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The Role of Parkin in the Differential Susceptibility of Tuberoinfundibular and Nigrostriatal Dopamine Neurons to Acute Toxicant Exposure

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Abstract

Parkinson Disease causes degeneration of nigrostriatal dopamine (DA) neurons, while tuberoinfundibular DA neurons remain unaffected. A similar pattern is observed following exposure to 1-methy-4-phenyl-1, 2, 3, 6-tetrahydropyridine (MPTP). The mechanism of tuberoinfundibular neuronal recovery from MPTP is associated with up-regulation of parkin protein. Here we tested if parkin mediates tuberoinfundibular neuronal recovery from MPTP by knocking-down parkin in tuberoinfundibular neurons using recombinant adeno-associated virus (rAAV), expressing a short hairpin RNA (shRNA) directed toward parkin. Following knockdown, axon terminal DA and tyrosine hydroxylase (TH) concentrations were analyzed 24 hours post-MPTP administration. rAAV-shRNA-mediated knockdown of endogenous parkin rendered tuberoinfundibular neurons susceptible to MPTP induced terminal DA loss, but not TH loss, within 24 hours post-MPTP. To determine if the neuroprotective benefits of parkin up-regulation could be translated to nigrostriatal neurons, rAAV expressing human parkin was injected into the substantia nigra of mice and axon terminal DA and TH concentrations were analyzed 24 hours post-MPTP. Nigral parkin over-expression prevented loss of TH in the axon terminals and soma of nigrostriatal neurons, but had no effect on terminal DA loss within 24h post-MPTP. These data show that parkin is necessary for the recovery of terminal DA concentrations within tuberoinfundibular neurons following acute MPTP administration, and parkin can rescue MPTP-induced decreases in TH within nigrostriatal neurons.

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Keywords

parkin; dopamine; tyrosine hydroxylase; MPTP; tuberoinfundibular; nigrostriatal; parkinson's disease

1. Introduction

Parkinson disease (PD) is associated with the progressive degeneration of midbrain nigrostriatal dopamine (DA) neurons. Within 4–5 years of clinical diagnosis of PD there is an approximate 70–90% loss of nigrostriatal DA fibers in the striatum¹. By the 5 year time point, loss of functional DA fibers in the striatum is relatively stable and complete, and this loss of DA innervation to the striatum ultimately results in the motor disturbances observed in PD¹. Interestingly, during this same stage of PD, 40–70% of DA neurons of the substantia nigra remain intact¹, demonstrating a temporal progression of PD pathology in which functional DA fiber loss precedes actual loss of nigrostriatal neurons. The severe behavioral manifestations of dopaminergic terminal loss and the dissonance between the degree of fiber loss and actual cell death underscore the crucial need for early interventions in PD, designed to provide recovery and mitigate further degeneration. Although nigrostriatal neurons display severe degeneration and terminal dysfunction in PD, other central DA neuronal populations are affected to lesser extents. For example, mesolimbic DA neurons show an intermediate degree of degeneration while tuberoinfundibular DA neurons are completely spared by the disease^{2–5}. The molecular machinery responsible for DA handling, metabolism and release are similar between tuberoinfundibular and nigrostriatal neurons, and as such, characterization of the properties unique to PD-resistant tuberoinfundibular neurons may shed light on mechanisms that could mediate recovery from nigrostriatal neuronal degeneration.

This same pattern of differential susceptibility of central dopaminergic neurons is also observed in neurotoxicant-based animal models of PD. Specifically, nigrostriatal neurons display both axon terminal degeneration and cell loss following exposure to the neurotoxicant 1-methyl, 4-phenyl, 1, 2, 3, 6-tetrahydropyridine (MPTP), while tuberoinfundibular neurons are resistant to MPTP induced cell death, and fully recover terminal DA concentrations following an initial MPTP-induced loss^{6,7}. By examining the differential responses of tuberoinfundibular and nigrostriatal neurons to low-dose, one-time exposure to MPTP it may be possible to identify initial cell-specific mechanisms following toxicity, which mediate early events in dopaminergic terminal recovery in tuberoinfundibular neurons and degeneration in nigrostriatal neurons. Identification of such early mechanisms could be exploited in the development of translational therapeutics aimed at mediating recovery and halting degeneration of nigrostriatal neurons that have sustained fiber loss but are not yet dead at the time of PD diagnosis. One such potential mechanism underlying the differential susceptibility of tuberoinfundibular and nigrostriatal neurons is the ability to up-regulate the expression of neuroprotective proteins.

One of the major findings of our laboratory is the correlation between synthesis of key PD related proteins and the ability of tuberoinfundibular neurons to recover from acute MPTP toxicity. Specifically, tuberoinfundibular neurons recover axon terminal DA stores within 24

h following a single injection of MPTP, and this recovery is correlated with rapid and sustained up-regulation of the protein parkin^{6,7}. Conversely, nigrostriatal neurons show sustained terminal DA depletion along with decreased parkin protein concentrations following MPTP administration^{6,8}. The ability of tuberoinfundibular neurons to recover from the same toxicity that severely damages nigrostriatal neurons is not due to extrinsic factors such as decreased MPTP exposure, bioactivation of MPTP to MPP⁺, or MPP⁺ accumulation within tuberoinfundibular neurons⁶. Rather, it appears that tuberoinfundibular neuronal recovery is mediated by an intrinsic ability to up-regulate neuroprotective proteins, such as parkin, following cellular stress. The fact that tuberoinfundibular neuronal recovery from MPTP is protein synthesis dependent supports the role of neuroprotective protein expression in the recovery process⁶.

