THE ROLE OF PARKIN IN MAINTAINING PROTEASOME ACTIVITY FOLLOWING ACUTE NEUROTOXIC INSULT

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ABSTRACT

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The motor symptoms of Parkinson disease are primarily caused by the progressive degeneration of nigrostriatal dopamine neurons of the substantia nigra pars compacta. While these neurons are susceptible, tuberoinfundibular DA neurons are not affected. Exposure to the neurotoxicant 1-methyl-4-phenyl-1,2,3,6-tetrahyropyridine (MPTP) results in a similar pattern of DA neuronal susceptibility. The recovery of tuberoinfundibular DA neurons is dependent upon the increased expression of parkin following MPTP exposure. Parkin is an E3 ligase that contains a ubiquitin like domain that can bind to the 26S proteasome and directly enhance its activity. The purpose of this dissertation was to test the hypothesis that increased parkin expression is both necessary and sufficient to maintain proteasome activity following oxidative insult by MPTP. MPTP caused similar oxidative damage to proteins in regions containing axons terminals of nigrostiatal (striatum; ST) and tuberoinfundibular (median eminence; ME) DA neurons, however proteasome activity followed the differential pattern of parkin expression and was decreased in the ST and maintained in the ME. The ST of parkin knockout mice had decreased proteasome activity, accumulation of ubiquitinated TH, and increased oxidatively modified proteins, while these were not affected in the ME. Proteasome activity in the ME of parkin deficient mice was decreased following MPTP but oxidatively modified proteins were not changed in either the ME or ST. To determine if parkin up-regulation could rescue proteasome activity following exposure to MPTP, rAAV expressing Flag-tagged human parkin was injected

into the substantia nigra containing cell bodies of nigrostriatal DA neurons and proteasome activity was measured 24 h post MPTP. Parkin overexpression in the substantia nigra increased basal proteasome activity and prevented MPTP-induced loss of proteasome activity in the ST. rAAV-mediated parkin expression resulted in activation of 26S proteasome activity that was due to an increase in the amount of assembled 26S proteasome. Furthermore, increased parkin expression resulted in the rescue of proteasome mediated turnover of ubiquitinated TH. Although parkin over-expression was not sufficient to rescue NSDA axon terminal DA stores following MPTP exposure, DA turnover was decreased. The data presented here reveal that parkin is necessary and sufficient for the maintenance of 26S proteasome activity in dopaminergic axon terminal regions following acute MPTP exposure. Over-expression of parkin may be beneficial in protecting nigrostriatal DA neurons from cytosolic DA related oxidative damage. In addition, the discovery of small molecules that positively modulate the 26S proteasome may have potential therapeutic applications in the treatment of Parkinson disease.

This dissertation is dedicated in loving memory to my father, Robert H. Lansdell.
Thank you for always having my back.

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KEY TO ABBREVIATIONS

AADC	Aromatic amino acid decarboxylase
AAV	Adeno-associated virus
AC	Adenylate cyclase
ADP	Adenosine diphosphate
AIPM2	Aminoacyl-tRNA synthase interacting multifunctional protein type 2
ALDH	Aldehyde dehydrogenase
ALP	Autophagy lysosome pathway
ANOVA	Analysis of variance
ARC	Arcuate nucleus
AR-JP	Autosomal recessive juvenile Parkinson disease
ATP	Adenosine triphosphate
ATP13A2	ATPase type 13A2
BCA	Bicinchchonic acid
Casp-L	Caspase-like activity of the proteasome
CMA	Chaparone mediated autophagy
CNS	Central nervous system
COMT	Catechol O-methyl transferase
CPu	Caudate-Putamon
CT-L	Chymotryptic-like activity of the proteasome
2,4-D	2-4-dichlorophenoxyacetic acid
DA	Dopamine

DAergic	Dopaminergic
DAT	Dopamine transporter
DDT	dichloro-diphenyl-trichloro-ethane
DHP	1-methyl-4-phenyl-1,4-dihydropyridine
DJ-1	Parkinsonism Associated Deglycase
DOPA	Dihydroxyphenylalanine
DOPAC	3,4-dihydroxyphenylacetic acid
DOPAL	3,4-dihydroxyphenylacetaldehyde
EIF4G1	Eukaryotic translation initiation factor 4 gamma 1
ETC	Electron transport chain
FBP1	Fuse binding protein 1
FCx	Frontal cortex
F-hParkin	Flag-tagged human parkin
FBX07	F-box only protein 7
GABA	Gamma-amino butyric acid
GAPDH	Glyceraldehyde phosphate dehydrogenase
Glu	Glutamate
GPex	Globus pallidus externa
GPi	Globus pallidus interna
GSH	reduced glutathione
GSSG	oxidized glutathione
Hsc70	Heat shock protein 70
HNE	Hydroxy-2-nonenal

HTRA2	High temperature requirement protein A2
HVA	Homovanillic acid
hParkin	Human parkin
HPLC-ED	High pressure liquid chromatography coupled to electro-detection
IBR	In between RING
IHC	Immunohistochemistry
K	Lysine
Lamp-2A	Lysosomal Associated Membrane Protein 2A
LAT-1	Large neutral amino acid transporter 1
LC3	Microtubule-associated protein 1A/1B-light chain 3
Levodopa	Levo-dihydroxyphenylalanine
L-Tyr	L-tyrosine
LRRK2	Leucine rich repeak kinase 2
Maneb	Manganese ethylenebis-dithiocarbamate
MAO-B	Monoamine oxidase-B
MCDA	Mesocortical dopamine
ME	Median eminence
MLDA	Mesolimbic dopamine
MPDP	1-methyl-4-phenyl-2,3-dihydropyridine
MPP^+	1-methyl-4-phenylpyridinium
MPPP	1-methyl-4-phenyl-4-propionoxypiperidine
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
MSN	Medium spiny neuron

MT	microtubule
3-MT	3-methoxytyramine
NAc	Nucleus accumbens
NADH	Nicotinamide adenine dinucleotide
NS	Nigrostriatal
NSD-1015	3-hydroxybenylhydrazine
NSDA	Nigrostriatal dopamine
OCT3	Organic Cation Transporter 3
P_i	Inorganic phosphate
Paris	Parkin interacting substrate
Park2	parkin gene
$Park2^{-/-}$	Parkin knockout mouse $Park2^{tm1Shn}$
PD	Parkinson disease
PINK1	PTEN putative induced kinase
PKA	Protein kinase A
PLA2G6	Phospholipase A2 Group VI
PGC-1a	Peroxisome proliferator-activated receptor-Y-coactivator-1a
PRL	Prolactin
rAAV	Recombinant adeno-associated virus
RING	Really interesting new gene
ROS	Reactive oxygen species
Ser	Serine
SN	Substantia nigra

SNc	Substantia nigra pars compacta
SNCA	alpha synuclein gene
SNr	Substantia nigra reticulata
SOD1	Superoxide dismutase 1
DOS2	Superoxide dismutase 2
ST	Striatum
STN	Subthalamic nucleus
TH	Tyrosine hydroxylase
TH-ir	Tyrosine hydroxylase immunoreactivity
TI	Tuberoinfundibular
TIDA	Tuberoinfundibular dopamine
T-L	Trypsin-like proteasome activity
UCH-L1	Ubiquitin C-terminal hydrolase-L1
Ub	Ubiquitin
UPS	Ubiquitin proteasome system
VMAT-2	Vesicular monoamine transporter 2
VPS35	Vacuolar protein sorting 35 homolog
VTA	Ventral tegmental area

Chapter 1. General Introduction

Parkinson Disease

Parkinson disease (PD) is the second most common neurodegenerative disease after Alzheimer's disease and affects approximately seven million people worldwide and nearly one million people in the United States (Parkinson's Disease Foundation, 2017a). The disease is categorically an age-related disease. The mean age of onset is about 65 years and is rare before the age of 50 (Pringsheim, Jette, Frolkis, & Steeves, 2014). In the United States, PD affects approximately 0.3% of the entire population, but with aging, the prevalence of PD increases. Prevalence increases to 1% for those individuals over the age of 60 (de Lau & Breteler, 2006; Pringsheim et al., 2014).and affects 3.5% of those between the ages of 85 and 89 (Benito-Leon et al., 2003; Bharucha, Bharucha, Bharucha, Bhise, & Schoenberg, 1988; C. C. Chen et al., 2009; Clarke & Moore, 2007; Claveria et al., 2002; de Lau & Breteler, 2006; de Rijk et al., 1995; de Rijk et al., 1997; Fall et al., 1996; S. C. Li et al., 1985; Morgante et al., 2008). As our aging population demographics shift to a more advanced age, we can expect to see the prevalence of neurodegenerative diseases such as PD rise (de Lau & Breteler, 2006; Pringsheim et al., 2014)

The primary symptoms of PD are motor in nature and include slowness of movement (bradykinesia), resting tremor, rigidity, and impaired balance and coordination (Fahn, 2003). While PD is thought of as a motor disease, many patients experience a range of non-motor symptoms that include decreased ability to smell (hyposmia), sleep disturbances, depression, constipation and other autonomic dysfunctions (Fahn, 2003). In many cases, the appearance of these non-motor symptoms can precede the appearance of motor symptoms (Parkinson's Disease Foundation, 2017b). In patients with advanced disease there may be neuropsychiatric symptoms that include dementia, hallucinations and risk-taking behavior (Aarsland et al., 1999). The onset

of PD for each patient has varied onset of symptomology and disease severity. Disease progression may be slow for one patient and rapid for another (Parkinson's Disease Foundation, 2017b).

