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Therapeutic attenuation of mitochondrial dysfunction and oxidative stress in neurotoxin models of Parkinson's disease

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Abstract

Parkinson's disease (PD) is a progressive neurodegenerative disorder for which there is no current therapy preventing cumulative neuronal loss. There is substantial evidence that mitochondrial dysfunction, oxidative stress, and associated caspase activity underlie the neurodegeneration observed. One potential drug therapy is the potent free radical scavenger and antioxidant cystamine, which has demonstrated significant clinical potential in models of neurodegenerative disorders and human neurological disease. This study examined the oral efficacy of cystamine in the MPTP and 6-hydroxydopamine neurotoxin models of PD. The neuroprotective effects of cystamine treatment significantly ameliorated nigral neuronal loss, preserved striatal dopaminergic projections, and improved striatal dopamine and metabolite levels, as compared to MPTP alone. Cystamine normalized striatal 8-hydroxy-2′-deoxyguanosine levels and ATP concentrations, consistent with reduced oxidative stress and improved mitochondrial function. Cystamine also protected against MPTP-induced mitochondrial loss, as identified by mitochondrial heat shock protein 70 and superoxide dismutase 2, with concomitant reductions in cytochrome *c* and caspase-3 activities. The neuroprotective value of cystamine was confirmed in the 6-hydroxydopamine model. Together these findings show cystamine's therapeutic benefit to reduce neuronal loss through attenuation of oxidative stress and mitochondrial dysfunction, providing the rationale for human clinical trials in PD patients.
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Keywords: Cystamine; MPTP; 6-OHDA; Basal ganglia; ATP; Capsase-3; Dopamine

1. Introduction

Parkinson's disease (PD) is a devastating neurological disorder characterized by motor and behavioral disturbances resulting from selective neurodegeneration within the basal ganglia. PD affects approximately 2% of the population with incidence variation associated with age, gender, and race [1–3]. It is the second most common age-related neurodegenerative disorder after Alzheimer's disease. Clinical manifestations of PD initially present as motor abnormalities, consisting of a resting tremor, postural instability, and bradykinesia [4]. As disease progresses, these motor symptoms worsen, with concurrent behavioral and cognitive disruptions, including depression, anxiety, akinesia, and ultimately dementia [5]. The neuropathology of idiopathic PD is distinguished by a significant loss of dopaminergic neurons within the substantia nigra pars compacta (SNpc) [6]. Loss of dopaminergic nigral neurons is coincident with a decrease in dopamine, the dopamine transporter (DAT), and the dopamine metabolites dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) in PD brain [7–9].

Mitochondrial dysfunction and oxidative stress are widely held to play pivotal roles in the observed neurodegeneration in

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PD and are supported by several experimental findings in human PD [10–12]. There is significant oxidative damage to lipids, proteins, and DNA observed in postmortem PD striatum and within the substantia nigra, along with decreased glutathione levels [13–19]. In addition to reduced brain ATP levels, mitochondrial alterations in peripheral tissue and postmortem PD brain are a major source of reactive oxygen species [20–23]. Studies of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), which produces parkinsonism in humans, and MPTP and rotenone toxicity in laboratory animals, show that the selective nigral neurodegeneration acts through inhibition of complex I of the electron transport chain. In addition, the neurotoxin 6-hydroxydopamine (6-OHDA) has also been used as an experimental animal model of parkinsonism to induce selective dopaminergic neuronal loss in the substantia nigra. Consistent with the human PD findings, toxin models in both non-human primates and mice recapitulate the biochemical and cellular changes observed in human PD [24–26], with a loss of dopaminergic nigral neurons and dopamine, DAT, DOPAC, and HVA levels, along with significant increases in reactive oxygen species [25,27–30]. It is of great interest to note that substantia nigra dopaminergic neurons accrue mtDNA deletions with normal aging, resulting in neuronal death [31,32].

Mitochondrial injury also activates apoptotic signaling pathways that result in neuronal death [33,34]. The release of cytochrome *c* results in the activation of a family of cysteine proteases known as caspases [35]. Cytochrome *c* activation of caspase-9 induces the subsequent activation of caspase-3, the downstream executioner molecule of this death signaling cascade, resulting in both destruction of the intracellular architecture as well as activating additional signaling cascades that contribute to neuronal death [36]. Support for the role of cytochrome *c*-mediated neuronal death in PD has been reported in both PD patients and murine models of PD [37,38].

Existing medical care for PD primarily focuses on symptom management, optimizing functions that are in continual decline, and providing ever-increasing levels of assistance. Given the lack of an effective therapeutic intervention that can halt or slow the progression of PD, compounds with antioxidant capacities and the ability to reduce mitochondrial dysfunction may be efficacious. Among several promising compounds is cystamine, a potent antioxidant, free radical scavenger, and caspase inhibitor [39–41]. Cystamine has demonstrated significant neuroprotection in multiple models of neurodegenerative disease [42–46] and its reduced form, cysteamine, is safe, tolerable, and effective in human diseases [47,48]. While the efficacy of cystamine administration has previously been reported in the MPTP model of parkinsonism [45], mechanisms of neuroprotection for cystamine were not addressed. In addition, the doses used in the above study were inconsistent with those previously reported in other models of neurodegeneration. As such, the present studies sought to determine a dose-dependent therapeutic benefit with respect to neuronal loss and other neuropathological sequelae along with how cystamine may ameliorate the pathophysiological events associated with MPTP- and 6-OHDA-induced neurodegeneration.

2. Materials and methods

2.1. Animals

Male wild type mice with the B6CBA background were obtained from Jackson Laboratories (Bar Harbor, ME) and maintained at the Bedford Veterans Affairs Medical Center. A total of 135 mice were used in the MPTP and 6-OHDA studies. All mice were held in a temperature-controlled room that was maintained under a 12-hour light/dark cycle and had access to food and water *ad libitum*. All animal experiments were performed in accordance with the NIH Guide for the Care and Use of Laboratory Animals and were approved by the Boston University School of Medicine and Veterans Administration Animal Care Committees.

2.2. MPTP and cystamine treatment

Adult mice ($N=15$/group) were randomly distributed into one of six treatment groups at 8.5 months of age: untreated (no cystamine and no MPTP), MPTP-treated (no cystamine), 120 mg/kg cystamine+MPTP, 250 mg/kg cystamine+MPTP, 500 mg/kg cystamine+MPTP, or 750 mg/kg cystamine+MPTP. Cystamine was prepared daily and administered in drinking water, with intake monitored to maintain adequate dosing throughout the experiment. Using a dosing strategy similar to previous neuroprotective studies in the MPTP model of PD [49–51], all cystamine groups received cystamine treatment for one week prior to MPTP administration. During the second week, cystamine treatment continued and all MPTP treatment groups received intraperitoneal injections of 20 mg/kg MPTP b.i.d., over five days. Cystamine treatment continued for seven days after the last MPTP treatment with mice euthanized at that time point for biochemical and neuropathological analysis.