The ability of tuberoinfundibular neurons to up-regulate parkin expression could account for recovery from the neurotoxicant MPTP. Parkin is a multifunctional ubiquitin ligase that is neuroprotective against many of the cellular pathologies produced by MPTP, including; proteasomal stress, mitochondrial dysfunction, and DA associated toxicity^{9–11}. Further, exogenous over-expression of parkin can protect against some forms of MPTP or 6-hydroxydopamine toxicity^{12–14}, while decreased parkin concentrations are associated with increased nigrostriatal neuronal toxicity in PD and associated models^{6,7,15}. Accordingly, parkin is an attractive candidate for a mechanism that could mediate the recovery and homeostatic maintenance of DA neurons following an initial cytotoxic insult.

Evidence linking parkin to the recovery of tuberoinfundibular neurons from MPTP toxicity is, however, largely correlative. Here we aimed to test the hypothesis that the recovery of tuberoinfundibular neurons from single-acute MPTP toxicity is mediated by the ability to up-regulate parkin protein expression following toxicant exposure. Further, if parkin was found to mediate tuberoinfundibular recovery from MPTP toxicity, we also sought to determine if this unique response of tuberoinfundibular neurons could be translated to nigrostriatal neurons. To test these hypotheses, endogenous parkin expression was knocked-down in tuberoinfundibular neurons using a recombinant adeno-associated virus (rAAV) type 2/5 expressing a short hairpin RNA (shRNA) directed toward the murine parkin gene. In parallel, parkin was exogenously over-expressed within nigrostriatal neurons using a rAAV vector expressing a FLAG-tagged human parkin (F-hParkin) transgene. Four-weeks following rAAV administration, mice received a single injection of MPTP or saline vehicle. Importantly, this MPTP dosing paradigm does not result in any overt cell death within either of the neuronal populations being examined^{1,7}, thus the ability of parkin to maintain, or recover, dopaminergic terminal integrity was determined by analyzing axon terminal DA and tyrosine hydroxylase (TH) protein concentrations of tuberoinfundibular and nigrostriatal neurons within 24 h post-MPTP.

2. Materials and Methods

2.1. rAAV 2/5 Construction

The parkin shRNA was designed based on the public RNAi Consortiums mouse parkin shRNA TRCN0000041143. The target parkin sequence was CGTTTCATTATCTGCAACTTT and the scrambled siRNA sequence was

GTCGACAATTCATATTTGCATGTCGC. All sequences were BLASTriatum confirmed for specificity. Synthetic DNA encoding shRNA sequences targeting mouse parkin and the scrambled control were cloned under control of the H1 promoter and flanked by AAV 2 terminal repeats as previously described¹⁶. The shRNA expression cassette also contained a humanized enhanced green fluorescent protein (GFP) transgene under control of the chicken beta actin/Cytomegalovirus promoter hybrid (CBA) promoter as a reporter for vector transduction within the brain.

Full-length human parkin (MJH 115) was identical to that used previously¹⁴. The FLAG-hParkin construct was generated by PCR using primers encoding a 5' in-frame FLAG epitope. The F-hParkin sense primer was 5'-GCG GCC GCA TGG ATT ATA AAG ATG ATG ATG ATA AAA TAG TGT TTG TCA GGT TCA ACT-3' and antisense was 5'-GCG GCC GCC ACG TCG AAC CAG TGG TCC C-3'. The F-hParkin transgene was then cloned into the rAAV genome behind the CBA promoter as previously described¹⁴. The entire transcription cassette was flanked by AAV 2 terminal repeats.

Briefly, viruses were created by co-transfection of AAV plasmid with the helper plasmid pXYZ5 and purified using an iodixanol step gradient and sepharose Q-column chromatography as described previously¹⁷. Virus titers were determined using dot blot assays as described¹⁷. The final rAAV2/5 titers were 3.4×10^{13} viral genomes/milliliter (vg/ml) for rAAV F-hParkin, 3.55×10^{13} vg/ml for rAAV scrambled shRNA and 1.42×10^{13} vg/ml for the rAAV parkin shRNA. rAAV expressing the mouse parkin and scrambled control shRNAs were normalized to 1.4×10^{13} using balanced salt solution (Alcon Laboratories). Virus stock was confirmed to be at least 99% pure by silver-stained sodium dodecyl sulfate acrylamide gel fractionation.

2.2. Animals

All experiments were conducted in 8–10 week old male C57Bl/6J mice purchased from Jackson Laboratories (Bar Harbor, MA). Animals were housed two to four per cage, maintained in a light-controlled (12 h light/dark cycle; lights on 0600 h) and temperature-controlled ($22 \pm 1^\circ\text{C}$) room, and provided with food and water *ad libitum*. The Michigan State University Institutional Animal Care & Use Committee approved all experiments using live animals (AUF 08/08-123-00).

2.3. Stereotaxic Surgery

Mice were induced using 4–5% isofluorane while the surgical site was shaved and the animal was placed in the stereotaxic frame. Isofluorane was reduced to 2% for the remainder of the surgery. The surgical site was scrubbed with a Betadine swab prior to incision. A single incision was made along the rostrocaudal axis of the skull and tissue overlying the skull was retracted to expose the skull surface. A Hamilton syringe (Hamilton, Reno, NV) with a 30 gauge blunt-tip needle was fitted with a siliconized pulled glass micropipette with an opening of 60–80µm to use for injections. Axons of tuberoinfundibular neurons terminate within the median eminence, which is a medial structure receiving projections from both the left and right arcuate nuclei. As there is no way to analyze changes occurring within the axon terminals originating specifically from either the left or right arcuate, mice received

bilateral injections of rAAV into the left and right arcuate nuclei. Bilateral arcuate nucleus injections of 250nl/side were performed at a 10 degree angle in the following coordinates from Bregma: Anteroposterior (AP) -1.75mm, mediolateral (ML) 1.25 and -1.25 mm, and dorsoventral (DV) -6.2 mm from the skull surface. Unlike tuberoinfundibular neurons, axons from nigrostriatal neurons do not converge into a single terminal field, rather neurons originating from the right substantia nigra project to the right striatum, and vice versa. The discrete nature of nigrostriatal projections enables experimental manipulation within one hemisphere and the use of the opposite hemisphere as an internal control. Unilateral substantia nigra injections of 500nl were performed using the following coordinates from Bregma: AP: -3.0 mm, ML: -1.4 mm, DV: -4.4 mm from the skull.