Dopamine (DA) Neuronal Pathways in the Brain

There are four major dopaminergic (DA)ergic neuronal populations in the brain (**Figure 1-1**). Mesolimbic DA neurons originate in the ventral tegmental area and project to the nucleus accumbens and regulate reward, motivation, reinforcement learning, and fear (Felten & Shetty, 2010). Mesocortical DA neurons also originate in the ventral tegmental area but project to the frontal cortex where DA innervation is involved in cognitive control, motivation and emotional response (Felten & Shetty, 2010). Tuberoinfundibular (TI) DA neurons originate in the arcuate nucleus (ARC) and terminate in the median eminence (ME) and are involved in the regulation of prolactin secretion (Felten & Shetty, 2010). TIDA neurons are not affected in PD (Braak & Braak, 2000; J. W. Langston & Forno, 1978). Both the MLDA and MCDA neurons are affected in PD, but to a lesser extent than nigrostriatal (NS) DA neurons which originate in the substantia nigra pars compacta (SN) and terminate in the striatum (ST) (Fearnley & Lees, 1991). NSDA neurons are severely affected in PD and it is the loss of these neurons that results in the loss of the ability to initiate movement (Dauer & Przedborski, 2003).

NSDA neuronal degeneration results in reduced DA innervation to the ST and thus, loss of neurotransmission to the basal ganglia circuitry that controls the initiations of movement. Following the loss of approximately 85% of NSDA neurons, motor symptoms begin to appear and include: slowness of movement (bradykinesia), resting tremor, rigidity, and impaired balance and coordination (Bernheimer, Birkmayer, Hornykiewicz, Jellinger, & Seitelberger, 1973; Cheng, Ulane, & Burke, 2010; Fahn, 2003).

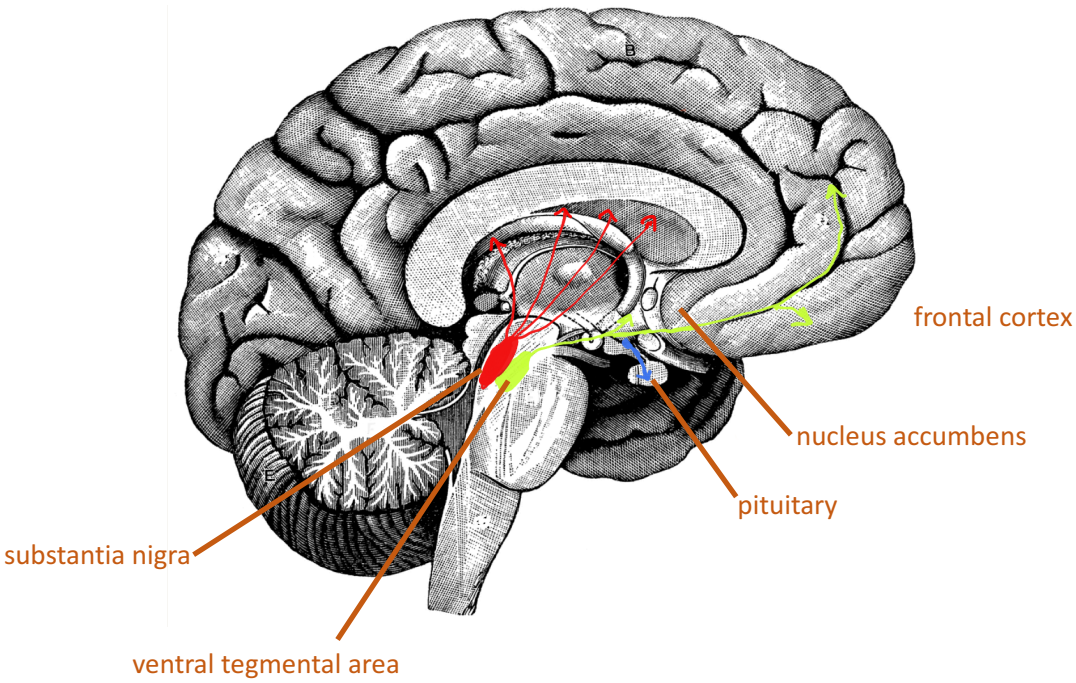

Figure 1-1 Location of major DA pathways in the brain. NSDA neurons (Red) cell bodies are located in the SNc and project to the ST. TIDA neurons (blue) have cell bodies in the the(Bernheimer et al., 1973) ARC and project to the ME. Mesocortical and Mesolimbic DA neurons (green) project from the Ventral tegmental area to the NAc or to the FCx Abbreviations: NSDA, nigrostriatal dopamine; SN, substantia nigra pars compacta; TIDA, tuberoinfundibular dopamine; ARC, arcuate nucleus; ME, median eminence; VTA, ventral tegmental area; NAc, nucleus accumbens; FCx, frontal cortex. Image modified from Sagital Section of Human Brain (Anatomy Chart Body, 2016).

Synthesis of DA and Regulation of Tyrosine Hydroxylase

NSDA neurons function to synthesize DA and release it into the ST where it modulates the basal ganglia circuitry (Hersch et al., 1995). The synthesis and release of DA is depicted in **Figure 1-2**. L-tyrosine (L-Tyr) from the diet is actively transported across the blood brain barrier and into the DA neuron by the large amino acid transporter, LAT-1(Kageyama, Imura, Matsuo, Minato, & Shimohama, 2000). Once in the cytosol, L-Tyr is converted to L-DOPA by tyrosine hydroxylase (TH), the biopterin dependent and rate-limiting enzyme for DA synthesis (Levitt, Spector, Sjoerdsma, & Udenfriend, 1965). L-DOPA is decarboxylated by aromatic L-amino acid decarboxylase (AADC) to produce DA (Jonkers, Sarre, Ebinger, & Michotte, 2001). Cytosolic DA is transported by the vesicular monoamine transporter (VMAT2) into the synaptic vesicle, where it is sequestered until an action potential signals its release into the DA synapse (Lohr & Miller, 2014).

