blinded to the treatment groups. Forepaw step length was measured as the number of grid squares/openings transversed. The average forepaw distance was calculated by summing the distances for each step then dividing by the total number of steps. The number of unsuccessful forepaw steps divided by the total number of attempted forepaw steps was recorded as foot faults. An unsuccessful step was defined as an attempt to step or place the forepaw in which the paw clearly slipped from its destined position and had to be replaced on the grid by the animal.

**Statistical analysis.** All cell culture experiments represent three to four independent experiments performed in triplicate. The in vivo studies represent 3–12 independent animals for each treatment. Data were calculated as mean ± standard error of the mean (SEM), and statistical analyses were performed on raw data. Differences between groups were determined by Student’s t-test for analyses involving only two groups or by analysis of variance (ANOVA) followed by mean separation by the Student-Newman-Keuls (SNK) method for analyses involving more than two groups. Significance is reported at the level of p < 0.05.

### RESULTS

**MPP⁺ Toxicity is Prevented by Replacement of Complex I with Rotenone-Inensitive NDI1**

In an earlier study, we demonstrated that stable expression of NDI1 in SK-N-MC neuroblastoma cells abolished rotenone toxicity, providing evidence of an obligatory role for complex I inhibition in rotenone toxicity (Sherer et al., 2003). Here, we used the same cell line to determine whether complex I is required for MPP⁺ toxicity. NDI1 was properly incorporated into the mitochondria as evidenced by fluorescence micrographs demonstrating mitochondrial colocalization (far right panel) of NDI1 (left panel) and the F1-ATPase (middle panel) in SK-N-MC cells stably expressing NDI1 (Fig. 1A). These

![FIG. 1. NDI1 expression abolishes MPP⁺ toxicity in vitro. (A) Fluorescence micrographs demonstrating mitochondrial colocalization (far right panel) of NDI1 (left panel) and the F1-ATPase (middle panel) in SK-N-MC cells stably expressing NDI1. For cell death and uptake measurements, SK-N-MC and SK-N-MC cells stably expressing NDI1 were transfected with the DAT in 100-mm dishes and transferred 24 h later into 24-well plates for toxicity or uptake assays. (B) Cell death was analyzed by Sytox green 48 h after exposure to 100 μM MPP⁺. Results represent mean ± SEM (n = 3–4) with the maximum percent cell death of MPP⁺. ***Indicates significantly different from maximum inhibition (p < 0.001) by Student’s t-test. (C) 3H-MPP⁺ uptake in SK-N-MC and NDI1 cells transfected with DAT. Results represent mean ± SEM (n = 3–4). Absence of error bars indicates that the SEM was less than the size of the symbol.](http://toxsci.oxfordjournals.org/).
cells were then transfected with the human DAT to allow MPP⁺ to gain access to the cells (Richardson et al., 2005). Following exposure to 100 μM MPP⁺ for 48 h, 50–60% cell death was observed and this value was set as the maximum % of cell death of 100%. However, cell death was totally abolished in cells overexpressing NDI1 (Fig. 1B), demonstrating the requirement of interaction of MPP⁺ with complex I to cause toxicity in this system. This effect appears to be dependent on the presence of NDI1 rather than altered uptake of the toxin, as there were no differences in ³H-MPP⁺ uptake between NDI1 cells and SK-N-MC cells transfected with the DAT (Fig. 1C).