rAAV vectors were injected at a titer of 1.4×10^{13} vg/ml for shRNAs and 3.4×10^{13} vg/ml for parkin cDNA. Volumes were injected at a rate of 125 nl/min using an automated micropump (World Precision Instruments). The needle was left in place for an additional 5 min to prevent reflux. The hole in the skull was filled with sterile bonewax, and the skin replaced and closed using surgical staples. Mice were kept on a heating pad until recovery from anesthesia and returned to their home cages. Mice were checked daily for signs of infection/distress.

2.4. Drugs

MPTP was purchased from Sigma-Aldrich (St. Louis, MO) and dissolved in 0.9% sterile saline on the day of the experiment. Doses were calculated as the free base of MPTP. Mice received a single injection of either saline vehicle (10 ml/kg; s.c.) or MPTP (20 mg/kg; s.c.) and the experiment was terminated 24 h post-MPTP injection.

2.5. Tissue Preparation

For neurochemical and protein analyses mice were sacrificed by decapitation and brains were rapidly removed and placed on an ice-cooled glass stage. Under a dissecting microscope, the axon terminal region of tuberoinfundibular neurons (median eminence; median eminence) was collected and the remaining brain was quickly frozen on dry ice. Consecutive frozen coronal sections (500 μ m) were prepared throughout the rostrocaudal extent of the brains using a cryostat set at -10 °C (CTD-Model Harris, International Equipment Co., Needham, MA) and the regions of interest were microdissected using a modification of the method described previously [1]. Brain regions microdissected include the axon terminal region of nigrostriatal neurons (striatum; striatum), the cell body region of nigrostriatal neurons (substantia nigra; substantia nigra) and the cell body region of tuberoinfundibular neurons (arcuate nucleus; arcuate nucleus). These tissue samples were used for neurochemical and Western blotting analyses, and were processed according to the appropriate protocols described below.

For immunohistochemical (IHC) analysis, mice transcardially perfused with 0.9% saline followed by 4% paraformaldehyde. Brains were then removed and post fixed in 4% paraformaldehyde and cryoprotected in 20% sucrose. Coronal sections (35 μ m) through the entire rostrocaudal axis of the brain were prepared with a cryostat (-19°C).

2.6. Neurochemical Analyses

Microdissected brain tissue samples were placed into cold tissue buffer (0.1M phosphate-citrate buffer pH 2.5) and sonicated with three consecutive 1 sec bursts (Heat Systems Ultrasonics, Plainville NY). Protein was pelleted by centrifugation at 12,000 x g (Beckman Coulter Microfuge, Palo Alto, CA) for 1 min. The content of DA and 3, 4-dihydroxyphenylacetic acid (DOPAC) in supernatants was determined with high pressure liquid chromatography coupled with electrochemical detection (HPLC-ED) and expressed as a concentration in nanogram (ng) per milligram (mg) protein as previously described⁶

2.7. Fluorescence Immunohistochemistry

Immunohistochemistry (IHC) was performed on free-floating sections through injected brain regions in order to visualize the location and spread of rAAV-mediated expression, in addition to providing an estimation of transduction efficiency. Sections were washed in 0.05M phosphate buffer containing 0.1% Triton x-100 (PB-TX) and incubated overnight in either 1:2000 rabbit antiTH (Millipore AB152), 1:1000 chicken anti-GFP (Abcam 13970) or 1:2000 goat anti-FLAG (Abcam 1257). Following overnight incubation in primary antibody, sections were washed in PB-TX and incubated in either 1:500 Cy-3 conjugated goat anti-rabbit (Jackson Immune Research 111-165-003), 1:1000 Alexafluor 488 donkey anti-goat (Invitrogen A11055) or 1:1000 Alexafluor 488 goat anti-chicken (Invitrogen A11039) secondary antibodies for 1h at room temperature and protected from light. Sections were then washed and covered slipped using ProLong Gold antifade reagent (Molecular Probes, Eugene, OR). Sections were viewed on a Nikon TE-2000-U-Fluorescence Microscope (Melville, NY)

2.8. Western Blot Analyses

Microdissected brain samples were sonicated in ice cold homogenization buffer (TBS containing 1% SDS, 0.1mM PMSF, 1mM DTT with Complete Mini Protease Inhibitor Cocktail Tablets, (Roche Diagnostics, Mannheim, Germany) pH 7.4, and centrifuged (10,000 x g 10 min). The supernatants containing total cytoplasmic protein were removed and placed into fresh microcentrifuge tubes. The pellets were assayed for protein content using the Bradford protein method (Thermo Fisher, IL, USA). Protein (15–20 µg) from each sample was run on polyacrylamide gels and transferred to 0.45 µm FL-PVDF membrane (Millipore, Pittsburgh, MA, USA) by electrophoresis. Following, PVDF membranes were cut using protein standard ladders as a guide (to probe bound proteins separately) and immersed in buffer containing either 1:1000 rabbit anti-parkin (Cell Signaling 4211), 1:2000 rabbit anti-TH (Millipore AB152), 1:2000 rabbit anti-FLAG (Sigma F7425) and 1:20,000 mouse anti-GAPDH (Sigma G8795) primary antibodies overnight at 4°C. Membranes were subsequently incubated with IR Dye 800-conjugated goat anti-rabbit (Li-Cor 926-68021) or 680-conjugated goat anti-mouse (Li-Cor 926-32210) secondary antibodies (1:15,000 dilution in blocking buffer) for 1 h at room temperature.