2.3. 6-OHDA and cystamine treatment

An additional cohort of mice were weighed and randomly placed into one of three cystamine treatment groups at 8.5 months of age: untreated (no cystamine and no 6-OHDA), 6-OHDA-treated (no cystamine), and 750 mg/kg cystamine+6-OHDA ($N=15$/group). Cystamine was administered as noted above. After one week of cystamine treatment, 6-OHDA (Sigma-Aldrich, USA) (2.5 μg) was stereotactically injected into the right striatum (coordinates: 0.5 mm anterior to bregma, −2.0 mm lateral to the midline, and 3.1 mm ventral) [52] under pentobarbital anesthesia (50 mg/kg, i.p.). A Hamilton syringe was stereotactically inserted and allowed to equilibrate for 30 s followed by injection over 60 s. Dwell time was 30 s. Cystamine administration continued for 1 week after surgery. Mice were then euthanized for neuropathological and biochemical analyses.

2.4. Histological evaluation

Mice ($N=15$/group) were deeply anesthetized and transcardially perfused with 4% buffered paraformaldehyde at 90 days, with care to avoid the introduction of any physical or perfusion artifact, post-fixed in the perfusate for 2 h, rinsed in buffer and then cryoprotected in a graded series of 10 and 20% glycerol/2% dimethyl sulfoxide. Frozen serial sections were cut at 50 μm, stored in six well plates, and stained for Nissl substance using cresyl violet and for immunocytochemical detection of tyrosine hydroxylase (TH), DAT, mitochondrial heat shock protein 70 (mtHSP70), cytochrome *c* (cyt *c*), active caspase-3, or superoxide dismutase 2 (SOD2). Sections were incubated overnight in mouse anti-TH (1:2000; Chemicon, Temecula, CA), rabbit anti-DAT (1:500; Serotec, Raleigh, NC), mouse anti-mtHSP70 (1:200; Affinity BioReagents, Golden, CO), rabbit anti-cyt *c* (1:200; Santa Cruz, Santa Cruz, CA), anti-rabbit active caspase-3 (1:200; Abcam, Cambridge, MA), or anti-rabbit SOD2 (1:500, Abcam). Sections were incubated in a goat anti-mouse or goat anti-rabbit peroxidase-conjugated secondary antibody to detect TH and DAT and visualized using diaminobenzidene. For immunofluorescent visualization, sections were incubated in donkey anti-rabbit Cy3-congugated or donkey anti-mouse FITC-conjugated secondary antibodies to detect mtHSP70, cyt *c*, active caspase-3, and SOD2 and counterstained with DAPI. An additional series of sections were processed for Fluoro-Jade immunofluorescence (IF) (Histo-Chem Inc., Jefferson, AZ) staining as previously described [53]. All immunofluorescent procedures were carried out in the dark.

2.5. Microscopy and image analysis

Light and fluorescent microscopy was performed using a Nikon Eclipse E800 microscope with a Spot RT digital camera. Digital imaging analysis of cyt *c* and active caspase-3 puncta, representing apoptotic figures [54] was performed using an IX81 microscope (Olympus, Inc.) equipped with a IX2-DSU3 spinning confocal disk unit and a PLAPON 60XOTIRFM objective (NA 1.45; Olympus, Inc.) and FL-CUBE dichromatic mirror and emission filter sets (Semrock). Images were captured at 0.25 μm intervals, and stacks were deconvoluted with a constrained iterative algorithm, using a cooled charge-coupled device camera (Orca-ERGA; Hamamatsu). Optical sections and 3D images were captured using IPLab (BD Biosciences, Bethesda, MD) and processed using Auto Deblur (Media Cybernetics, Inc.). Rendered images were processed further for Isosurface and 3 dimensional optics. The spatial distribution of MnSOD (SOD2) in substantia nigral neurons was determined using confocal microscopy and an image analysis program (Scanalytics BD Biosciences-Bioimaging and AQI-X-COMBO-CWF, Media cybernetics Inc. Bethesda, MD). Isosurface images were reconstructed after a deconvolution of the confocal image by the AQI-X-COMBO-CWF program (Media cybernetics Inc. Bethesda, MD). "Isosurfaces" are a graphical rendering technique that creates 2D contours in 3D space by interpolating between stacked sequential images, such as the 2D cellular maps that comprise a cross section of the 3D data volume. We analyzed a series of 40 confocal layers representing fluorescence data from substantia nigral neurons and subsequently developed an abstract image that provided the results seen in the Fig. 5.

2.6. Quantification/stereology

Absolute neuronal counts of Nissl and TH-immunoreactive (IR) neurons were performed in the substantia nigra pars compacta (SNpc) from serial sections throughout the rostro-caudal plane within the SNpc (from Interaural 0.88/bregma −2.92 through Interaural 0.00/bregma −3.80) [52]. To quantify cyt *c* and caspase 3, immunofluorescence puncta representing apoptotic figures [54] (>8 pixels) and SOD2 or mtHSP70 labeled mitochondria, rendered images from the SNpc were analyzed using ImageJ and the ITCN module [55].

2.7. HPLC analysis

Identification of dopamine, DOPAC, and HVA in mouse brain was performed as previously described [27]. To determine ATP levels, brain samples were sonicated in cold (4 °C) perchloric acid and centrifuged. The supernatant was neutralized with 2 M NaH_2PO_4, and samples were run on 16 channel HPLC-EC Coularray (ESA, Chelmsford, MA) with 2 M NaH_2PO_4, 0.1% methanol and 120 mg/L of tetrabutyl ammonium phosphate used for the A mobile phase, and 0.1 M NaH_2PO_4, 0.1% methanol, 20% acetonitrile and 200 mg/L of tetrabutyl ammonium phosphate used for the B mobile phase. A liquid chromatographic electrochemical carbon column switching technique was employed for the detection of 8-hydroxy-2′-deoxyguanosine (8-OH_2dG), as previously described [56]. To determine MPTP and MPP+ levels, brain tissue homogenates were incubated with either 10 μg MPTP/ml or 10 μg MPTP and 4 mg cystamine/ml. The samples were mixed and 100 μl were removed and added to 400 μl of ice-cold acetonitrile/0.4% HAC. Samples of 100 μl were removed for each time point (0 30, 60, and 120 min). Samples were centrifuged and decanted, evaporated by speedvac and reconstituted in 100 μl mobile phase A (0.1 M LiPO4 pH 2.9), and run on a 16 channel HPLC-EC Coularray (ESA, Chelmsford, MA) with UV detection set at 240 nm and florescence set 210–310, with a 40 min gradient. Buffers were mobile phase A and mobile phase B (0.1 M LiPO4, 55% acetonitrile, pH 2.9). Both MPTP and MPP+ (Sigma-Aldrich) were detected by UV and florescence using Shiseido C18 4.6×25 cm and Capcell pak C18 4.6 mm×7.5 mm columns with the auto sampler set to 4 °C.

2.8. Statistics

Interval scale data involving more than two groups were analyzed using one-way analysis of variance. If an overall significant difference was detected, multiple comparisons were performed with Fishers least significant difference test. For the MPTP conversion experiment, regression analyses were performed to determine whether the slopes for the two conditions (MPTP vs. MPTP–cystamine) were significantly different. For each analysis, we included MPTP or MPP+ levels as the dependent variable, and three predictor variables: Minutes (0, 30, 60, 120), Group (MPTP, MPTP–cystamine), and the interaction term Group×Minutes expressed as a product vector (The Group×Minute vector in multiple regression is equivalent to a linear trend interaction component×group in ANOVA). For all analyses, a significance level of $p \leq 0.01$ was observed.