**Viral-Mediated Expression of NDI1 In Vivo**

To confirm our *in vitro* findings, we employed a bilateral injection to deliver NDI1 in a viral vector to the brain, which we have previously demonstrated to confer expression in dopaminergic terminals in the striatum (Seo et al., 2004). NDI1 expression did not alter basal dopamine levels when compared to animals which received vehicle at the time of surgery (data not shown) and these values were combined to form the control group. Administration of a moderate dose of MPTP (2 × 15 mg/kg) resulted in an 84% decrease of striatal dopamine 7 days after administration in animals receiving vehicle at surgery. This effect was greatly attenuated in mice expressing NDI1, with only a 48% decrease of striatal dopamine observed (Fig. 2A). There was a wide range of variance observed as some animals (n = 3) showed a small amount of protection (74% decrease of striatal dopamine), some (n = 6) that showed a moderate level of protection (60% decrease of striatal dopamine), and some (n = 3) that showed total protection as evidenced by no significant loss of dopamine. These results are consistent with variation in expression of NDI1 between animals and our previous observation that not all dopamine neurons express NDI1 after injection of rAAV-NDI1 (Seo et al., 2006). To confirm this variation, we attempted to perform immunohistochemical analysis of NDI1 expression in each individual animal. Unfortunately, technical limitations of the NDI1 antibody prevented successful quantification of NDI1 levels. However, overall significant neuroprotection was observed, suggesting that viral-mediated expression of NDI1 is a plausible means for providing neuroprotection against MPTP.

In addition to measures of striatal dopamine depletion, we also assessed neurochemical markers in the striatum of these same animals to determine the degree of dopaminergic damage (Tillerson et al., 2002). Similar to the effects observed on striatal dopamine depletion, NDI1 expression provided a significant level of neuroprotection against the loss of DAT (Fig. 2B), TH (Fig. 2C), and VMAT2 (Fig. 2D) protein levels as determined by immunoblotting. MPTP treatment caused a 70% reduction of DAT in animals receiving vehicle at surgery. This was significantly attenuated by NDI1, with only a 41% loss observed in animals receiving NDI1 at surgery. A similar protective effect was observed when we measured levels of TH and VMAT2. MPTP caused a 63 and 45% decrease in TH and VMAT2 levels in vehicle-injected mice. However, TH and VMAT2 levels were only decreased by 32 and 23% in NDI1-injected mice. As can be seen in the representative Western blots (Fig. 2E), the neuroprotection observed ranged from partial to complete. NDI1 expression did not alter DAT or VMAT2 levels as compared to animals that received vehicle at surgery (data not shown), suggesting that the neuroprotective effect of NDI1 against MPTP toxicity is likely not the result of altered uptake of or enhanced sequestration of MPP⁺. No significant effect of NDI1 expression was observed on TH protein levels. We observed a similar range of neuroprotection when TH immunoreactivity in the striatum was assessed by immunohistochemistry (Fig. 3A–F). Finally, we performed immunohistochemical staining of TH in the cell bodies in the substantia nigra pars compacta and found a similar range of neuroprotection (Fig. 3G–L). Although immunohistochemical staining as performed here is qualitative, these data support the ability of NDI1 to protect both the dopaminergic terminals and cell bodies from MPTP-induced neurodegeneration.

**Viral-Mediated Expression of NDI1 In Vivo Attenuates Glial and Microglial Activation**

Astrogliosis and microgliosis have been used extensively as markers of MPTP-induced neurotoxicity (Francis et al., 1995; O’Callaghan et al., 1990). Here, we determined GFAP levels as an indicator of astrogliosis (O’Callaghan et al., 1990) and Glut5 levels as an indicator of microgliosis (Payne et al., 1997) to determine whether NDI1 expression *in vivo* would attenuate the glial response in response to MPTP administration. Basal levels of GFAP and Glut5 as assessed by Western blot were not significantly different between animals administered vehicle and those administered NDI1 and were combined to form a pooled control group. MPTP treatment increased GFAP levels by 42% in animals receiving vehicle as the time of surgery, whereas those receiving NDI1 and subsequent MPTP treatment exhibited only a 17% increase in GFAP levels (Fig. 4A). A similar protective effect was observed for microglial proliferation as assessed by determining Glut5 levels. MPTP treatment increased Glut5 levels by 34% in animals receiving vehicle at surgery. This increase was attenuated in animals receiving NDI1, which only exhibited a 15% increase in Glut5 levels (Fig. 4B). As with the other measures of MPTP-induced neurotoxicity, there was a range of neuroprotection observed with these markers (Fig. 4C). However, NDI1 treatment provided a significant overall level of neuroprotection.