Membranes were washed and cut membranes were reassembled after immunoblotting. Bound antibodies were visualized with the Odyssey infrared imager (Li-Cor Biosciences, Lincoln NE). The density of each band was quantified by measuring the infrared absorbance using the Odyssey infrared imager and Odyssey software (Version 3.0, Li-Cor Biosciences).

Relative density was obtained by normalizing the band density of parkin to that of the control protein used to account for variations in loading of samples onto the gel. GAPDH was used as control protein. Detection and visualization of control proteins was linear relative to estimated protein loaded. Expression levels of GAPDH were similar amongst the compared brain regions regardless of treatment. Each FL-PVDF membrane contained representative samples from all experimental conditions.

2.9. Statistical Analyses

Power analyses were conducted to determine optimal sample size required to detect a statistical difference at $p < 0.05$ with a power of 0.8. The experimenter was blind to all experimental conditions during data collection and analysis. One-way analysis of variance (ANOVA) tests were used to detect statistical significance between two or more groups on a single independent variable. Two-way ANOVAs were used to detect statistical significance between two or more groups when there were two independent variables in the experiment. Repeated measures ANOVA was used when comparing brain regions within the same animal. A *p* value of less than or equal to 0.05 was considered statistically significant. If the ANOVA revealed an interaction of statistical significance Tukey's test was used for multiple comparisons among groups.

3. Results

3.1. Characterization of rAAV Parkin-shRNA Vector Expression and Knockdown of Endogenous Parkin in the Arcuate Nucleus

Mice received bilateral stereotaxic injection of rAAV expressing the parkin shRNA and a green fluorescent protein (GFP) reporter into the cell body regions of tuberoinfundibular neurons, the arcuate nucleus, and 4 weeks post-surgery transduction of DA neurons in the arcuate nucleus was assessed by visualization of the GFP reporter gene in TH expressing neurons. Fig. 1 shows immunohistochemical (IHC) characterization of TH (Figs. 1A, D, G; red) and GFP (Figs. 1B, E, H; green) expressing neurons. DA neurons that have been successfully transduced express both TH and GFP, and appear yellow (Figs. 1C, F, I). The bilateral injection of rAAV transduced the entire rostrocaudal axis of the mediobasal hypothalamus, and GFP expression was observed in $51.8 \pm 6\%$ of dopaminergic neurons in the arcuate nucleus. GFP expression was observed in both the soma (box in Figs. 1A–C and corresponding higher magnification in Figs. 1D–F) and in the axon terminals of tuberoinfundibular neurons located in the median eminence (Arrow heads in Figs. 1A–C and corresponding high magnification images in Figs. 1G–I). Further, GFP expression was observed in both TH and non-TH expressing cells of the mediobasal hypothalamus as previously described^{1,18}.

Four-weeks following rAAV-shRNA injection, parkin protein concentrations in the arcuate nucleus were measured by quantitative immunoblotting to validate knockdown of endogenous parkin expression. Following rAAV-mediated parkin shRNA expression, endogenous parkin protein levels were decreased to approximately half of non-injected controls (Fig. 1J, K). Expression of the parkin shRNA in the arcuate nucleus decreased expression of both the 44kDa and 53kDa isoforms of parkin (Fig. 1J). The 53kDa MW

isoform, which is the primary native form of the protein^{2-5,19}, was used as the index of parkin concentrations in all future analyses.

3.2. Tuberoinfundibular Neuronal Recovery from Single-Acute MPTP Toxicity following Knockdown of Parkin in the Arcuate Nucleus

To determine the role of parkin in the ability of tuberoinfundibular neurons to recover from MPTP, mice received either the parkin shRNA, the scrambled control shRNA, or remained naïve to surgery. Four-weeks following surgery, mice received a single injection of MPTP or saline vehicle and were sacrificed 24 h later. Fig. 2 shows changes in parkin protein expression in the arcuate nucleus 24 h following MPTP administration. Replicating previous findings^{6,7}, parkin protein concentrations were increased in the arcuate nucleus of MPTP treated mice that were naïve to surgery, as compared to saline treated controls (Figs. 2A, B). Injection of the scrambled shRNA had no effect on the MPTP-induced increase in parkin protein within the arcuate nucleus 24 h following MPTP treatment (Figs. 2A, B). The rAAV parkin-shRNA decreased parkin expression by approximately forty-percent compared to animals that remained naïve to surgery in both saline and MPTP treated mice (Figs. 2A, B). These data demonstrate that the parkin shRNA is able to effectively decrease parkin protein expression in the arcuate nucleus and abrogate the ability of tuberoinfundibular neurons to up-regulate parkin expression following MPTP-induced injury.

Median eminence (location of tuberoinfundibular axon terminals) DA and DOPAC concentrations were measured 24 h post-MPTP in mice that had previously received rAAV expressing the parkin shRNA or the scrambled shRNA, in addition to a non-injected control group (Fig. 3). Within the non-injected surgery group, median eminence DA concentrations in MPTP treated animals were no different than saline treated controls 24 h post-MPTP (Fig. 3A), reflecting recovery of terminal DA stores as shown in our prior work^{6,7}. In contrast, knockdown of parkin in the arcuate nucleus significantly attenuated the ability of tuberoinfundibular neurons to recover axon terminal DA concentrations (Fig. 3A). The scrambled shRNA had no effect on median eminence DA concentrations following MPTP (Fig. 3A). There was no change in the DA metabolite DOPAC (Fig. 3B), nor the DOPAC to DA ratio (an index of DA turnover; Fig3C) following any of the treatment paradigms examined.