3. Results

An analysis of MPTP conversion to MPP+ in the presence of cystamine showed no differences between MPTP and MPTP–cystamine treated brain homogenates (Fig. 1). Comparison of the curves for MPTP levels in MPTP and MPTP–cystamine treated brain using regression analysis (Fig. 1A) revealed no differences in the rate of MPTP degradation ($t(20)=0.197$, $p=0.846$). Similarly, comparison of the curves for MPP+ levels in MPTP- and MPTP–cystamine-treated brain (Fig. 1B) revealed no differences in the rate of MPP+ formation ($t(20)=0.646$, $p=0.526$). These results provide evidence that cystamine does not disrupt MPTP metabolism or the conversion of MPTP to MPP+.

Neuropathological examination of MPTP-treated mice revealed bilateral lesions and a striking loss of Nissl-stained SNpc neurons, as compared to untreated mice (Fig. 2A and B; Table 1). This was coincident with a conspicuous reduction in TH-IR neurons and a marked increase in fluoro-jade-IF-positive

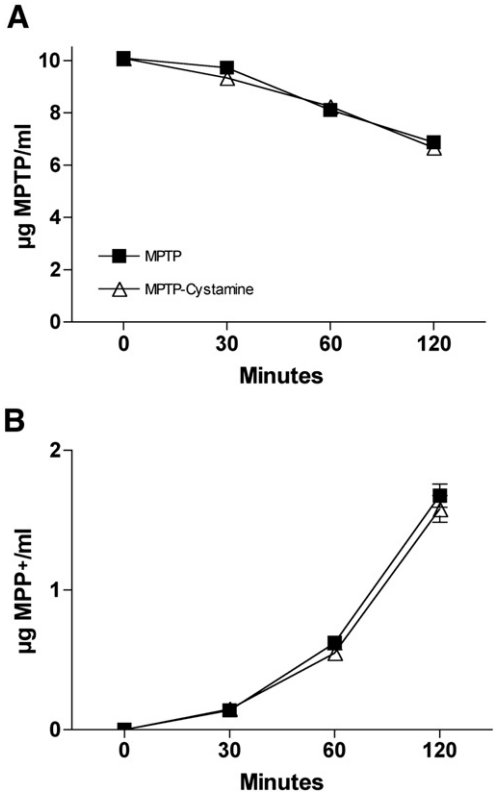

Fig. 1. Effect of cystamine on MPTP degradation and MPP+ formation. A. Brain MPTP levels were not significantly altered in MPTP-treated brain compared to MPTP–cystamine-treated brain. B. Similarly, levels of MPP+ were not significantly altered in MPTP-treated brain compared to MPTP–cystamine-treated brain.

neurons. A dose-dependent improvement in neuropathology was observed using cystamine administration. Treatment with cystamine (250 mg/kg) resulted in a decrease in SNpc neuronal loss (Fig. 2C), along with improved numbers of TH-IR neurons and a simultaneous decrease in fluoro-jade-IF-positive neurons. Treatment with the highest dose of cystamine (750 mg/kg) resulted in the greatest neuroprotection (Fig. 2D), combining a relative sparing of TH-IR neurons with a near complete absence of fluoro-jade-IF. Cell counts confirmed these observations (Table 1). There was a significant reduction in the SNpc neuronal number in Nissl-stained sections from MPTP-treated mice, as compared to untreated mice ($F_{(3,16)}=881.04$, $p<0.01$). A similarly significant reduction in the number of TH-positive SNpc neurons in MPTP-treated mice was also observed ($F_{(3,15)}=311.6$, $p<0.01$). Treatment with cystamine resulted in a dose-dependent improvement in SNpc neuronal number. Cystamine (250 mg/kg) significantly improved Nissl neuronal cell counts and TH-positive neurons by 4.8% and 12.2%, respectively.

Table 1
Effects of cystamine on cell number within the substantia nigra in MPTP-treated mice

Group	Estimated cell count	
	Cresyl violet	TH+
Untreated	951.2±6.64 *	711.8±13.05 *
MPTP	473.8±9.35 †	261.0±20.17 †
MPTP+cystamine, 250 mg	693.5±5.32 #	394.5±2.87 #
MPTP+cystamine, 750 mg	896.6±3.44 **	647.6±8.32 **

*$p<0.0001$ vs. all, †$p<0.0001$ vs. all, #p,0.0001 vs. all, **$p<0.0001$ vs. all.

Treatment with the highest dose of cystamine (750 mg/kg) resulted in a significant (39%) improvement in Nissl SNpc neuron cell numbers with a concomitant 84.8% neuroprotection of TH-positive neurons compared to mice treated with MPTP alone.

An analysis of striatal dopaminergic projections showed a clear reduction in DAT-IR terminals in MPTP-treated mice