**Viral-Mediated Expression of NDI1 In Vivo Abolishes MPTP-Induced Behavioral Deficits**

The loss of motor control and associated behavioral dysfunctions is the most debilitating symptom of PD. In previous
studies, we established the grid performance test as a sensitive indicator of dopamine-associated behavioral impairment in the MPTP mouse model of PD (Tillerson and Miller, 2003; Tillerson et al., 2002). Here we sought to determine whether the amelioration of dopaminergic damage by NDI1 would result in prevention of MPTP-induced behavioral deficits. Administration of MPTP to animals given vehicle during surgery reduced forepaw step length on the grid to 50% of control

FIG. 2. Viral-mediated expression of NDI1 attenuates MPTP-induced dopaminergic damage in vivo. Mice were stereotaxically injected with either vehicle (n = 14) or rAAV-NDI1 (n = 17) and treated with saline (n = 4 for vehicle and n = 5 for NDI1) or 2 × 15 mg/kg MPTP (n = 5 for vehicle and n = 12 for NDI1) 6 months later. One week after MPTP administration mice were sacrificed for determination of striatal dopamine by HPLC and protein levels of the DAT, TH, and VMAT2 by Western blot. (A) There were no significant differences between DA levels in vehicle and NDI1-injected animals, and the data from these groups were collapsed to represent the control group. Results represent mean ± SEM. ***Indicates significantly different from control (p ≤ 0.001) by ANOVA followed by means separation by SNK. *Indicates significantly different from vehicle mice administered MPTP (p ≤ 0.05). Western blot data of (B) DAT, (C) TH, and (D) VMAT2 reveal a similar range of neuroprotection by NDI1. There were no significant differences between protein levels of DAT, VMAT2, or TH in vehicle and NDI1-injected animals, and the data from these groups were collapsed to form the control group. Results represent mean ± SEM. ***Indicates significantly different from control (p ≤ 0.001). ** and * indicates significantly different from vehicle mice administered MPTP (p ≤ 0.01 and 0.05). (E) Representative Western blots of DAT, TH, VMAT2, and β-actin, which was used to ensure equal protein loading.
values (Fig. 5) while animals expressing NDI1 did not exhibit a significant reduction in forepaw step length. In contrast to the variation in protective effect observed at the neurochemical level, NDI1-expressing animals were totally resistant to the behavioral deficit caused by MPTP neurotoxicity, suggesting that even a small amount of neuroprotection is sufficient to prevent behavioral impairment. NDI1 expression also decreased the percentage of foot faults in MPTP-treated animals but this effect did not quite reach statistical significance ($p = 0.0579$; data not shown).

**DISCUSSION**

There is accumulating evidence suggesting that oxidative damage to and dysfunction of complex I of the mitochondrial electron transport chain may be involved in PD pathogenesis (Dawson and Dawson, 2003; Greenamyre et al., 2001). The observation of reduced complex I activity in PD patients provided some of the first clinical evidence of the potential involvement of complex I dysfunction in PD (Mizuno et al., 1989; Parker et al., 1989; Schapira et al., 1989). Most recently, Jin et al. (2006) reported three-fold decreases of two subunits of complex I in the substantia nigra of PD patients compared to age-matched controls using a proteomic approach. Furthermore, the development of the rotenone rat model of PD provided direct experimental evidence that complex I inhibition alone is sufficient to induce a parkinsonian syndrome (Betarbet et al., 2000). Although PD is not typically considered a mitochondrial disease, these studies cited above and recent work demonstrating that genes linked to PD affect mitochondrial function or localize to the mitochondria suggest that mitochondrial dysfunction, especially at complex I, may play a larger role in the development of PD than previously realized (Schapira, 2006).