The hydroxylation of L-Tyr by TH is dependent upon the cofactors tetrahydrobiopterin and molecular oxygen (LeWitt et al., 1984; K. M. Roberts & Fitzpatrick, 2013). The enzyme exists as a tetramer covalently trapping ferrous iron at the active sites (Ramsey, Hillas, & Fitzpatrick, 1996). While one of the oxygen atoms is used to hydroxylate L-Tyr, the other is used to hydroxylate tetrahydrobiopterin (K. M. Roberts & Fitzpatrick, 2013). The activity of TH is tightly regulated at both the transcriptional and post-transcriptional level (**Figure 1-3**; allosteric control, covalent modifications, and protein-protein interactions). Short-term regulation of TH occurs via multiple serine phosphorylations of the N-terminal regulatory domain (Nakashima et al., 2011). Ser19 phosphorylation does not directly affect TH activity, but this phosphorylation must be in place before TH is phosphorylated at Ser40 (Dunkley, Bobrovskaya, Graham, von Nagy-Felsobuki, & Dickson, 2004). Ser40 phosphorylation significantly increases TH activity

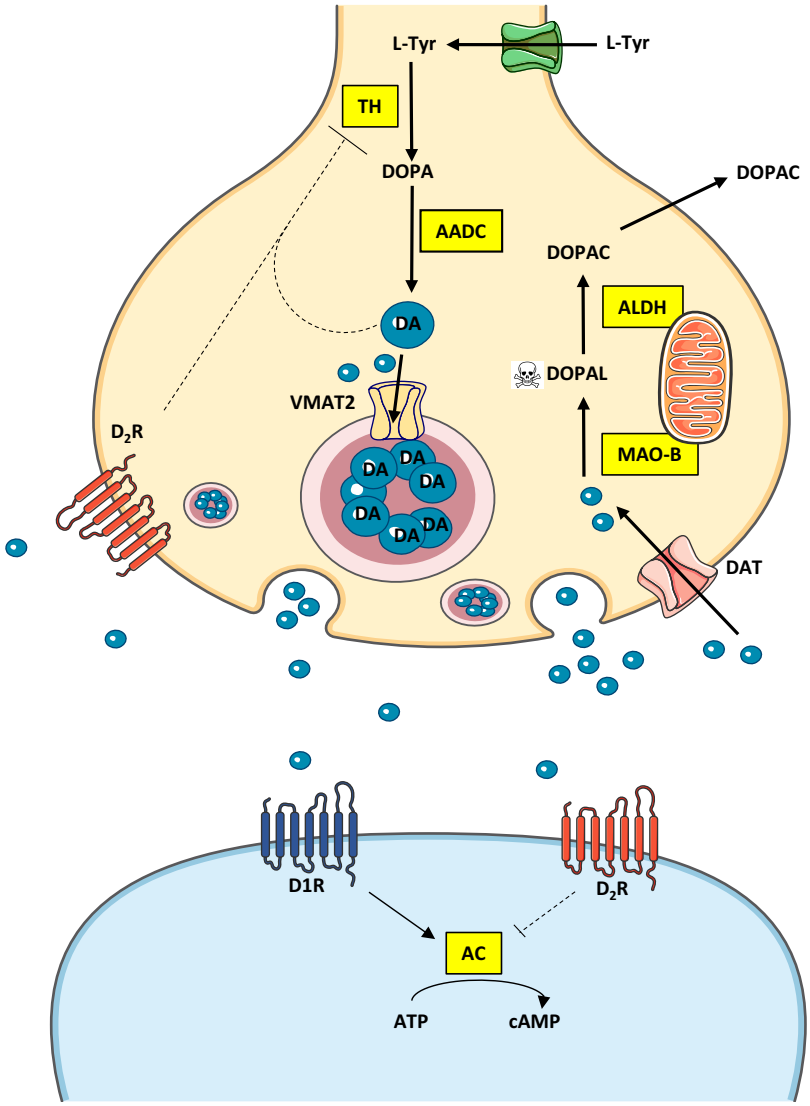

Figure 1-2 DA metabolism in NSDA neurons. L-Tyr is transported by LAT-1 into the DA neuron and is hydroxylated by TH to form DOPA. DOPA is decarboxylated by AADC to produce DA. Cytosolic DA is packaged into vesicles by VMAT2. An action potential causes the release of DA into the synapse where it binds to D_1R or D_2R. Synaptic DA is taken back up into the DA neuron by DAT and is either repackaged into DA vesicles or is metabolized. DA is deaminated by MAO-B to form DOPAL, which is oxidized by ALDH to form DOPAC. Abbreviations: L-Tyr, L-tyrosine; LAT-1, large amino acid transporter; NSDA, nigrostriatal dopamine; SNc, substantia nigra pars compacta; TIDA, tuberoinfundibular dopamine; ARC, arcuate nucleus; ME, median eminence; TH, tyrosine hydroxylase; DOPA, dihydroxy-phenylalanine; AADC, aromatic amino acid decarboxylase; DA, dopamine; DAT, dopamine transporter; MAO-B, monoamine oxidase B; DOPAL, dihydroxyphenylacetyladehyde; ALDH, aldehyde dehydrogenase; DOPAC, dihydroxyphenylacetic acid. Image made using Servier (Les Laboratoires, 2017).

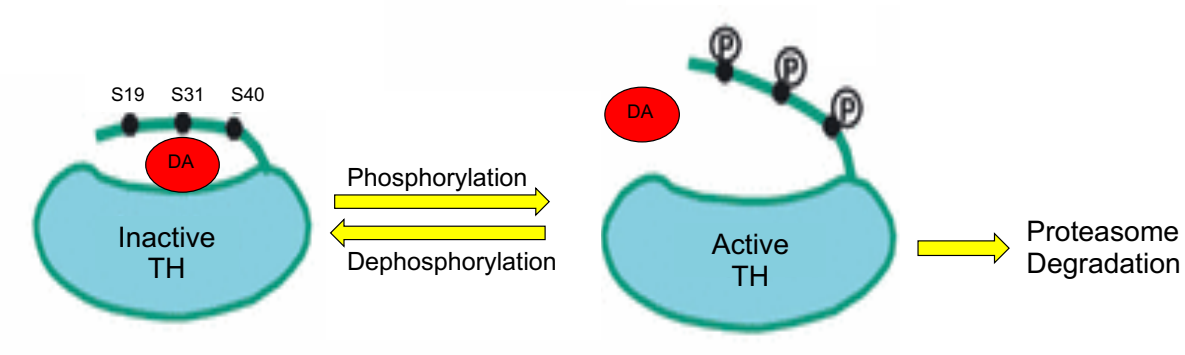

Figure 1-3 Phosphorylation of Ser residues in the N-terminus of TH controls the catalytic activity and intracellular stability of TH. In the inactive state, DA traps Fe (III) at the catalytic site, rendering the enzyme inactive. Phosphorylation of Ser19 allows the phosphorylation of Ser40 allowing the release of DA and binding of Fe (II) to the catalytic site. Ser19 and Ser31 phosphorylation promote the degradation of TH by the ubiquitin proteasome system. Abbreviations: TH, tyrosine hydroxylase; S19, Serine 19; S31, Serine 31; S40, Serine 40. Figure adapted from Nakashima et al. (2011).

because it relieves feedback inhibition by cytosolic DA. DA traps the active-site iron in the ferric state, which prevents enzymatic activity (Muga, Arrondo, Martinez, Flatmark, & Haavik, 1998). Ser40 phosphorylated TH is also regulated by the ubiquitin proteasome system (UPS) as inhibition of the proteasome results in increased abundance of phosphorylated Ser40 TH (Doskeland & Flatmark, 2002; Nakashima et al., 2011).

Release and Reuptake of DA

Once released into the synapse, DA can bind to several different receptors located on either the presynaptic terminal or on the post synaptic neuron. Binding of DA to D2 autoreceptors results in a negative feedback loop that results in both decreased TH activity and decreased release of DA (Timmerman, De Vries, & Westerink, 1990; Westerink & de Vries, 1989). Binding to postsynaptic DA receptors can either activate or inhibit the GABAergic medium spiny neuron depending on the type of DA receptor that is present.

DA receptors are G-protein coupled receptors (Boyd & Mailman, 2012). Based on their ability to stimulate adenylate cyclase (AC) via $G\alpha_s$ coupling, or inhibit AC via $G\alpha_{i/o}$ coupling, DA receptors are considered either D1-like or D2-like. D_1-like receptors (D1R) are coupled to $G\alpha_s$ (Beaulieu & Gainetdinov, 2011). Binding of DA to D1R stimulates AC and elevates cyclic adenosine monophosphate and activates protein kinase A (PKA). PKA phosphorylates ion channels, which allows sodium ions to enter the neuron and increases the chance of the channels opening, and increases the chances of setting off an action potential. Conversely, DA binding to D2-like receptors (D2R) results in the inhibition of AC and decreases the chances of setting off an action potential (Beaulieu & Gainetdinov, 2011). Most medium spiny neurons in the ST

express either D1R or D2R however, a small proportion express both D1R and D2R (Hersch et al., 1995; Surmeier et al., 1996).