Within nigrostriatal neurons, the MPTP-induced loss of axon terminal DA occurs with a concomitant loss of TH protein in the striatum⁶⁻⁸. To determine if preventing the toxicant-induced increase in parkin expression would render tuberoinfundibular neurons susceptible to MPTP-induced loss of TH protein concentration, median eminence TH concentrations were measured 24 h following MPTP administration. TH protein concentrations were unaltered by MPTP, an observation that was consistent across non-injected mice and mice previously injected with rAAV expressing parkin shRNA (Fig. 4). A scrambled control shRNA was not included in the analyses since neither rAAV nor toxicant administration affected median eminence TH protein concentrations.

3.3. Characterization of rAAV F-hParkin Expression in the Substantia Nigra

Mice received unilateral injections of rAAV expressing F-hParkin into the left substantia nigra, allowing the use of the contralateral substantia nigra as an internal control. Fig. 5 shows a representative characterization of TH-immunoreactive (Figs. 5A, D, G; red) and FLAG-immunoreactive (Figs. 5B, E, H; green) neurons in the ventral mesencephalon. DA neurons that have been successfully transduced show immunoreactivity for both FLAG and TH and appear yellow (Figs. 5C, F, I). Successful transduction of DA neurons was observed in the left, ipsilateral midbrain, leaving the contralateral hemisphere devoid of exogenous parkin expression (Figs. 5A–C). The rAAV-mediated FLAG expression was observed in both TH and non-TH expressing cells in the midbrain. Four weeks post-surgery, $45.9 \pm 2.9\%$ of dopaminergic neurons in the injected substantia nigra were transduced and exhibited FLAG immunoreactivity (Figs. 5F, I).

To further confirm the expression of F-hParkin specifically within regions containing nigrostriatal neurons, quantitative immunoblotting was performed on tissue from the striatum and substantia nigra. F-hParkin was observed in the striatum and substantia nigra of the injected hemisphere (Fig. 5J), demonstrating transgene expression in the soma, as well as anterograde transport of F-hparkin to striatal terminals. Injection of rAAV expressing F-hParkin into the midbrain produced a large increase (7.6 ± 0.58 fold) in F-hParkin protein concentrations, relative to endogenous mouse parkin levels (data not shown), and this expression of F-hParkin was limited to the striatum and substantia nigra of the ipsilateral hemisphere only (Fig. 5J), confirming that the rAAV FLAG-hParkin did not transduce the non-injected contralateral substantia nigra. These data show successful expression of FLAG-hParkin within nigrostriatal neurons, and also supports the use of the contralateral, non-injected hemisphere as an internal control.

3.4. Nigrostriatal Neuronal Recovery from Single-Acute MPTP Toxicity Following Over-expression of F-hParkin in the Substantia Nigra

To determine if decreases in protective proteins following MPTP administration contributes to the pathology observed in nigrostriatal neurons, the ability of parkin to rescue MPTP-induced loss of striatal DA and TH was examined. Four-weeks post rAAV injection, mice were injected with a single dose of MPTP or saline vehicle and sacrificed 24 h later. Fig. 6 shows striatal DA, DOPAC, and the DOPAC/DA ratio, in the rAAV injected and non-injected hemispheres of saline or MPTP treated mice. Despite the successful expression of exogenous F-hParkin in the ipsilateral substantia nigra (Fig. 6A), striatal DA (Fig. 6B) and DOPAC (Fig. 6C) concentrations remain significantly depleted in both the injected and non-injected hemispheres 24 h post-MPTP. MPTP administration produced an increase in DA turnover as indexed by the DOPAC to DA ratio, in both the ipsilateral and contralateral hemispheres (Fig 6D). Interestingly, over-expression of human parkin in nigrostriatal neurons completely rescued the MPTP-induced decrease in TH protein concentrations within the striatum (Figs. 7A, B) and substantia nigra (Figs. 7C, D) within 24 h following toxicant exposure.

4. Discussion

At the onset of clinically observable symptoms of PD, the degree of nigrostriatal DA fiber loss exceeds that of actual cell death^{1,6}. Accordingly, there are likely nigrostriatal neurons that are dysfunctional, but could recover provided the necessary machinery or therapeutics. Here we sought to uncover early mediators of DA neuron recovery by examining the responses of separate dopaminergic subpopulations to single acute MPTP toxicity. There are noteworthy limitations to the use of neurotoxins in studying dopaminergic dysfunction as it relates to PD^{20,21}, however MPTP does recapitulate many of the molecular pathologies observed in PD^{22–30}. The single injection MPTP dosing paradigm has been extensively characterized, and primarily produces loss of the TH phenotype, with a corresponding loss of axon terminal DA, in the absence of any overt cell death^{6,7}. Although this type of toxicity may be similar to very early stage PD, within the current work the use of MPTP is not meant as a comprehensive model of PD *per se*, but rather a tool to examine early events in dopaminergic toxicity and recovery. Tuberoinfundibular neurons are resistant to PD pathology and have a unique ability to recover from neurotoxicant insult. As such, examining the response of tuberoinfundibular neurons to MPTP exposure provides a useful and innovative platform to dissect mechanisms of endogenous DA neuronal recovery that could potentially be translated to damaged nigrostriatal neurons. Here we show that one such mediator of dopaminergic terminal recovery is the protein parkin.

There is currently a growing body of evidence that suggests parkin is crucial in the homeostatic maintenance of central DA neurons^{9–11,31}. Mutations in the parkin gene cause nigrostriatal neuronal degeneration in autosomal recessive juvenile parkinsonism^{12–15}, while oxidative and nitrosative post-translational modifications of parkin are thought to contribute to nigrostriatal neurodegeneration in idiopathic PD^{6,7,15,32–34}. Conversely, the natural ability of tuberoinfundibular neurons to up-regulate parkin following cellular stress has been shown to correlate with the recovery and/or resistance of tuberoinfundibular neurons to several neurotoxins^{6–8,13,14,35}.