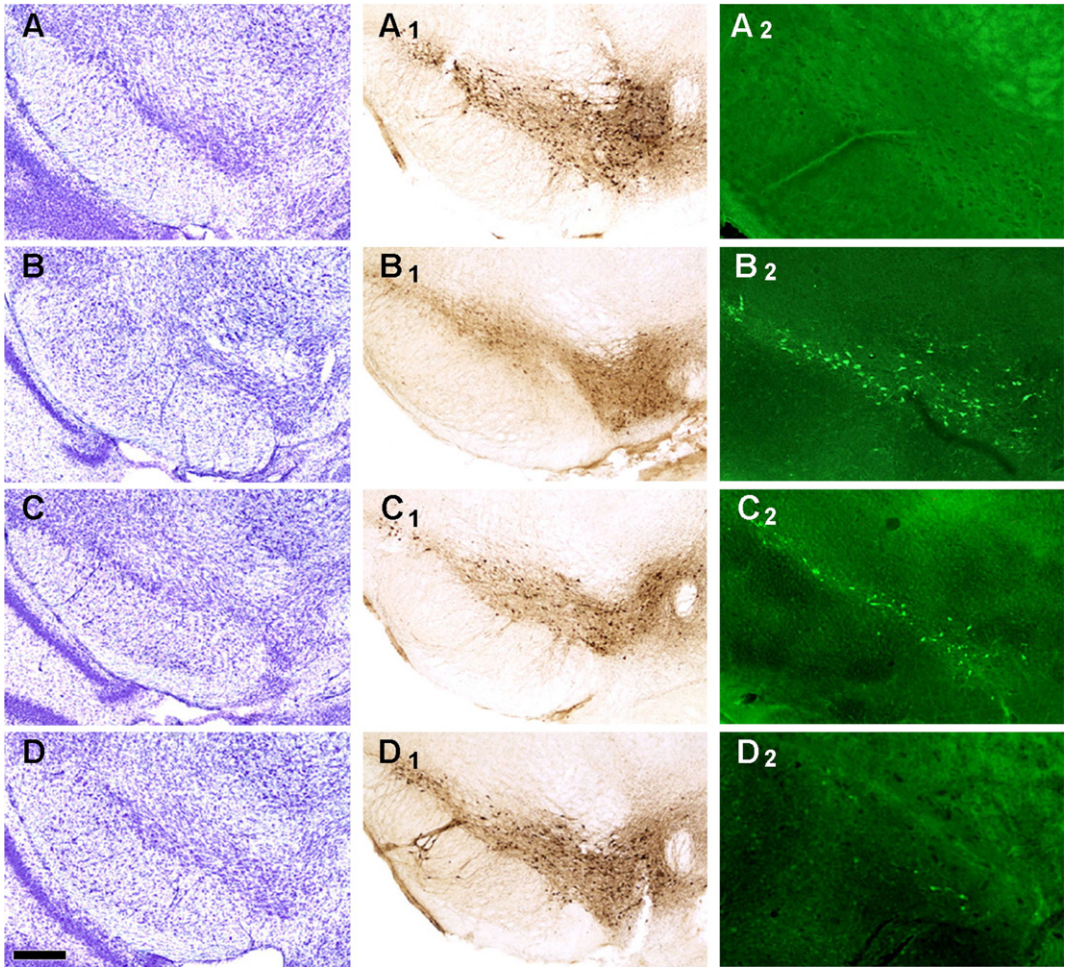

Fig. 2. Neuroprotective effects of cystamine within the substantia nigra in MPTP-treated mice. Cresyl violet staining of the substantia nigra in non-lesioned control mice (A), MPTP-treated mice (B), MPTP and 250 mg/kg cystamine (C), and MPTP and 750 mg/kg cystamine (D) reveals marked neuronal loss in MPTP-treated mice (B) compared to non-lesioned mice (A). Pretreatment with 250 mg/kg cystamine (C) provides significant protection against SNpc neuronal loss, while the most marked and significant neuroprotection is observed after pretreatment with 750 mg/kg cystamine (D). Loss of dopaminergic neurons was confirmed through tyrosine hydroxylase immunoreactivity (TH-IR). There is a significant reduction in TH-IR after MPTP treatment (B1), as compared to non-lesioned control mice (A1). Pretreatment with 250 mg/kg cystamine significantly rescued neuronal TH-IR (C1), with greater improvement after administration of 750 mg/kg cystamine (D1). Confirmation of neuronal death using fluoro-jade-IF revealed no fluoro-jade-IF in untreated mice (A2). There was a marked increase in fluoro-jade-IF in MPTP-treated mice (B2), which was reduced in a dose-dependent manner by the administration of 250 mg/kg cystamine (B3) and 750 mg/kg cystamine (D2). Bar in D = 200 μm.

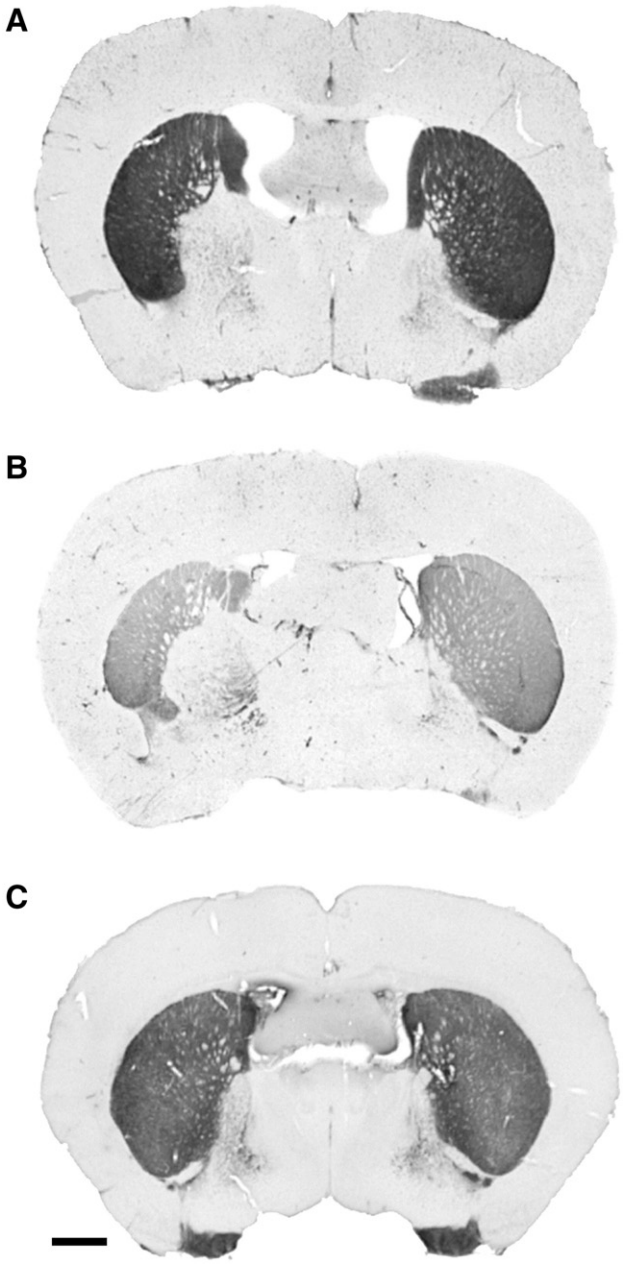

Fig. 3. Neuroprotection of striatal dopaminergic projections after cystamine administration in MPTP-treated mice. A. DAT-IR within the striatum in non-lesioned control mice. B. Treatment with MPTP resulted in a marked reduction of DAT-IR. C. Pretreatment with cystamine (750 mg/kg) ameliorated striatal DAT-IR loss in MPTP-treated mice. Bar in C = 1 mm.

(Fig. 3), consistent with the loss of nigral TH-positive neurons. Treatment with the highest dose of cystamine (750 mg/kg) resulted in a marked improvement in DAT-IR terminal fibers in the striatum. An analysis of striatal levels of dopamine, DOPAC and HVA (Fig. 4) showed that levels of dopamine ($F_{(2,22)}$= 33.19, $p<0.01$), DOPAC ($F_{(2,21)}$=31.73, $p<0.01$), and HVA ($F_{(2,22)}$=20.78, $p<0.01$) were all significantly reduced in MPTP-treated mice compared to untreated controls, and that cystamine treatment significantly improved striatal levels of dopamine, DOPAC and HVA.

To assess mitochondria, the select mitochondrial markers mtHSP70 and SOD2 were employed. Mitochondrial HSP70 is a resident mitochondrial protein involved in protein translocation, while SOD2 is a select marker of mitochondria that is an essential component of the mitochondrial defense against oxidative damage by reactive oxygen species. Loss in mitochondrial number has not been previously reported in MPTP toxicity. In comparison to untreated mice, MPTP treatment resulted in a significant reduction in mitochondrial number in SNpc neurons, as identified