Synaptic DA levels from NSDA neurons are modulated by two neuronal activity states: tonic and phasic transmission (Rice, Patel, & Cragg, 2011). Tonic transmission is related to slow irregular release of small amounts of DA into the extracellular space where it is metabolized by catechol-O-methyl transferase (COMT) either in or on glial cells[1] (J. Chen et al., 2011; Schott et al., 2010). Tonic DA firing is not driven by action potentials but by L-type calcium channel dependent pacemaker-like membrane currents that maintains a constant background level of DAergic activity. Electrochemical studies indicate that the small amount of extracellular DA that is released is not enough to activate post-synaptic neurons but is enough to bind to and activate presynaptic autoreceptors and decrease phasic DA release (May & Wightman, 1989; Suaud-Chagny, Ponec, & Gonon, 1991). Phasic DA transmission is caused by burst spike firing in response to an action potential. Unlike tonic release of DA, phasic release of DA results in synaptic DA that can cross the synapse and binds to postsynaptic DA receptors.

NSDA neurons remove DA from the synapse by reuptake into the presynaptic neuron by the DA transporter (DAT) or by low affinity high volume transport (Cass, Zahniser, Flach, & Gerhardt, 1993; Giros, Jaber, Jones, Wightman, & Caron, 1996; Hensler et al., 2013; Jaber, Jones, Giros, & Caron, 1997). Once back in the cytosol, VMAT2 can transport DA back into the synaptic vesicle for later release, or cytosolic DA can be metabolized by monoamine oxidase B (MAO-B) to DOPAL and subsequently by aldehyde dehydrogenase to the less toxic metabolite,

[1] Controversy exists as to the orientation of COMT on postsynaptic neurons and glial cells. Data by Chen et al. support COMT being oriented outward on the membrane and acting extracellularly on synaptic and extracellular DA. However, data by Schott et al. support COMT being oriented inward suggesting DAT dependent uptake and degradation of DA by COMT.

DOPAC (Marchitti, Deitrich, & Vasiliou, 2007). DOPAC is then metabolized by glial COMT to homovanillic acid (HVA) which is sulfated by Phase II conjugation and excreted. Alternatively, DA that is not taken back up by the neuron can be metabolized to 3-methoxytyramine (3-MT) by glial COMT which is subsequently metabolized to HVA by MAO-B (Mannisto et al., 1992).

Basal Ganglia Circuitry and Control of Voluntary Movement

The basal ganglia circuitry (**Figure 1-4, Left panel**) enables the initiation of learned motor programs that are stored in the motor cortex. The basal ganglia are a group of brain nuclei that include the ST, globus pallidus (internal and external; GPi and GPex), SN (pars reticulata and pars compacta; SNr and SNc) and the subthalamic nucleus (STN). These nuclei are interconnected to form the basal ganglia circuitry within the cerebral cortex, the thalamus, the midbrain and specific brainstem nuclei.

The ST is the main recipient of excitatory input from several areas of the cortex and sends output to nuclei within the basal ganglia. Major output nuclei in the basal ganglia circuitry include the internal segment of the GPi and the SNr. Outputs from the GPi and SNr make GABAergic inhibitory connections to their target nuclei in the thalamus that prevent the excitation of cortical neurons that would otherwise initiate movement.

The inputs to the ST are received by the medium spiny neurons. Medium spiny neurons are of two types: those that project directly to the GPi and modulate the so-called direct pathway, and those that project to the GPex and establish an indirect pathway to the GPi, via the STN, and modulate the so-called indirect pathway. The direct and indirect pathways have opposite net effects on their respective thalamic target structures.

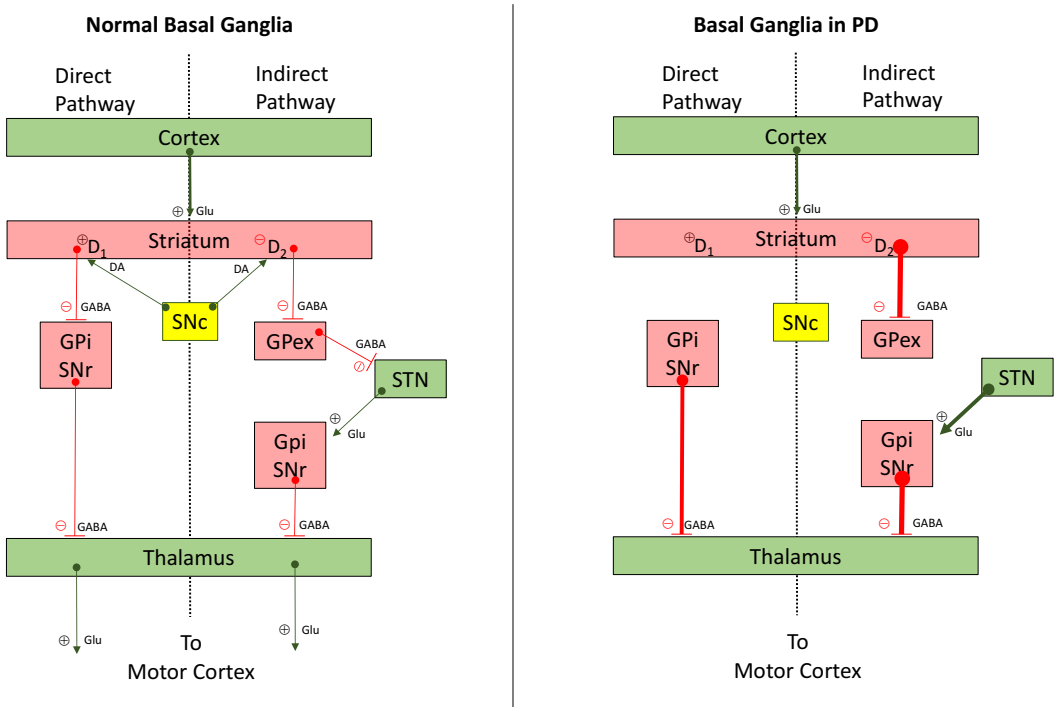

Figure 1-4 The basal ganglia in normal and PD brains. Left panel, Prior to the initiation of movement, the thalamus is inhibited directly and indirectly by GABA innervation from the GPi/SNr. Disinhibition occurs when DA is released from NSDA neurons. DA activates the direct pathway and inhibits the indirect pathway to initiate movement. **Right panel,** Loss of NSDA neurons in PD results in loss of DA innervation to the ST. Decreased activation of the direct pathway and increased activation of the indirect pathway leads to continued inhibition of the thalamus and an inability to initiate movement. Abbreviations: GABA, gamma-aminobutyric acid; GPi, globus pallidus interna; SNr, substantia nigra reticulata; DA, dopamine; NSDA, nigrostriatal dopamine; STN, subthalamic nucleus; GPex, globus pallidus externa; Glu, glutamate.

Activation of the direct pathway excites thalamic neurons that make excitatory connections onto cortical neurons. In the direct pathway, striatal neurons are excited, and when they fire, they inhibit GPi firing which is tonically inhibiting its target in the thalamus. Inhibition of GPi firing to the thalamus results in disinhibition and further excitation of cortical neurons (Pollack, 2001).

Activation of the indirect pathway has an inhibitory effect on thalamic neurons and prevents excitation of cortical neurons. In the indirect pathway, excitatory neurons originating in the STN act to increase GABAergic firing from the GPi. However, GABAergic neurons from the GPex prevent the subthalamic neurons from firing and make the thalamus more active. When neurons from the ST are firing, they inhibit the GPex and disinhibit the subthalamic neurons. With the subthalamic neurons free to fire, GPi neurons prevent the activation of the thalamus (Pollack, 2001).

NSDA neurons originate in the SNc and have long axons that project along the median forebrain bundle and terminate in the ST where they release DA and modulate the direct and indirect pathways. Post synaptic medium spiny neurons within the ST possess either D1R or D2R dopamine receptors (Surmeier, Ding, Day, Wang, & Shen, 2007). Medium spiny neurons with D1R modulate the direct pathway, while medium spiny neurons with D2R modulate the indirect pathway. Activation of the direct pathway results in disinhibition of the thalamus and facilitates movements while activation of the indirect pathway acts to further inhibit the thalamus and acts to inhibit movement (Pollack, 2001).

Depending on which receptor is present, the medium spiny neuron can modulate its respective pathway. Medium spiny neurons within the direct pathway expressing $G\alpha_s$ coupled D1R are excited by DA while medium spiny neurons within the indirect pathway expressing

$G\alpha_{i/o}$ coupled D2R are inhibited by dopamine (Surmeier et al., 2007). In effect, DA released from NSDA neurons in the ST will activate the direct pathway and facilitate movement and will also inhibit the neurons of the indirect pathway, facilitating movement. In this regard, DA release in the ST is critical in the modulation of motor control. The loss of NSDA neurons results in basal ganglia circuitry that is tipped in favor of the indirect pathway resulting in inhibition of the motor cortex (**Figure 1-4, Right panel**).