The data presented here support the role of parkin in neuronal maintenance of axon terminal dopaminergic homeostasis following acute MPTP toxicity. Specifically, we have shown for the first time that endogenous parkin expression is necessary for the cell autonomous ability of tuberoinfundibular neurons to recover terminal DA concentrations following acute neurotoxicant exposure. Further, over-expression of parkin in the substantia nigra was sufficient to rescue the MPTP-induced loss of TH in both the substantia nigra and striatum. This is in line with previous findings showing that parkin over-expression enhances the dopaminergic tone of nigrostriatal neurons *via* elevated TH expression¹⁴. Taken together, these results support the exploration of mechanisms regulating parkin expression, and pathways downstream of parkin, as targets for disease modifying therapies aimed at restoring normal DA neurotransmission in early stage PD.

The administration of rAAV expressing parkin shRNA transduced a large number of neurons in the mediobasal hypothalamus. However, though a large number of GFP positive cells were observed throughout the hypothalamus, the degree of transduction within target TIDA neurons was modest (roughly 50%). Further, though the parkin-shRNA significantly

decreased endogenous parkin protein concentrations in the arcuate nucleus, the extent to which this decrease represents the degree of parkin knockdown specifically within TIDA neurons remains unclear. Nonetheless, parkin protein concentrations were decreased within the arcuate nucleus, and possibly more importantly, the MPTP-induced upregulation of parkin in the arcuate nucleus was abolished. In this context of blunted parkin expression, tuberoinfundibular neurons were unable to recover terminal DA concentrations within 24 h of a single-acute MPTP challenge. It should be noted that the present studies are not able to determine if tuberoinfundibular neurons were rendered susceptible to MPTP toxicity due to an actual decrease in parkin expression or an inability to up-regulate parkin following toxicant exposure. The fact that tuberoinfundibular neurons show a similar inability to recover median eminence DA following exposure to a protein synthesis inhibitor (without directly reducing parkin expression) supports the latter of these two possibilities⁶. Further, though the current MPTP dosing paradigm used does not cause cell death in either of the neuronal populations examined^{6,7,36}, it is possible that the observed loss of DA following the combination of parkin knock-down and MPTP exposure could represent cell loss. Further experimentation is needed to uncover the mechanism of parkin's protection from toxicant exposure. These data confirm that parkin is necessary for mediating the recovery of tuberoinfundibular terminal DA concentrations following acute MPTP toxicity.

Unlike tuberoinfundibular neurons, nigrostriatal neurons show an inability to up-regulate parkin expression following MPTP exposure along with a corresponding susceptibility to toxicity⁶. Here we sought to determine if over-expression of parkin within the substantia nigra could rescue MPTP-induced decreases in axon terminal DA concentrations. Despite successful over-expression of F-hParkin within the substantia nigra, there was no difference in the concentrations of striatal DA between the injected and non-injected hemispheres within 24 h post-MPTP. This may be due to the modest transduction of dopaminergic neurons in the substantia nigra. Within the current set of experiments, an average of 44% of nigrostriatal neurons were transduced, and while there was a large increase in F-hParkin protein within the substantia nigra and the striatum, again, the amount of protein over-expression specifically within nigrostriatal neurons remains unknown. Despite these limitations, these results are in line with prior reports. Using an alternative dosing paradigm, Paterna et al., 2007, found that over-expression of parkin in the substantia nigra significantly rescued MPTP-induced TH-immunoreactive cell loss but had no effect on DA depletion within the striatum. Nonetheless, it is possible that in general, the limited transduction achieved in the current study may underestimate the contribution of parkin's neuroprotective effects (both in tuberoinfundibular and nigrostriatal neurons). More over, the effect of parkin manipulation within non-target cells (i.e. transduced non-dopaminergic neurons in the arcuate nucleus or substantia nigra) is also unknown and may be contributory to the effects observed in the current study.

The observed differences in the ability of parkin upregulation to rescue loss of terminal DA concentrations within tuberoinfundibular and nigrostriatal neurons may indicate that endogenous murine parkin upregulation differs from that of exogenously administered human parkin over-expression. The human parkin construct used in the current experiments contained an N-terminal FLAG tag. The FLAG moiety is essentially a string of charged aspartic acid residues, which could potentially impair intramolecular interactions of the

parkin protein. Although, this same FLAG tagged parkin construct has been functionally validated and used in many mechanistic studies^{37–40}, the possibility remains that, in terms of axon terminal DA recovery, the FLAG tag interfered with the normal function of parkin. The work by Paterna et al., 2007, in which parkin over-expression was unable to rescue striatal DA depletion following MPTP, supports the possibility that N-terminal interactions are important for parkin mediated protection from MPTP-toxicity. Although the parkin construct used by Paterna et al., 2007 did not have an N-terminal FLAG tag, it did have an N-terminal HA tag. The N-terminal of parkin contains an ubiquitin like domain that is integral for the autoubiquitination of parkin, a process that regulates the activity and concentrations of parkin within the cell^{41,42}. Recent studies have found that the addition of small N-terminal tags, such as FLAG or HA, to parkin affect the stability of parkin and increase the autoubiquitination activity of the protein⁴³. Accordingly, it is possible that the N-terminal tags used in the current, as well as previous studies, modified the E3 ligase activity of parkin or the interactions of the N-terminal domain of parkin. N-terminal, tag-induced modifications may underlie the dissonance between the ability of endogenous parkin upregulation to mediate rescue of axon terminal DA concentrations within tuberoinfundibular neurons, and the inability of exogenous N-terminal tagged-parkin to rescue terminal DA concentrations in nigrostriatal neurons.