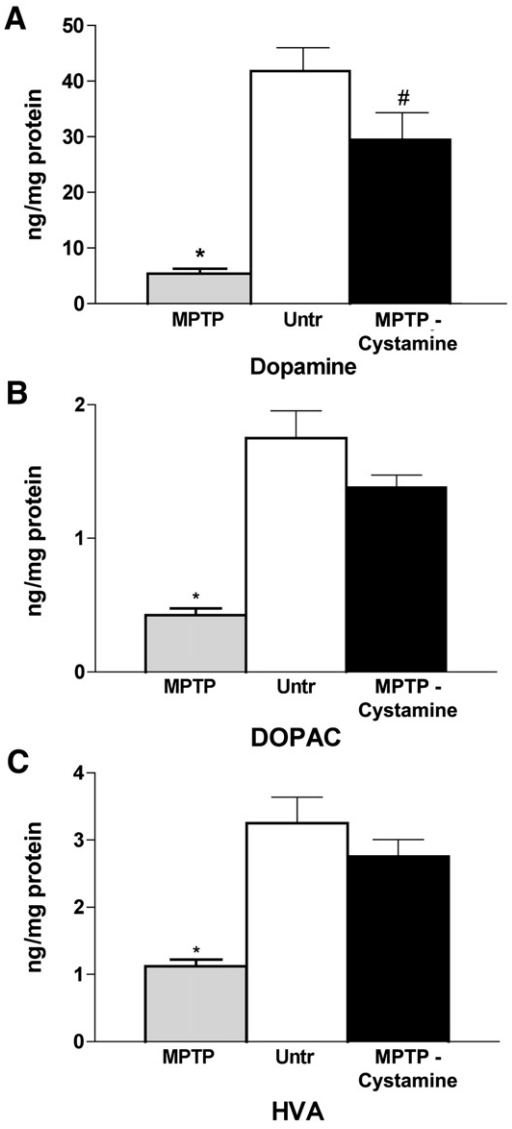

Fig. 4. The effect of cystamine administration on the levels of brain dopamine and dopamine metabolites in MPTP-treated mice. A. MPTP treatment resulted in a significant decrease in striatal dopamine levels compared with non-lesioned control mice. Cystamine administration (750 mg/kg) significantly improved striatal dopamine levels compared with MPTP-treated mice. B. Consistent with an MPTP-mediated reduction in striatal dopamine, there was a significant reduction in striatal DOPAC. Cystamine improved striatal DOPAC to levels indistinguishable from untreated mice. C. MPTP treatment significantly reduced HVA within the striatum, as compared to non-lesioned mice. Cystamine administration significantly improved striatal HVA to comparable levels in non-lesioned mice. *$p<0.01$ vs. Untreated, MPTP–cystamine; #$p<0.05$ vs. Untreated, MPTP.

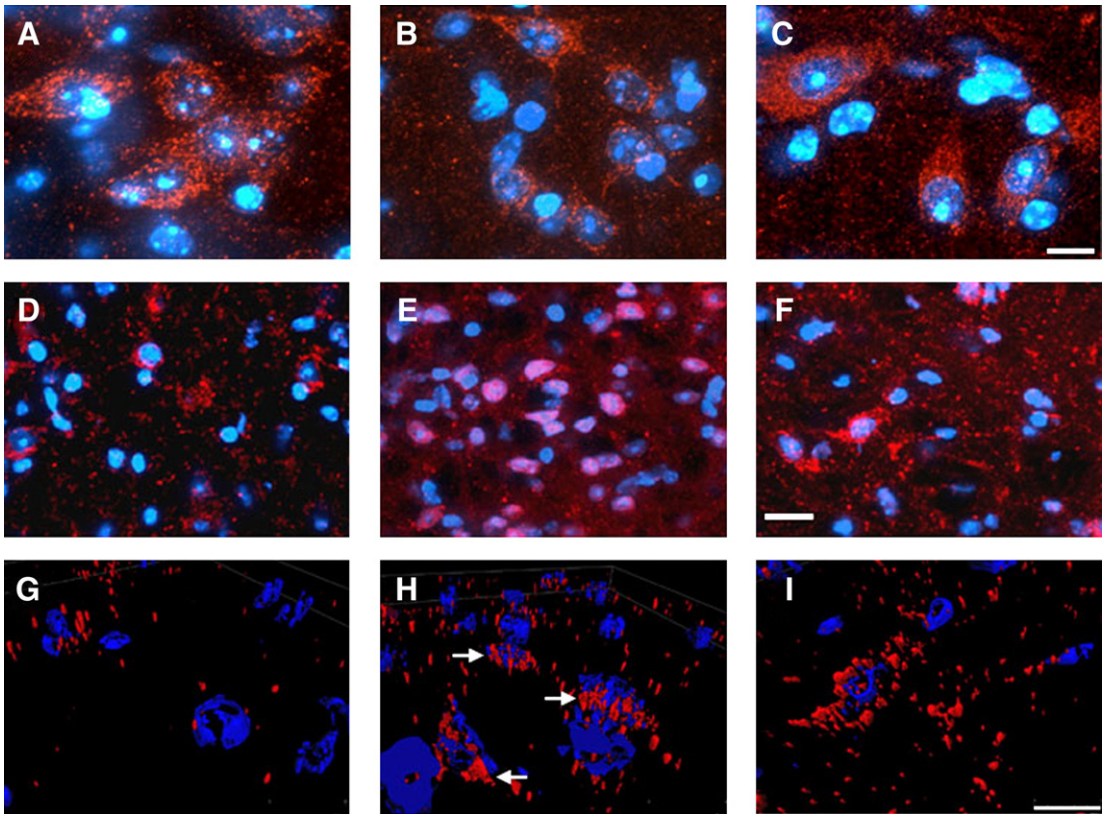

Fig. 5. Cystamine reduced mitochondrial loss in the SNpc of MPTP-treated mice. Examination of mtHSP70-IF within the SNpc of a non-lesioned control mouse (A) reveals mitochondria (red puncta) and nuclei (DAPI: blue). There is a significant MPTP-induced decrease in mtHSP70-positive mitochondria within SNpc neurons (B). Treatment with cystamine significantly improved mtHSP70-positive mitochondrial number in MPTP-treated mice (C). Cystamine also improves SOD2-positive mitochondria in the SNpc of MPTP-treated mice. The profile of SOD2-positive mitochondria within the SNpc of a non-lesioned control mouse (D) reveals mitochondria (red puncta) and nuclei (blue). There is a marked MPTP-induced decrease in SOD2-positive mitochondria within the SNpc (E). Cystamine treatment markedly improved the integrity of neuronal mitochondria in MPTP-treated mice. Examination of SOD2-IF within the SNpc of a cystamine-treated mouse (F) reveals increased numbers of mitochondria. In comparison to non-lesioned mice (G), confocal 3 dimensional isosurface analysis of SOD2-IF shows distinct MPTP-induced SOD2-positive mitochondrial translocation tightly formed around the nucleus (arrows) (H), which is reduced after pretreatment with cystamine in MPTP-treated mice (I). Bar in C = 15 μm. Bar in F = 20 μm. Bar in I = 10 μm.

by mtHSP70-IF ($F_{(2,9)}=27.953$, $p<0.01$) (Fig. 5A–C). Cystamine treatment significantly ameliorated mitochondrial loss in MPTP-treated mice ($p<0.01$). These results were confirmed using an antibody against SOD2 (Fig. 5D–F). There was a marked reduction in SOD2-positive mitochondria in MPTP-treated mice compared with non-lesioned controls (non-lesioned control: $9.67\pm0.16/5$ μm^2; MPTP-treated: $6.12\pm0.57/5$ μm^2; MPTP/cystamine-treated: $9.50\pm0.23/5$ μm^2; $F_{(2,9)}=9.341$, $p<0.01$), that was significantly improved by cystamine treatment ($p<0.001$). The degree of mitochondrial preservation in cystamine-treated mice was consistent with the observed neuronal sparing. Further analysis of SOD2-IF-positive mitochondria, using a novel 3 dimensional confocal method, revealed that the mitochondria had tightly translocated around the nucleus, and that this phenomenon was reduced by cystamine treatment. (Fig. 5G–I).

Consistent with the observed mitochondrial alterations, confocal analysis of neurons from the SNpc showed a marked MPTP-mediated increase in cyt *c*-IF, as compared to non-lesioned mice ($F_{(2,6)}=90.973$, $p<0.01$; Fig. 6). Treatment with cystamine (750 mg/kg) resulted in a significant decrease in cyt *c*-IF compared with MPTP alone ($p<0.01$). Consonant with the MPTP-mediated increase in cyt *c*-IF, there was a concomitant significant MPTP-mediated increase in caspase-3 activation ($F_{(2,9)}=143.69$, $p<0.01$; Fig. 6) that was significantly reduced after treatment with cystamine (750 mg/kg) ($p<0.01$). Together, these results demonstrate an MPTP-induced mitochondrial injury and activation of apoptotic signaling cascades within the substantia nigra.