Current Therapies for PD

There is currently no cure for PD and despite our ever-growing aging population with an increased prevalence of PD there are limited therapies available. Current therapies of PD address the motor symptoms of PD, which are caused by the loss of NSDA innervation to the ST and resulting dysfunction of the basal ganglia circuitry. These therapies work by either replacing DA, protecting existing pools of DA from metabolism, or bypassing dopaminergic input to the basal ganglia circuitry all together by stimulating targets downstream of the degenerating NSDA neurons. These treatments are often effective in managing the motor symptoms of PD, however they do not prevent further neurodegeneration. Ongoing degeneration makes these treatments only temporarily effective.

Often patients with early disease progression are treated to prevent the metabolism of existing DA. MAO-B is the first enzyme involved in the metabolism of cytosolic DA. MAO-B converts DA to a toxic metabolite, DOPAL which is subsequently metabolized, as previously described, through several steps to homovanillic acid (HVA), a product that can be cleared by excretion. MAO-B inhibitors include selegiline and rasagiline (Engberg, Elebring, & Nissbrandt,

1991; Finberg, 2010). These drugs are well tolerated, but they are not as effective as treatment for more advanced disease.

Treatment of patients with the DA precursor levo-dihydroxyphenylalanine (levodopa) is a successful strategy in managing the motor symptoms of PD (LeWitt, 2015). Levodopa is administered in combination with a peripheral decarboxylase inhibitor such as carbidopa to prevent conversion of levodopa to DA before it can cross the blood brain barrier (Fermaglich, 1974). Levodopa crosses the blood brain barrier while the decarboxylase inhibitor cannot. Once in the brain, levodopa is decarboxylated by AADC to produce DA. The newly synthesized DA compensates for the lack of presynaptic DA in the ST and restores neurotransmission to the medium spiny neurons. While levodopa must be converted in the brain to DA by existing AADC, DA agonists mimic the effects of DA without the need to be metabolized. Current DA agonists used to treat PD include pramipexole, ropinerole, rotigotine, and apomorphine (Bennett & Piercey, 1999; Konta & Frank, 2008; Morgan & Sethi, 2006).

While both levodopa and DA agonist therapy are often highly effective in the treatment of the motor symptoms of PD, they do not abate NSDA neuron degeneration. As the disease progresses, both treatments lose their efficacy. Patients that have been on levodopa for greater than six years often experience motor fluctuations (Stocchi, Jenner, & Obeso, 2010). These patients find that their motor symptoms return before it is time for the next dose. One reason for this "wearing off" is that levodopa has a short half-life and as the disease progresses, there are fewer DA neurons to store the newly made DA (Reichmann & Emre, 2012). Because of this, the DA is more quickly catabolized and cleared from the brain. In addition to "wearing off", these drugs can have unwanted side effects such as dyskinesia, hallucinations, confusion and psychosis (Hersch et al., 1995; Surmeier, Song, & Yan, 1996).

For a limited group of patients with advanced progression of PD that have developed drug induced dyskinesias, surgical treatment is an option. This option is both expensive and invasive. Surgical treatment works by bypassing the degenerating NSDA neurons and directly stimulating the affected brain nuclei within the basal ganglia. In deep brain stimulation, a pulse generator, a device similar to a heart pacemaker, is surgically implanted into the patient's chest wall. The pulse generator sends small electrical currents through wires placed under the skin that lead to permanent electrodes that have been placed in the brain nuclei that are lacking the correct input due to the loss of NSDA neurons (GPi, thalamus or STN). By bypassing NSDA neurons, deep brain stimulation can restore normal neuronal activity downstream of the degenerating NSDA neurons (Almeida et al., 2017).

Protection of existing DA, DA replacement, and deep brain stimulation can all be effective treatments used in the management of motor symptoms caused by PD. Unfortunately, none of the available treatments prevent the ongoing degeneration of NSDA neurons. In developing new therapeutic strategies for the treatment of PD, it would be of great use to identify new targets that would aid in the prevention of further neuronal degeneration.

Pathological Hallmarks of PD

There are two major pathological findings in the brains of patients with PD (**Fig 1-5**): Depigmentation of the SNc and the presence of Lewy bodies and Lewy neurites (Braak & Braak, 2000). Loss of pigmentation in the SNc is caused by loss of NSDA neurons. NSDA neurons contain a darkly pigmented substance called neuromelanin and because of this the SNc normally appears dark in color (Sulzer & Surmeier, 2013). In patients who have NSDA neurodegeneration the loss of these pigmented neurons results in discoloration of the SNc. In most cases of PD,

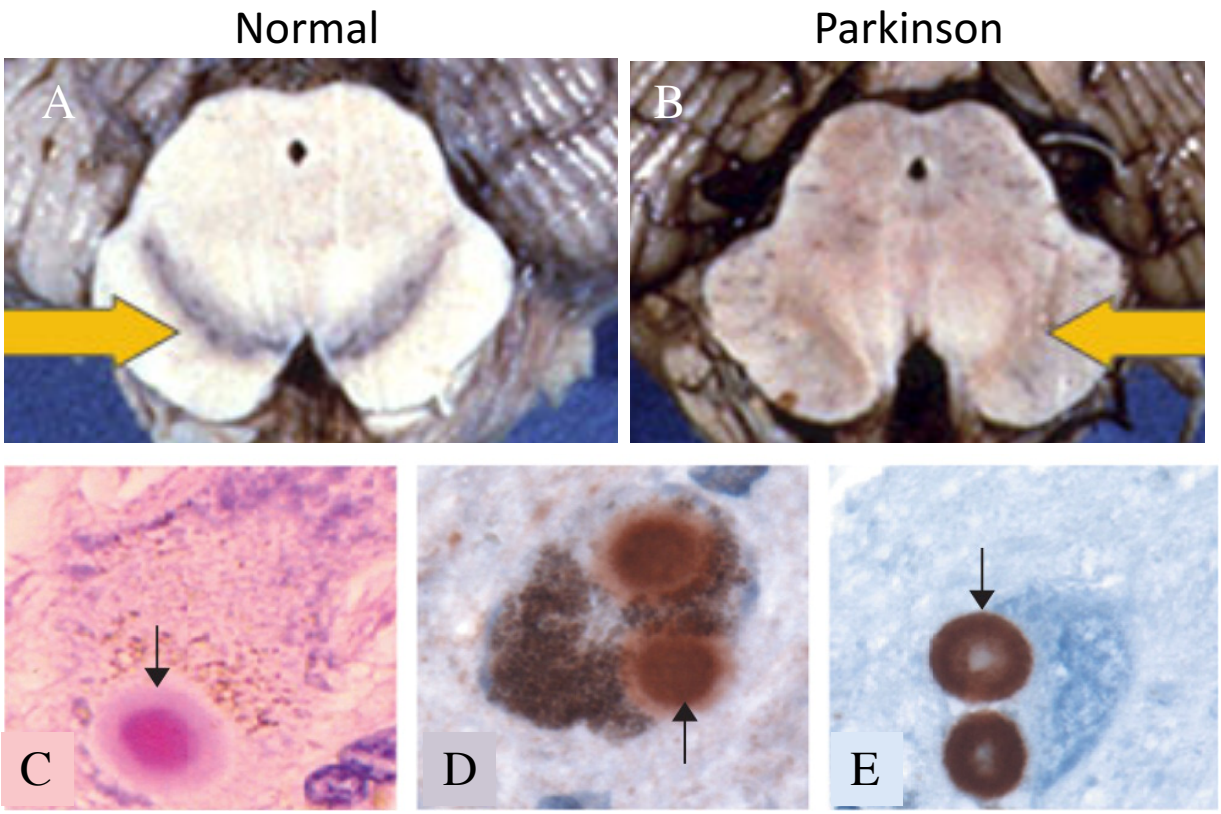

Figure 1-5 Pathological hallmarks of PD. **A and B**, Pigmentation of the SN adapted from http://emedicine.medscape.com. The SN is darkly pigmented in a normal brain while loss of NSDA neurons results in depigmentation of the SN in the Parkinson's disease. **C**, Hemotoxylin and eosin staining of a Lewy body in the SN. **D**, Immunohistochemical staining for ubiquitin reveals a large amount of ubiquitin in the core of the Lewy body. **E**, Immunohisto-chemical staining for α-synuclein reveals α-synuclein in the halo of the Lewy body. Modified images from Olanow et al. (Olanow, Perl, DeMartino, & McNaught, 2004).

intracellular inclusion bodies, termed Lewy bodies, are present in the surviving neurons. Lewy bodies are intracytoplasmic proteinaceous inclusions that contain ubiquitinated proteins in its core and are enriched with the polymerized form of the presynaptic protein α-synuclein. It is not clear if Lewy bodies play a role in killing or protecting the surviving neurons. However, the presence of Lewy bodies suggests that there is a disturbance in protein homeostasis and this disturbance could be an underlying mechanism for NSDA neuron loss.