By 24 h post-MPTP administration, nigrostriatal neurons exhibit a loss of terminal DA and TH concentrations that corresponds to a loss of parkin in the substantia nigra. As such, it was hypothesized that by decreasing parkin expression in tuberoinfundibular neurons, a similar pattern of susceptibility would be seen following MPTP. However, the decrease in TH was not observed following MPTP treatment, regardless of whether endogenous parkin expression was reduced or remained intact. It is possible that the time-course of TH depletion and recovery occurs prior to, or after, the 24 h time point analyzed in the present studies. It is also possible that a partial reduction in parkin expression was not sufficient to render tuberoinfundibular neurons susceptible to terminal TH loss. Further experiments are needed to differentiate between these two alternate explanations and would require examining the time course of TH concentrations following MPTP in mice treated with parkin shRNA, in addition to examining MPTP-induced loss of TH following a more efficacious knockdown of endogenous parkin expression.

Although parkin over-expression was unable to rescue striatal DA depletion, it was able to completely rescue MPTP-induced loss of TH in both the striatum and substantia nigra. The pathological mechanism mediating MPTP-induced loss of TH is unknown, but has been attributed to nitrosative damage of TH within the active phase of MPTP induced degeneration^{27,44–46}. Parkin over-expression has been shown to decrease levels of nitrated proteins⁴⁷. It is possible that parkin is preserving TH concentrations in nigrostriatal neurons by suppressing oxidative and nitrosative protein damage. Alternatively, it is possible that the rescue of nigrostriatal terminal TH following MPTP is caused by parkin actually enhancing TH production within nigrostriatal neurons¹⁴. However, the sustained striatal DA depletion, and the lack of an increase in DA turnover in the face of normal TH concentrations, calls into question the functionality of preserved TH protein. Regardless of the mechanism, loss of TH within this active phase of degeneration is one of the earliest pathologies observed following MPTP exposure and consistently precedes further cytotoxicity and

degeneration^{27,48,49}. As such, the ability of parkin to prevent this initial loss of TH is likely important and may translate into a more pronounced protective benefit given time.

5. Conclusion

The data presented here shows for the first time that parkin is necessary for the ability of tuberoindubular neurons to recover axon terminal DA concentrations following acute MPTP administration. Additionally, parkin was shown to be sufficient to rescue MPTP-induced decreases in TH within the striatum and substantia nigra. These results are consistent with the hypothesis that parkin is integral in maintaining DA neuronal homeostasis and promoting recovery from toxicity. This work supports further investigation into parkin-mediated events, both up- and downstream of parkin expression, that promote DA neuronal recovery.

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Abbreviations

TH	Tyrosine hydroxylase
GFP	green fluorescent protein
DA	dopamine
MPTP	1-methyl, 4-phenyl, 1, 2, 3, 6-tetrahydropyridine
MPP⁺	1-methyl, 4-phenylpyridinium
F-hParkin	FLAG-human parkin
rAAV	recombinant adeno-associated virus
shRNA	short hairpin RNA
GAPDH	glyceraldehyde 3-phosphate dehydrogenase

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Fig. 1. Characterization of rAAV expression and parkin-shRNA mediated knockdown of endogenous parkin in the arcuate nucleus

Mice received bilateral stereotaxic injections (250 nl) of rAAV-parkin shRNA (1.4×10^{13} vg/ml) into the arcuate nucleus or remained naïve to surgery. Four-weeks following rAAV injection mice were sacrificed and fixed brain sections were processed for fluorescent IHC to visualize rAAV spread (A–I). Tuberoinfundibular neurons are shown as TH expressing cells (red; A, D, G) surrounding the third ventricle (3V), with axons terminating in the median eminence (ME; arrow head). Transduction of tuberoinfundibular neurons was determined by examining colocalization of the GFP reporter (green; B, E, H) in TH expressing cells (colocalization appears yellow; C, F, I). (D–F) show higher magnification images of the arcuate nucleus (ARC) outlined in the box in (A–C), respectively. Tissue was also obtained from frozen brains and processed for Western blotting to determine knockdown of endogenous parkin protein in the arcuate nucleus (J, K). Columns in (K) represent total parkin (53 and 44kDa isoforms) normalized to GAPDH, in non-injected (white columns) and rAAV-parkin shRNA (black columns) injected mice, and expressed as percent of control. Vertical lines represent ± 1 SEM ($n=4$ /group). (*) Indicates parkin concentrations significantly different ($p < 0.05$) from non-injected controls. (J) Representative blots showing the 53kDa and 44kDa isoforms of parkin. Scale bar in (C) represents 100 μ m and applies to (A, B). Scale bar in (F and I) represents 50 μ m and applies to (D, E, G, H). Abbreviation: ARC, arcuate nucleus; ME, median eminence; 3V, third ventricle.

Fig. 2. Parkin protein concentrations in the arcuate nucleus following administration of scrambled or parkin shRNA in saline and MPTP treated mice

Mice received bilateral stereotaxic arcuate nucleus injections (250 nl) of rAAV (1.4×10^{13} vg/ml) expressing a scrambled shRNA or the parkin shRNA. Non-injected control animals remained naïve to surgery. Four-weeks following rAAV injection, mice were treated with a single injection of saline (white columns, 10ml/kg; s.c.) or MPTP (black columns, 20mg/kg; s.c.) and were sacrificed 24 h later. Arcuate nucleus tissue samples were obtained from frozen brains and parkin protein concentrations were determined by Western blotting and normalized to GAPDH. **(B)** Columns represent mean parkin concentrations and vertical lines represent +1 SEM ($n=8$ /group). (*) Indicates parkin concentrations significantly different ($p < 0.05$) from saline-treated controls. (**) Indicates parkin concentrations significantly different than saline and MPTP treated mice in the non-injected or scrambled shRNA surgery groups. Representative blots from all groups are shown in **(A)**.