Improved mitochondrial function was further demonstrated in the analysis of ATP levels. Striatal ATP levels were significantly reduced in MPTP-treated mice, as compared to non-lesioned control mice ($F_{(2,28)}=55.93$, $p<0.01$) (Fig. 7A), with significantly improved ATP levels in cystamine-treated mice (750 mg/kg), such that they were statistically indistinguishable from control mice. These findings were coupled with an attenuation of oxidative damage (Fig. 7B). Biochemical examination revealed significantly elevated levels of 8-OH$_2$dG in the striatum of MPTP-treated mice, compared to non-lesioned control mice ($F_{(2,12)}=11.22$, $p<0.01$), that were normalized by cystamine administration.

In order to provide additional support for the neuroprotective potential of cystamine, a series of unilateral intracerebral 6-OHDA injections were performed. 6-OHDA injections into the right

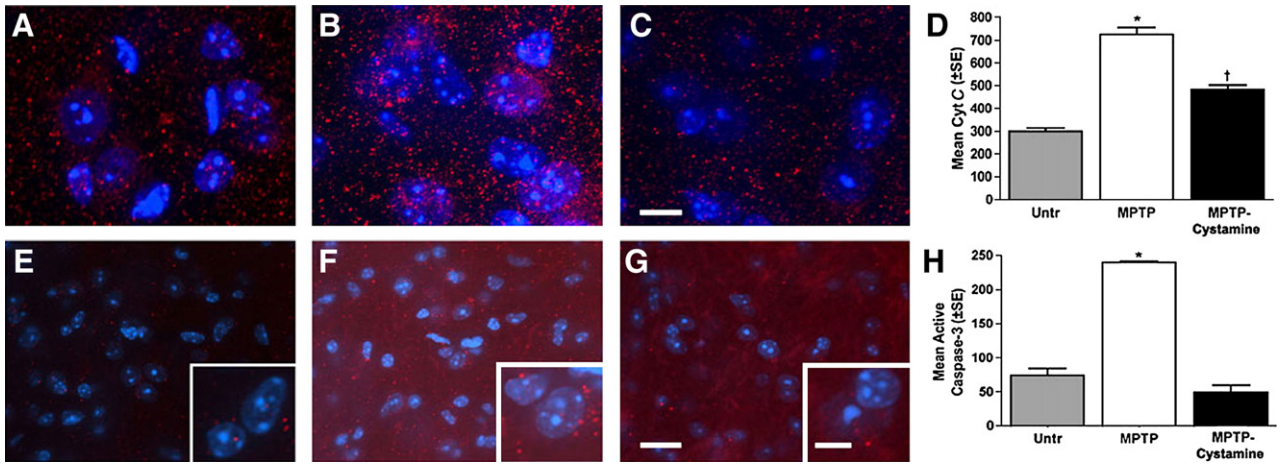

Fig. 6. Cystamine administration preserved neuron mitochondrial integrity within the substantia nigra and reduced both cyt *c* release and caspase-3 activation in MPTP-treated mice. The profile of cyt *c* within the SNpc of a non-lesioned control mouse reveals mitochondria (red) surrounding neuronal nuclei (DAPI; blue) (A). There is a marked MPTP-induced increase in cyt *c*-IF within the substantia nigra compared to non-lesioned control mice (B). Cystamine treatment significantly improved the integrity of neuronal mitochondria in MPTP-treated mice, by significantly reducing cyt *c*-IF (C). Quantitative analysis of mitochondrial cyt *c*-IF confirms the histological findings (D). Cystamine administration significantly ameliorates active caspase-3-IF in MPTP-treated mice. Active caspase-3 in the substantia nigra of in non-lesioned control mice reveals active caspase-3-IF (red) surrounding neuronal nuclei (blue) (E). There is a marked MPTP-induced increase in active caspase-3 within the substantia nigra compared to untreated control mice (F). Treatment with cystamine significantly reduced caspase-3 activity within the substantia nigra in MPTP-treated mice (G). Quantitative analysis of active caspase-3-IF demonstrates a significant increase in MPTP-treated mice (H). Cystamine administration significantly reduced caspase-3 activity in MPTP-treated mice. Bar in C = 15 μm. *$p<0.01$ vs. non-lesioned and MPTP–Cystamine, †$p<0.01$ vs. non-lesioned and MPTP. Bar in G = 25 μm. Bar in inset (G) = 10 μm *$p<0.01$ vs. non-lesioned, MPTP–cystamine.

striatum resulted in a prominent ipsilateral reduction of TH-IR within the substantia nigra along with a significant reduction in the number of TH-positive SNpc neurons, in comparison to non-lesioned mice ($F_{(3,16)}=273.4, p<0.01$) (Fig. 8 and Table 2). Using the optimal dose of cystamine (750 mg/kg), there was a marked improvement in TH-positive neurons in the SNpc ($p<0.01$). Consistent with these findings, DAT-IR terminals in the striatum, measured using densitometric analyses, were markedly reduced by 76% after 6-OHDA administration and rescued by cystamine treatment (96% of control) (Fig. 8). There were significantly elevated levels of 8-OH_2dG in the striatum of 6-OHDA-treated mice compared to non-lesioned control mice ($F_{(2,10)}=9.31$, $p<0.01$) (Table 2) that were normalized by cystamine administration (750 mg/kg), consistent with the MPTP findings.

4. Discussion

Therapeutic strategies that target the pathophysiological indices of neuronal death in PD hold significant promise in the treatment of patients with this neurological disorder. We show that treatment with cystamine exerts a dose-dependent therapeutic benefit in the murine MPTP and 6-OHDA models of PD, providing significant neuroprotection, while ameliorating biomarkers of oxidative stress and improving mitochondrial dysfunction and loss.

Mitochondrial dysfunction in idiopathic PD (Fig. 9) has been demonstrated through a reduction in complex I activity in substantia nigra homogenates from PD brain [23,57]. This reduction in complex I activity is associated with decreased immunoreactivity for complex I [58]. While the underlying causes of reduced complex I activity in PD are not well understood, alterations in mtDNA have been suggested [59], along with