Etiology of PD

The cause of PD is largely unknown, however it is thought that a combination of an individual's genetics and environmental exposures contribute in the etiology of this disease. Environmental risk factors include: rural living, consumption of well-water, exposure to pesticides and herbicides, and proximity to industrial plants or quarries (Wirdefeldt et al., 2011). In addition, there are approximately 16 genes that are associated with PD and cause either autosomal dominant or autosomal recessive forms of familial disease (Klein & Westenberger, 2012). The combination of known genetic and environmental factors suggests that some common pathogenic mechanisms exists across the spectrum of PD and are beginning to shed some light on some common themes in the pathogenesis of PD.

Environmental Factors

Although at times inconsistent, results of epidemiological studies suggest an important role for environmental factors in the development of PD. Incidents of PD are associated with rural living, and the associated features of rural living such as consumption of well-water, gardening, and exposure to pesticides and herbicides (Barbeau, Roy, Bernier, Campanella, &

Paris, 1987; Wirdefeldt et al., 2011). Of all the chemical exposures that have been linked to PD, herbicides and pesticides have been the most widely reported. Meta-analyses of 89 studies found that exposure to pesticides may increase the risk for PD by 30 to 80% (Ahmed, Abushouk, Gabr, Negida, & Abdel-Daim, 2017; Pezzoli & Cereda, 2013; van der Mark et al., 2012). Furthermore, the increased risk associated with pesticides seems to increase with the amount and duration of exposure. Among these chemicals, exposure to rotenone, paraquat, dichlorodiphenyltrichloro-ethane (DDT), dieldrin and organophosphates have been extensively examined. Of 31 specific agricultural chemicals studied, only a few were found to increase the risk (Odds ratio = 2.5) of developing PD: paraquat, manganese ethylenebis-dithiocarbamate (Maneb) and rotenone (Tanner et al., 2011).

2-4-Dichlorophenoxyacetic acid (2,4-D) is one of the chemicals that comprise Agent Orange, a defoliant that was used in the Korean and Vietnam wars. Although it has not been definitively proven that Agent Orange can cause PD, the US Department of Veterans Affairs has determined that veterans with PD who served in Vietnam are eligible to receive disability compensation from the Veterans Administration (*Blue Water Navy Vietnam Veterans and Agent Orange Exposure*, 2011; "Parkinson's Disease and Agent Orange," 2016),

Besides agricultural exposure of pesticides and herbicides, other toxicants have been proposed to increase risk for PD. These include: polychlorinated biphenyls (Steenland et al., 2006; Weisskopf et al., 2012), solvents (Goldman et al., 2012; Pezzoli & Cereda, 2013; Segal, 2012), and air pollutants (Finkelstein & Jerrett, 2007; Genc, Zadeoglulari, Fuss, & Genc, 2012). Other studies suggest that there is a greater incidence of PD in urban areas that have high levels of industrial emissions of manganese and copper (A. W. Willis et al., 2010). In this vein, occupational exposure to several metals may be related to PD (Gorell et al., 1997; Gorell, Rybicki, Cole

Johnson, & Peterson, 1999). However, these studies on metal exposure are controversial because long-term exposure to metals is not easily measured and differences in methods may have caused inconsistencies from study to study.

There are some environmental exposures that appear to be protective for PD including: cigarette smoking (Kiyohara & Kusuhara, 2011; X. Li, Li, Liu, Shen, & Tang, 2015) and caffeine consumption (Moccia et al., 2016; Palacios et al., 2012; Qi & Li, 2014). . There have been several case-control studies that demonstrate a clear dose-response relationship with cigarette smoking and the risk of developing PD (Benedetti et al., 2000; Gorell et al., 1999; Hellenbrand et al., 1997). However, it is possible that studies on the effects of smoking on PD are biased by selective survival of non-smokers. Regardless, smokers appear to have reduced MAO-B enzymatic activity in the brain and this might account for the protective effect of cigarette smoking (Fowler, Logan, Wang, & Volkow, 2003; Fowler et al., 1996). Consumption of caffeinated beverages is also closely associated with a reduced risk of developing PD (Hernan, Takkouche, Caamano-Isorna, & Gestal-Otero, 2002). The benefit of caffeine is believed to be due to antagonism of the A2A receptors in the brain. These receptors are highly localized to the basal ganglia specifically within the indirect pathway (Bogenpohl, Ritter, Hall, & Smith, 2012). Antagonists of the A2AR can modulate GABA and glutamate levels that act in the indirect pathway to modulate motor activity(Mori, 2014; Mori & Shindou, 2003).

Genetic Factors

Most PD cases are idiopathic in nature and have no known origin. Of these patients, only 10-15% report having a family history of PD (Gasser, 2007) and only 5-10% of PD cases are caused by Mendelian inheritance (Antony, Diederich, Kruger, & Balling, 2013). . Genetic factors

appear to be important when there is early onset of PD (before the age of 50). Early PD twin studies found low to similar concordance rates for monozygotic and dizygotic twins, which would suggest that genetics do not play a role in PD. However, in 16 pairs of twins that had been diagnosed with PD before the age of 50, all four monozygotic pairs and only 2 of the 12 dizygotic pairs were concordant (Tanner & Ben-Shlomo, 1999).

To date, about 18 distinct chromosomal regions that are associated with PD (PARK loci) have been identified (Klein & Westenberger, 2012) and of these loci, only six genes have been identified that cause monogenic forms of PD (**Table 1-1**). Mutations in two genes (alpha synuclein; SNCA, and the leucine-rich repeat kinase 2; LRRK2) cause autosomal dominant forms of PD. Mutation in the LRRK2 gene shows variable penetrance (30-70% at age 80 years) but is the most common cause of autosomal dominant PD (Paisan-Ruiz et al., 2004; Zimprich et al., 2004).

Expression of mutated LRRK2 causes alterations in macroautophagy leading to excess mitochondrial clearance that can be prevented by protein kinase A phosphorylation of LC3 (Cherra et al., 2010). Mutations in the SNCA are rare and include point mutations and whole locus multiplications (Polymeropoulos et al., 1997; A. Singleton & Hardy, 2011). Mutations in SNCA are fully penetrant, and cause rapid progressing disease with abundant Lewy body formation. This is important because α-synuclein is the major component of Lewy bodies and Lewy bodies are present in both familial and sporadic forms of the disease. There appears to be a dose response relationship between SNCA gene dosage and disease severity (Ibanez et al., 2009; Ross et al., 2008). Patients with SNCA duplications commonly develop classical PD, while

Locus	Gene	Chromosome Position	Inheritance	Onset
PARK1	*SNCA*	4q21-22	Autosomal Dominant	Early
PARK2	*PRKN*	6q25.2-q27	Autosomal Recessive	Early
PARK3	*unknown*	2p13	Autosomal Dominant	Early
PARK4	*SNCA*	4q21-q23	Autosomal Dominant	Early
PARK5	*UCH-L1*	4p13	Autosomal Dominant	Early
PARK6	*PINK1*	1p35-p36	Autosomal Recessive	Early
PARK7	*DJ-1*	1p36	Autosomal Recessive	Early
PARK8	*LRRK2*	12q12	Autosomal Dominant	Early
PARK9	*ATP13A2*	1p36	Autosomal Recessive	Early
PARK10	*unknown*	1p32	Risk Factor	Early
PARK11	*unknown*	2q36-27	Autosomal Recessive	Early
PARK12	*unknown*	Xq21-q25	Autosomal Recessive	Early
PARK13	*HTRA2*	2p12	Risk Factor	Early
PARK14	*PLA2G6*	22q13.1	Autosomal Recessive	Early
PARK15	*FBX07*	22q12-q13	Autosomal Recessive	Early
PARK16	*unknown*	1q32	Risk Factor	Late
PARK17	*VPS35*	16q11.2	Autosomal Dominant	Late
PARK18	*EIF4G1*	3q27.1	Autosomal Dominant	Late