In 1983, astute epidemiological and clinical observation by Langston et al. (1983) led to the identification of MPTP as a compound capable of causing an acute permanent parkinsonian syndrome that was virtually indistinguishable from PD in a group of drug users in California. Since this time, almost 4000 articles have been published on MPTP according to PubMed. However, the critical issue of how MPTP exerts its toxicity on dopamine neurons has remained a topic of debate. It has been argued that complex I inhibition does not completely explain the neurotoxicity of MPTP (Lotharius and O’Malley, 2000; Nakamura et al., 2000; Obata, 2002). Previously, we have demonstrated that MPP$^+$ binds to the same site on complex I as rotenone (Richardson et al., 2005) and NDI1-expressing cells are resistant to rotenone toxicity because of the lack of this binding site (Sherer et al., 2003). Thus, NDI1-expressing cells provided a unique opportunity to define the role of interaction with complex I in the toxicity of MPP$^+$. Here, we transfected the DAT into this cell line to directly determine the role of complex I in the toxicity of MPP$^+$. MPP$^+$ toxicity was totally

![Image: Immunohistochemical evidence of the protective effect of NDI1 against dopaminergic neurodegeneration in the striatum and substantia nigra pars compacta. Mice were treated as described in Figure 2 and immunohistochemical staining of TH was performed on sections from the striatum and substantia nigra of the same animals described in Figure 2B and 2C. Representative images of TH immunostaining in the striatum and substantia nigra of mice administered vehicle during surgery and then saline 6 months later (A, G), NDI1-expressing mice administered saline (C, I), vehicle mice administered MPTP (B, H), and NDI1-expressing mice administered MPTP (D–F, J–L). The graded level of protection ranging from some protection to complete protection is readily observed in both the striatum (D–F) and substantia nigra (J–L) of NDI1 mice administered MPTP compared to the dopaminergic damage observed in vehicle mice administered MPTP (B, H).]
abolished in the NDI1 cells, demonstrating that the lack of the binding site for rotenone on NDI1 was sufficient to prevent the toxicity of MPP\(^+\) and providing further support for our previous finding that the binding site for rotenone and MPP\(^+\) are the same (Richardson et al., 2005). Although these in vitro studies do not rule out additional downstream effects of MPP\(^+\) that may occur in vivo, such as microglial activation or excitotoxicity (Beal, 1998; Wu et al., 2002), these findings suggest that complex I is likely the primary molecular site of action and initiator of MPP\(^+\) neurotoxicity. An alternate hypothesis for MPTP toxicity has proposed that displacement of dopamine from synaptic vesicles (Lotharius and O’Malley, 2000; Obata, 2002) and resultant oxidative damage may be largely responsible for MPTP toxicity. However, a recent study employing genetic and pharmacological depletion of dopamine in mice revealed that the absence of dopamine did not exacerbate MPTP toxicity (Hasbani et al., 2005), suggesting that dopamine displacement does not play a primary role in MPTP toxicity and reinforcing findings that complex I is the primary molecular target of MPTP.

Having established that NDI1 overexpression prevented MPP\(^+\) toxicity in vitro, we next sought to determine whether bilateral injection of an adeno-associated virus carrying the NDI1 gene into the substantia nigra could prevent the neurotoxicity of MPTP. Recently, we utilized unilateral administration of NDI1 to test the ability of NDI1 to protect against high-dose MPTP toxicity and found some degree of neuroprotection for all but three of the 13 mice examined (Seo et al., 2006). Although the use of a unilateral injection paradigm in our initial study allowed the untreated side to be able to serve as an internal control, this may not be the most optimal paradigm for assessing the dopaminergic toxicity of MPTP. First, there appears to be a natural imbalance in the dopamine system between the left and right side of the caudate putamen. Indeed, unilateral toxin lesions in experimental animals can result in sprouting of dopaminergic projections on both sides, which may affect the outcomes measured (Blanchard et al., 1996).