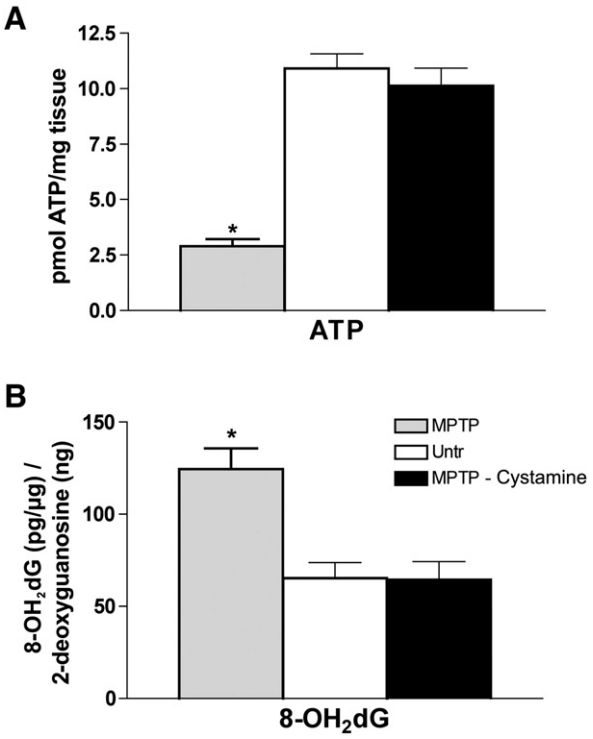

Fig. 7. Cystamine treatment significantly improved oxidative stress and mitochondrial dysfunction in MPTP-treated mice. A. Brain ATP levels were significantly reduced in MPTP-treated mice compared with non-lesioned mice. Cystamine administration significantly improved striatal ATP levels in MPTP-treated mice to levels observed in non-lesioned mice. B. In contrast, Striatal 8-OH_2dG levels were significantly elevated in MPTP-treated mice compared to non-lesioned controls. Cystamine treatment significantly reduced 8-OH_2dG in MPTP mice to levels indistinguishable from non-lesioned mice. *$p<0.001$ vs. non-lesioned, MPTP–cystamine.

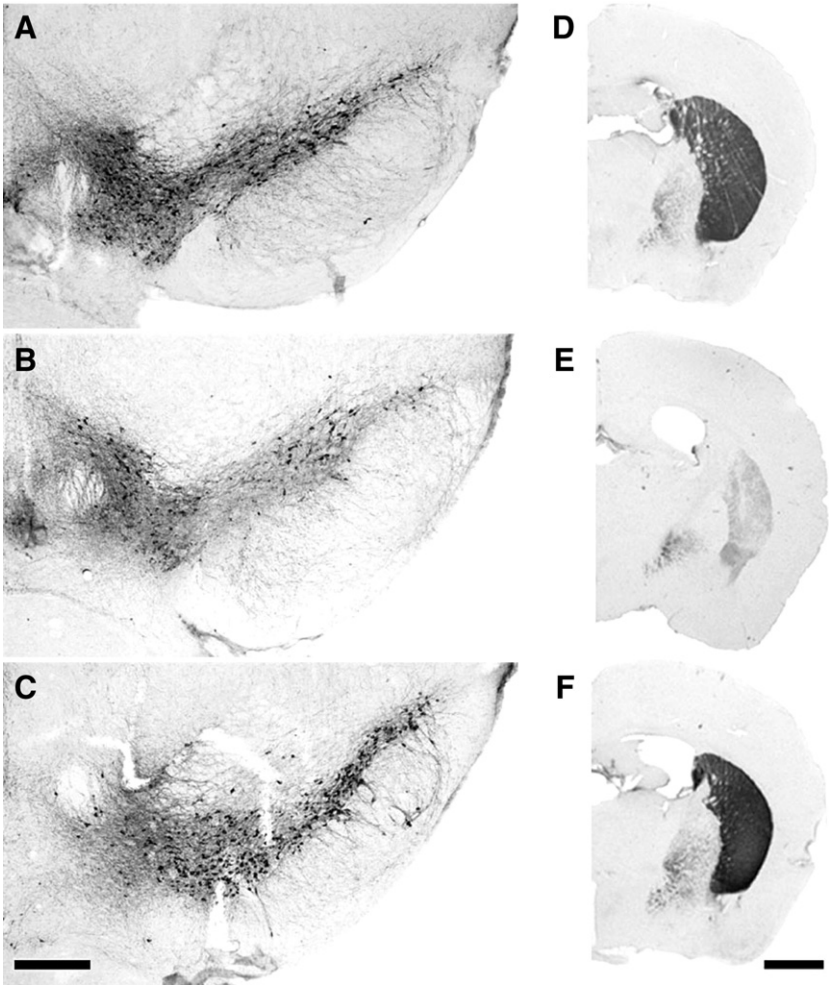

Fig. 8. Effects of 6-OHDA on tyrosine hydroxylase and DAT-IR in cystamine-treated mice. Intrastriatal microinjection of 6-OHDA resulted in a marked reduction of tyrosine hydroxylase immunoreactive-positive (TH-IR) neurons within the substantia nigra (B), as compared to non-lesioned control mice (A). Pretreatment with cystamine ameliorated TH-IR neuronal loss within the substantia nigra (C). Similar results were observed with DAT-IR within the striatum in non-lesioned mice (D), with a marked unilateral reduction of DAT-IR in 6-OHDA-treated mice (E). Pretreatment with cystamine markedly improved DAT-IR (F). Bar in C = 200 μm; F = 1 mm.

oxidative damage to mitochondrial complex subunits [60,61]. Indeed, alterations in mtDNA in the form of deletion mutations, as well as damage resulting from oxidative stress, have been observed in PD brain [19,32]. The mitochondrial genome encodes several RNA species, as well as 13 essential genes of the respiratory chain, including complex I [62]. In addition, deficits in complex I activity in PD platelets are associated with increased production of free radicals [63].

Whether oxidative stress contributes to mitochondrial injury or results as a consequence of mitochondrial dysfunction is not well established. It is well understood, however, that mitochondria, through oxidative phosphorylation and the electron transport chain, produce free radicals, such as peroxynitrite and the hydroxy radical [10,64]. Though an effective SOD2 scavenger system is present within normal mitochondria, damaged mitochondria may be less effective in combating free radical production, contributing to an increase in oxidative stress (Fig. 9). We show a significant numeral reduction of mitochondria in MPTP-treated mice that may indeed be dysfunctional, resulting in oxidative damage. Cystamine administration ameliorates this mitochondrial loss and decreases 8-OH_2dG, a biomarker of oxidative damage. It is also of interest to note that the MPTP-induced nuclear localization of SOD2 activity may be a consequence of both nuclear fragmentation and loss of mitochondrial integrity.

Within the SNpc, there is also another prominent cascade potentially responsible for the increase in oxidative stress observed in PD. Dopamine metabolism by monoamine oxidase results in the formation of hydrogen peroxide [13], and has been

Table 2
Effects of cystamine on substantia nigra cell number and 8-OH_2dG activity in 6-OHDA-treated mice

Group	TH+ cell count	8-OH_2dG level
Untreated controls	715.1±12.9	57.3±8.1 pg/μg
6-OHDA-treated	251.7±36.2*	114.7±15.2 pg/μg*
6-OHDA+cystamine, 750 mg	609.3±17.4	68.9±11.3 pg/μg

*$p<0.01$ vs. all.