Table 1-1 PARK designated PD related loci and associated genes. List of the genetic loci associated with PD with the corresponding gene name, type of inheritance, onset of symptoms, and chromosomal position. Abbreviations: SNCA, a-synuclein; PRKN, Parkin; UCH-L1, Ubiquitin Carboxy-Terminal Hydrolase-L1; PINK1, PTEN Induced Putative Kinase 1; LRRK2, leucine rich repeat kinase 2; ATP13A2, ATPase type 13A2; HTRA2, High Temperature requirement protein A2; PLA2G6, Phospholipase A2 Group VI; FBX07, F-box Only Protein 7; VPS35, Vacuolar Protein Sorting 35 Homolog; EIF4G1, Eukaryotic Translation Initiation Factor 4 Gamma 1. Modified from Klein et al. (2012).

patients carrying SNCA triplications have more severe disease (A. B. Singleton et al., 2003). Excess α-synuclein results in oligomerization, fibrilization, and aggregation which interferes with neuronal homeostasis (Devine, Gwinn, Singleton, & Hardy, 2011).

Mutations in parkin, PTEN putative induced kinase (PINK1), and DJ-1 cause early onset autosomal recessive forms of PD (Bonifati et al., 2003; Fu, Huang, Lin, & Tsai, 2017; Heutink, 2006; Kitada et al., 1998; Valente et al., 2001), and mutations in ATP13A2, PLA2G6, and FBXO7 cause rarer forms of recessive parkinsonism (Di Fonzo et al., 2009; Ramirez et al., 2006; Shi et al., 2011). Mutations in parkin explain about half of all the familial cases of PD (Lucking et al., 2000). Patients with parkin mutations generally have early onset of disease (on average in their 30s) and the phenotype of disease is not distinguishable from those patients with mutations in PINK1 or DJ-1(Bonifati, 2012). Interestingly, these patients typically lack Lewy body pathology, which may highlight the idea that Lewy bodies serve a protective function (Matsumine, 1998). It is interesting to note that Parkin, PINK1, and DJ-1 are all mitochondrial related and involved in oxidative stress mechanisms (Corti et al., 2015).

In addition to monogenic forms of PD, there are over 20 common variants that are recognized to increase the risk for PD (**Table 1-2**). As previously noted, rare mutations in SNCA and LRRK2 cause familial PD. Common variability within SNCA is also associated with risk for sporadic PD {Kruger, 1999 #1540} (Kruger et al., 1999; Hernandez et al., 2016). For example, patients with the combination of the APOE4 allele and NACP allele 1 polymorphisms of the SNCA promoter have a 12.8 fold increased risk for developing PD (Choraki et al., 2014; Maraganore et al., 2006). Common variability within LRRK2 doubles the risk of developing PD (Di Fonzo et al., 2006).

SNP	Chromosome	Nominated Gene	Odds Ratio
rs35749011	1	*GBA/SYT11*	1.824
rs114138760	1	*GBA/SYT11*	1.586
rs823118	1	*RAB7L1/NUCKS1*	1.122
rs10797576	1	*SIPA1L2*	1.131
rs6430538	2	*ACMSD/TMEM163*	0.875
rs1474055	2	*STK39*	1.214
rs12637471	3	*MCCC1*	0.842
rs34311866	4	*TMEM175/GAK/DGKQ*	0.786
rs34884217	4	*TMEM175/GAK/DGKQ*	1.105
rs11724635	4	*BST1*	1.126
rs6812193	4	*FAM47E/SCARB2*	0.907
rs356182	4	*SNCA*	0.76
rs7681154	4	*SNCA*	0.934
rs9275326	6	*HLA-DQB1*	0.826
rs13201101	6	*HLA-DQB1*	1.217
rs199347	7	*GPNMB*	1.11
rs591323	8	*FGF20*	0.916
rs117896735	10	*INPP5F*	1.624
rs329648	11	*MIR4697*	1.105
rs76904798	12	*LRRK2*	1.155
rs11060180	12	*CCDC62*	1.105
rs11158026	14	*GCH1*	0.904
rs2414739	15	*VPS13C*	1.113
rs14235	16	*BCKDK/STX1B*	1.103
rs11868035	17	*SREBF/RAI1*	0.939
rs17649553	17	*MAPT*	0.769
rs12456492	18	*RIT2*	0.904
rs8118008	20	*DDRGK1*	1.111

Table 1-2 Common variant risk loci for PD. List of the SNPs associated with PD with corresponding chromosome number, gene name, and odds ratio for the risk associated with developing PD (Hernandez et al., 2016).

Glucocerebrocidase is a gene associated with Gaucher's disease, a lysosomal storage disorder (Tsuji et al., 1987). Glucocerebrocidase is an enzyme required to breakdown glucocerebroside to ceramide. The knockdown of glucocerebrocidase results in the accumulation of α-synuclein. Interestingly, some patients with Gaucher's disease also have parkinsonism (Tayebi et al., 2001). A single heterozygous mutation in the glucocerbrocidase gene increases the risk for developing PD by five fold (Nalls et al., 2013).

The precise cause and mechanism of PD pathogenesis is currently unknown and although monogenic forms of PD are rare in all PD cases, studying the gene mutations involved in familial PD may provide some insight into common pathways underlying NSDA neuronal degeneration. In fact, many of the genetic mutations and environmental exposures that may lead to onset of PD result in the disruption of overlapping molecular pathways.

PD and Oxidative Stress

The exact cause of PD remains unknown. However, in both idiopathic and familial forms of PD, oxidative stress appears to be a common underlying mechanism that leads to NSDA neurodegeneration. Various pathogenic mechanisms are associated with NSDA neuronal degeneration including: mitochondrial dysfunction, increased DA synthesis, neuroinflammation, and impairment of the ubiquitin proteasome system (UPS). These mechanisms are all known to be involved in either the production of reactive oxygen species (ROS) or the removal of oxidatively damaged cellular components.

ROS are chemically reactive species containing oxygen and include (from least reactive to most reactive): singlet oxygen, superoxide radical, hydrogen peroxide, and hydroxyl radical (Hayyan, Hashim, & AlNashef, 2016). In the cell, ROS are produced as a byproduct of normal

aerobic respiration and have a role in cell signaling and homeostasis. Under normal conditions, the cell has sufficient antioxidant capacity to neutralize these reactive species and prevent damage. During biological impairment, increased ROS production can surpass the antioxidant capacity of the cell leading to significant damage to the cell contents. This is known as oxidative stress. ROS that is not neutralized are unstable and participate in the production of toxic peroxides and the generation of free radicals that modify and damage proteins, lipids and DNA. Additionally, some ROS act in redox signaling and the condition of oxidative stress can disrupt normal redox signaling within the cell.

NSDA neurons intrinsically have a high oxidative burden with limited antioxidant capacity. Due to the length of and the massive branching, these neurons have very high metabolic requirements which increases the risk of increased ROS production and susceptibility to oxidative stress (Braak, Ghebremedhin, Rub, Bratzke, & Del Tredici, 2004; W. Matsuda et al., 2009; Sulzer, 2007). Because of this, a moderate oxidative event could be sufficient to overwhelm the antioxidant capacity of the neurons and result in their degeneration. PD is associated with increased DA turnover, mitochondrial dysfunction, neuroinflammation, and increased iron, all of which favor the increased production of ROS (Gao et al., 2008; Mochizuki & Yasuda, 2012; Muftuoglu et al., 2004; Spencer et al., 1998). The oxidative stress hypothesis of DA neuronal degeneration suggests that increased ROS production such as what occurs during increased DA metabolism, mitochondrial dysfunction, and neuroinflammation can lead to a state of oxidative stress that results in the degeneration of these neurons (Fahn & Cohen, 1992). In support of this hypothesis, the SNc of PD patients have evidence of increased levels of 4-hydroxy-2-nonenal (HNE), a byproduct of lipid peroxidation (Jenner, 2003; Yoritaka et al., 1996), protein carbonylation (Floor & Wetzel, 1998), DNA and RNA oxidation products 8-

hydroxy-dioxyguanosine and 8-hydroxy-guanasine (Alam et al., 1997; J. Zhang et al., 1999)and decreased levels of reduced glutathione (Sechi, 2010; Bharath, Hsu, Kaur, Rajagopalan, & Andersen, 2002; Pearce, Owen, Daniel, Jenner, & Marsden, 1997)

Mitochondrial Dysfunction

Mitochondria are considered the "powerhouse" of the cell because they produce energy, in the form of adenosine triphosphate (ATP) to the cell. Mitochondria produce ATP through the process of oxidative phosphorylation. This process involves the coupling of redox reactions that occur within the enzymatic complexes of the electron transport chain.