Here, we used bilateral injections of NDI1 and systemic MPTP to more closely mimic the clinical condition observed in PD. Bilateral injection of NDI1 into the substantia nigra did indeed provide significant protection against striatal dopamine depletion following MPTP administration (48% decrease in NDI1-expressing animals and 84% in non–NDI1-injected animals), similar to that observed with unilateral injection. Because we have previously observed that there appears to be variance in the level of transgene expression between individual animals (Seo et al., 2006), it is possible that there is variance in the degree of neuroprotection between individual animals. Indeed, we found that out of the 12 animals injected with NDI1 and MPTP, there was a wide range of protection observed (10–100%). These results were corroborated by Western blots of

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**FIG. 4.** Viral-mediated expression of NDI1 attenuates MPTP-induced glial and microglial activation. Mice were treated as described in Figure 2, and Western blots for (A) GFAP and (B) Glut5 were used to determine the level of glial and microglial activation, respectively. There were no significant differences between vehicle and NDI1-injected animals, and the data from these groups were collapsed to form the control group. Results represent mean ± SEM. **Indicates significantly different from control (p ≤ 0.001) by ANOVA followed by means separation by SNK. ***Indicates significantly different from vehicle mice administered MPTP (p ≤ 0.01). (C) Representative Western blots of GFAP, Glut5, and β-actin, which was used to ensure equal protein loading.

**FIG. 5.** Viral-mediated expression of NDI1 prevents MPTP-induced behavioral deficits. Mice were treated as described in Figure 2. One week after MPTP administration, mice were tested on the grid test for behavioral deficits as determined by forepaw step distance. MPTP administration significantly reduced forepaw step distance in vehicle-treated animals (p ≤ 0.05). This behavioral deficit was prevented in NDI1-expressing animals as there was no significant difference between control animals and NDI1 animals administered MPTP.
DAT, TH, and VMAT2 in these same animals, which we have previously shown to be excellent indicators of MPTP-induced dopaminergic damage (Tillerson et al., 2002). DAT levels were not significantly different between NDI1-expressing animals and those not expressing NDI1, suggesting that altered uptake of MPP+ is not likely to have influenced the results similar to what we observed in vitro. We also observed qualitatively similar results when we analyzed dopaminergic damage by immunohistochemical methods for TH in the dopaminergic cell bodies of the substantia nigra and terminals in the striatum. Finally, a similar degree of neuroprotection was observed when we assessed the degree of astrogliosis and microgliosis, which have been shown to be excellent indicators of MPTP-induced neurotoxicity and known to be increased in PD brain. It should be noted that we assessed astrogliosis and microgliosis at 7 days following injection, which is not the peak of activation of these markers (Francis et al., 1995). Therefore, we cannot rule out that similar degrees of glial activation may occur between vehicle and NDI1-injected animals treated with MPTP at the time of peak effect. Nonetheless, the totality of our assessments clearly demonstrates a significant protective effect of NDI1 against MPTP neurotoxicity. Although we did observe variance in the protective effect of NDI1, recent advances in viral and nonviral methods for introducing transgenes into the brain may provide an avenue for achieving increased and more consistent transgene expression allowing for increased neuroprotection in future experiments (de Lima et al., 2005).

In PD, there is evidence that neurodegeneration begins long before patients are diagnosed and that the behavioral symptoms that are characteristic of PD and the most devastating part of the disease process do not fully manifest until there is greater than 80% loss of striatal dopamine (Koller, 1992). We have previously developed sensitive behavioral tests in MPTP-treated mice that correlate highly with loss of striatal dopamine and are able to detect behavioral dysfunction when there is less than 80% dopamine depletion (Tillerson and Miller, 2003; Tillerson et al., 2002). Here, we observed total prevention of MPTP-induced behavioral deficits in NDI1-expressing mice. Significant protection was observed even in the animals that were only marginally protected from the neurochemical deficits caused by MPTP. These data suggest that even a small level of neuroprotection is sufficient to ameliorate behavioral deficits in an animal model of PD. Taken in concert, our data provide support for the inhibition of complex I as a major contributor to the dopaminergic degeneration and behavioral deficits observed in animals treated with MPTP.

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