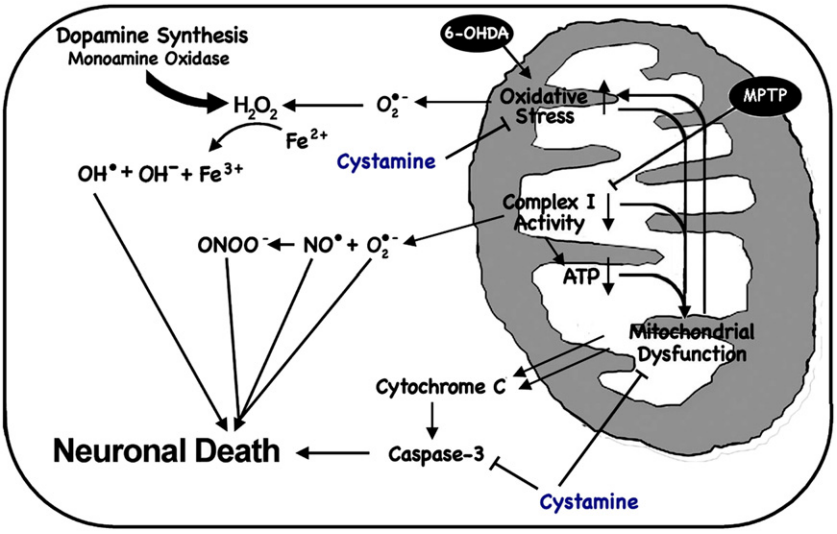

Fig. 9. Mitochondrial Dysfunction in PD. Both MPTP and 6-OHDA promote oxidative stress and mitochondrial dysfunction, which are linked in a feed-forward mechanism, increasing reactive oxygen species and the release of mitochondrial contents resulting in signaling cascades that promote neuronal death. Cystamine reduces oxidative stress and improves mitochondrial function. This results in normalized mitochondria, ATP, and cyt *c* levels, as well as reduced activity of caspase-3, improving neuronal viability.

suggested to inundate endogenous antioxidant mechanisms [65]. In the presence of iron, which is elevated in PD substantia nigra [14], peroxide is catalyzed and oxygen radicals are formed [66]. With the formation of reactive oxygen species, oxidative damage to proteins, lipids, and DNA results. Importantly, once oxidative damage occurs, it can promote additional oxidative injury [64].

In addition, apoptosis and caspase activity have been implicated in the neurodegeneration of PD [34]. Release of cyt *c* from mitochondria ultimately results in the activation of caspase-3, which executes cell death [33]. Importantly, increased caspase-3-IR has been demonstrated in both human PD and the MPTP murine model [37]. While the present series of experiments cannot rule out a direct effect of cystamine on caspase-3-induced neurodegeneration, previous work has demonstrated the ability of cystamine to inhibit capsase-3 activity *in vitro* [40]. It is important to note, however, that cystamine significantly reduced the MPTP-induced increase in cyt *c*-IF. Given that caspase-3 is a downstream target of cyt *c*, it is likely that cystamine-mediated improvement in mitochondrial function prevents the release of cyt *c* and the subsequent activation of caspase-3.

Altered protein–protein interactions leading to the formation of proteinaceous cytoplasmic aggregates, termed Lewy bodies, have also been suggested to play a role in PD pathogenesis [67]. A primary constituent of Lewy body inclusions is the protein α-synuclein, mutations of which are associated with an autosomal dominant form of PD [68]. Recent evidence suggests that α-synuclein aggregation is mediated in part by the activity of tissue transglutaminase, which results in cross linked α-synuclein [69,70]. Cystamine, in addition to possessing antioxidant and anti-caspase properties, is also a significant inhibitor of tissue transglutaminase. Acute MPTP administration is not associated with the formation of Lewy bodies in mice [71], and while the present study did not asses α-synuclein-IR, therapeutic benefit through inhibition of tissue transglutaminase cannot be ruled out, especially in light of recent findings demonstrating nigrostriatal neuroprotection in transglutaminase 2 knock-out mice treated with MPTP [72]. It is of importance to note that cross linking of α-synuclein has also been shown to occur via oxidative stress [73,74]. Thus, through antioxidant mechanisms, anti-caspase activity, and transglutaminase inhibition, cystamine has the potential to limit α-synuclein aggregation and thus promote neuroprotection in PD.

Mouse toxin models replicate many, but not all of the clinical, neuropathological, and molecular events observed in PD and, as such, their relevance to the human condition has been questioned. Postmortem PD tissues, however, only provide a static window of pathology at end stage disease and do not provide much understanding and characterization of early and moderate neurodegenerative events. As a consequence, the utility of toxin models cannot be underestimated. Given aspects of faithful disease recapitulation, toxin models that have been used over the past 20 years have provided a significant literature in identifying potential mechanisms of pathogenesis in the human condition and the molecular basis of select dopaminergic neuronal loss. Indeed, it has been shown that MPTP is a complex I inhibitor of the mitochondrial electron transport chain and that MPTP results in PD-like effects in humans [75–80]. The MPTP model also supports a role for oxidative stress and mitochondrial dysfunction in PD. Once metabolized, MPTP is converted to MPP+, which inhibits complex I. Inhibition of complex I results in increased oxidative stress [81,82]. There is also a decreased production of ATP in MPTP-treated mice, consistent with perturbed oxidative phosphorylation observed in human PD [23]. Oxidative stress and metabolic dysfunction are found in both human PD and MPTP models of PD [10,12,62]. As such, murine models that recapitulate human PD, such as MPTP, provide a useful platform to assess therapeutic potential.

Given the significant roles of oxidative stress and mitochondrial dysfunction in PD, cystamine is a very attractive candidate for therapeutic intervention. The long-term use of cysteamine bitartrate (Cystagon®, Mylan Laboratories, Inc.), the reduced form of cystamine and an orphan drug available in the US by prescription, has been well documented, with a starting dose of 10 mg/kg/day and increased weekly until a target dose of 60 – 90 mg/kg/day is reached. Side effects are restricted to nausea and vomiting. However, allergic rash, seizures and neutropenia have occurred in rare cases as a result of not incrementally increasing the dose [83]. These adverse events all resolve when the drug is withdrawn. A Phase I study using cysteamine in HD patients showed the drug to be safe and tolerable [47]. Previous studies assessing the therapeutic benefit of the antioxidant coenzyme Q_{10} in the MPTP model have demonstrated a significant neuroprotective potential [84]. This has lead to clinical trials of coenzyme Q_{10} in PD that have demonstrated therapeutic efficacy [85,86]. The antioxidant creatine has also showed significant neuroprotective potential in the MPTP model of PD [87], which led to a successful clinical futility trial in PD [88]. While previous work with cystamine in the MPTP model has been reported [45], no indication of the therapeutic neuroprotective mechanism was identified. In addition, this latter study employed cystamine doses that were markedly lower than effective doses employed in previous neurodegenerative contexts [43,44,46]. The doses of cystamine employed in the current study closely resemble those dosages found previously to be neuroprotective in other model systems. In addition, while the route of administration differs between the two studies (oral vs. intraperitoneal), it is worth noting previous work has demonstrated the significant therapeutic potential of oral cystamine in other murine models of neurodegeneration [89].

Importantly, while successful preclinical trials can demonstrate improved phenotype in murine models of PD, validating these results in PD patients is complicated by dose extrapolations between mouse and man. The reliance on Human Equivalent Dose extrapolation measurements derived from body surface area criteria in animals may not accurately predict the maximum-recommended safe dose in humans [90]. Evidence in support of this comes from human trials where human equivalent dosing of bioenergetic agents based on preclinical murine trials have not demonstrated similar efficacy in patients. The strengths of mouse models are in their utility to provide parallel pathophysiological targets that are present in patients, in their potential as sensitive predictors for therapeutic intervention, and their promise in the development of drug agents. While drug trials in mice confirm therapeutic direction, the challenge is in determining what dose might be of value in patients since the pharmacokinetics of mice and man is dissimilar. Notwithstanding, in concert with preclinical animal studies and human safety and tolerability trials in HD [47], the present findings provide the rationale for initiating clinical safety and tolerability trials in patients with PD using cystamine.

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