Fig. 3. The effects of parkin knockdown on tuberoinfundibular axon terminal DA and DOPAC concentrations following single-acute MPTP exposure

Mice received bilateral stereotaxic arcuate nucleus injections (250 nl) of rAAV (1.4×10^{13} vg/ml) expressing a scrambled shRNA or the parkin shRNA. Non-injected control animals remained naïve to surgery. Four-weeks following rAAV injection, mice were treated with a single injection of saline (white columns, 10ml/kg; s.c.) or MPTP (black columns, 20mg/kg; s.c.) and were sacrificed 24 h later. The median eminence was freshly dissected and DA (A) and DOPAC (B) concentrations were determined by HPLC-ED and expressed as ng/mg protein. DA turnover is represented by the index of the DA metabolite DOPAC to DA (C). Columns represent mean DA or DOPAC concentrations, or mean DOPAC/DA, and vertical lines +1 SEM ($n=8$ /group). (*) Indicates median eminence DA concentrations significantly different ($p < 0.05$) from saline-treated controls in all surgery groups, and MPTP treated animals in the non-injected and scrambled shRNA surgery groups.

Fig. 4. The effects of parkin knockdown on tuberoinfundibular axon terminal TH concentrations following single-acute MPTP exposure

Mice received bilateral stereotaxic arcuate nucleus injections (250 nl) of rAAV (1.4×10^{13} vg/ml) containing parkin shRNA or remained naïve to surgery. Four-weeks following injection, mice were treated with a single injection of saline (white columns, 10ml/kg; s.c.) or MPTP (black columns, 20mg/kg; s.c.) and were sacrificed 24 h later. The median eminence was freshly dissected and TH protein concentrations were determined by Western blotting and normalized to GAPDH. **(B)** Columns represent mean TH concentrations and vertical lines +1 SEM (n=8/group). Representative blots from all groups are shown in **(A)**.

Fig. 5. Characterization of rAAV F-hParkin expression in the substantia nigra
Mice received unilateral stereotaxic injections (500 nl) of rAAV expressing F-hParkin (3.4×10^{13} vg/ml) into the left, ipsilateral substantia nigra. The right, contralateral substantia nigra was un-injected and used as an internal control. Four-weeks following rAAV injection mice were sacrificed and fixed brain sections were processed for fluorescent IHC to visualize FLAG expression within the ventral mesencephalon (A–I). Nigrostriatal neurons are shown as TH expressing cells (red; A, D, G). Transduction of nigrostriatal neurons was determined by examining colocalization of FLAG immunoreactivity (green; B, E, H) in TH immunoreactive cells (colocalization appears yellow; C, F, I). (A–C) shows FLAG expression in the ipsilateral substantia nigra, leaving the contralateral substantia nigra devoid of F-hparkin expression. (G–I) show higher magnification images of the area shown within boxes in (D–F), respectively. Substantia nigra tissue samples were obtained from frozen brains and processed for Western blotting to determine expression of F-hparkin protein in the substantia nigra and striatum (J). F-hParkin protein was detected in the ipsilateral striatum and substantia nigra but not in the contralateral striatum or substantia nigra (J). Scale bar in (C) represents 100 μ m and applies to (A, B). Scale bar in (F)

represents 100 μm and applies to (**D**, **E**). Scale bar in (**I**) represents 50 μm and applies to (**G**, **H**).

Fig. 6. The effects of F-hParkin expression on nigrostriatal axon terminal DA and DOPAC concentrations following single-acute MPTP exposure

Mice received unilateral stereotaxic injections (500 nl) of rAAV expressing F-hParkin (3.4×10^{13} vg/ml) into the left, ipsilateral substantia nigra. The right, contralateral substantia nigra was un-injected and was used as an internal control. Four-weeks following rAAV injection, mice were treated with a single injection of saline (white columns, 10ml/kg; s.c.) or MPTP (black columns, 20mg/kg; s.c.) and were sacrificed 24 h later. Nigral tissue samples were obtained from frozen brains and processed for Western blotting to determine expression of F-hparkin protein (**A**). Striatal tissue samples were obtained and processed for neurochemical analyses using HPLC-ED to quantify terminal DA (**B**) and DOPAC (**C**) concentrations and are expressed as ng/mg protein. DA turnover is represented by the index of the DA metabolite DOPAC to DA (**D**). Representative blots depicting F-hParkin expression in the substantia nigra are shown in (**A**). Columns represent mean DA or DOPAC concentrations, or mean DOPAC to DA ratios, and vertical lines + 1 SEM ($n=8$ /group) in the ipsilateral (injected) and contralateral (non-injected) hemispheres. (*) Indicates striatum DA and DOPAC concentrations, or DOPAC/DA ratios, significantly different ($p < 0.05$) from saline-treated controls.

Fig. 7. The effects of F-hParkin expression on nigrostriatal TH concentrations following single-acute MPTP exposure

Mice received unilateral stereotaxic injections (500 nl) of rAAV-F-hParkin (3.4×10^{13} vg/ml) into the left, ipsilateral substantia nigra. The right, contralateral substantia nigra was un-injected and was used as an internal control. Four-weeks following rAAV injection, mice were treated with a single injection of saline (white columns, 10ml/Kg; s.c.) or MPTP (black columns, 20mg/Kg; s.c.) and were sacrificed 24 h later. Tissue samples from the striatum and the substantia nigra were obtained from frozen brains and processed for Western blotting. TH concentrations in the striatum (**A, B**) and substantia nigra (**C, D**) were quantified and were normalized to GAPDH. Columns represent mean TH concentrations and vertical lines + 1 SEM ($n=8$ /group) in the ipsilateral (injected) and contralateral (non-injected) hemispheres. (*) Indicates striatal (**B**) and nigral (**D**) TH concentrations significantly different ($p < 0.05$) from saline-treated controls. Representative blots are shown for the striatum in (**A**) and substantia nigra in (**C**).