The electron transport chain is comprised of a series of four enzymatic complexes (complex I-IV) that are embedded in the inner mitochondrial membrane (**Figure 1-6**). These complexes sequentially catalyze the transfer of single electrons from products of the Krebs Cycle to the terminal electron acceptor, oxygen. In the process, complexes in the electron transport chain use energy from the oxidation of NADH to transport protons across the inner membrane from the matrix to the intermembrane space to form a proton concentration gradient. The proton concentration gradient acts as a pool of free energy that is tapped by ATP synthase. The protons are channeled back down the proton gradient across the inner membrane through ATP synthase. As protons flow down the concentration gradient, they create proton motive force, which drives ATP synthase to catalyze the phosphorylation of ADP to form ATP.

The formation of ATP via the electron transport chain requires a series of redox reactions that result in the reduction of oxygen. Because of this, mitochondria are a primary source of ROS within the cell. Leakage of electrons can occur at complex I and complex III which leads to the transfer of a single electron to oxygen to form superoxide. Once generated, superoxide is

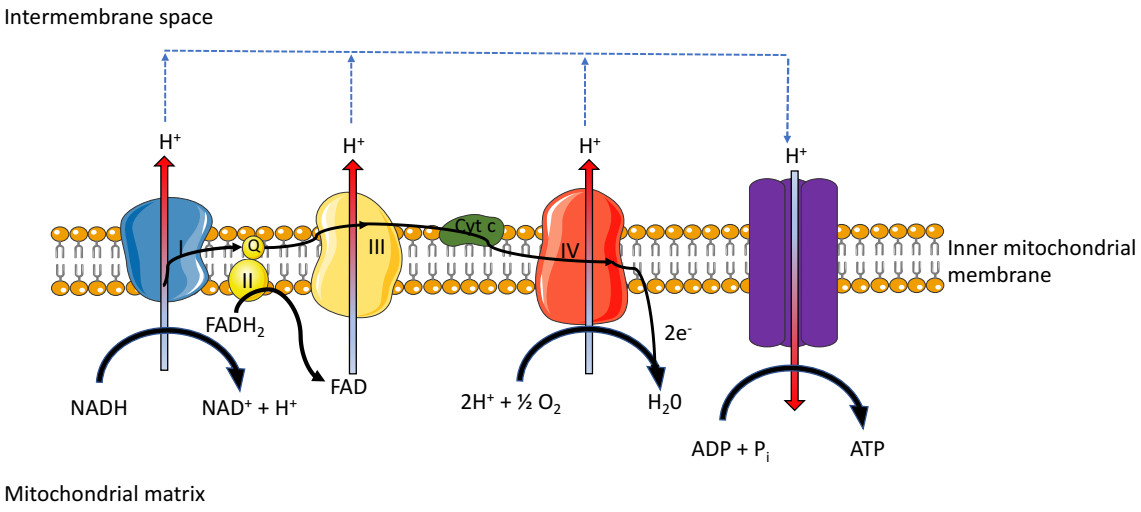

Figure 1-6 The electron transport chain. The electron transport chain is comprised of a series of four enzymatic complexes (complex I-IV) that are embedded in the inner mitochondrial membrane (**Figure 1-6**). These complexes sequentially catalyze the transfer of single electrons from products of the Krebs cycle to the terminal electron acceptor, oxygen. In the process, complexes in the electron transport chain use energy from the oxidation of NADH to transport protons across the inner membrane from the matrix to the intermembrane space to form a proton concentration gradient. The proton concentration gradient acts as a pool of free energy that is tapped to channel protons back across the inner membrane through ATP synthase. As protons flow back down the concentration gradient, they create a proton motive force which drives ATP synthase to catalyze the phosphorylation of ADP to form ATP. Image made using Servier (Les Laboratoires, 2017).

dismutated to hydrogen peroxide in healthy mitochondria by superoxide dismutase 2 (SOD2) in the mitochondrial matrix and superoxide dismutase 1 (SOD1) in the mitochondrial intermembrane space. The hydrogen peroxide is then reduced to water by catalase in peroxisomes. In the presence of transition metals such as Fe^{2+}, superoxide can be converted to a highly reactive hydroxyl radical. Hydroxyl radicals damage the cell through lipid peroxidation, DNA and protein damage.

DA neurons are highly dependent upon intact and functional mitochondria for their survival. Autonomous pacemaking activity of these neurons depends on Ca^{2+} influx from voltage dependent L-type calcium channels (Putzier, Kullmann, Horn, & Levitan, 2009). Ca^{2+} is involved in many cellular processes including the regulation of enzyme activity and programmed cell death (Orrenius, Zhivotovsky, & Nicotera, 2003). Disruption of calcium homeostasis is related to selective degeneration of NSDA neurons (C. S. Chan, Gertler, & Surmeier, 2009). To maintain Ca^{2+} homeostasis, ATP dependent transporters rapidly transport Ca^{2+} back across the plasma membrane (Demaurex, Poburko, & Frieden, 2009). DA neurons undergoing tonic DA transmission must constantly remove Ca^{2+} from the cytosol, and because of this, the energy demand of these cells is quite high. The increased production of ATP in these cells is at the cost of increased ROS production.

Mitochondrial complex I activity is decreased in the SN and ST of patients with idiopathic PD (Mizuno et al., 1989; Schapira, 1993).Additionally, complex I is impaired in the frontal cortex, fibroblasts, blood platelets, lymphocytes and skeletal muscle (Blin et al., 1994; Haas et al., 1995; Mytilineou et al., 1994). Furthermore, many of the environmental and genetic factors that appear to cause PD also influence mitochondrial function (mitochondrial quality control, bioenergetic capacity, calcium homeostasis, subcellular transport, regulation of cell

death pathways). Impaired mitochondrial function leads to increased ROS and, if not neutralized, can damage lipids, proteins and DNA. This damage can harm the integrity of the neuron and accelerate its degeneration.

Pesticides and other toxicants can inhibit the enzymatic complexes of the electron transport chain. In the early nineteen-eighties, a contaminant produced in the synthesis of a street drug related to meperidine was found to cause Parkinson-like symptoms in individuals who self-injected the drug (J.W. Langston & Palfreman, 2013). Post mortem brains from these patients revealed significant NSDA neuronal degeneration and the presence of Lewy bodies (J. W. Langston et al., 1999). The contaminant was identified as 1- methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). MPTP in the mitochondria inhibits Complex I, which leads to the production of ROS and decreased ATP production (Richardson et al., 2007). Because MPTP was found to inhibit Complex I and caused parkinsonian symptoms, mitochondrial impairment has been an important area of research in PD.

In experimental models, the pesticides paraquat (which causes oxidative stress through redox cycling) and rotenone (which inhibits mitochondrial complex I) both induce loss of NSDA neurons and behavioral changes associated with human PD (Henchcliffe & Beal, 2008). Tanner and coworkers (2011) found that farmers that farmers that used rotenone or paraquat were approximately 2 times more likely to develop PD. Two pesticides, dieldrin and Maneb, can selectively inhibit complex III of the electron transport chain (Bergen, 1971; J. Zhang et al., 2003). Dieldrin belongs to a class of pesticides that also includes dichloro-diphenyl-trichlorethane (DDT) and Maneb is an agricultural fungicide.

Many of the genetic mutations that are associated with familial PD also regulate different aspects of mitochondria function or physiology. Mitochondrial abnormalities have been observed

in mice over-expressing SNCA (Subramaniam, Vergnes, Franich, Reue, & Chesselet, 2014). The A53T SNCA mutation in mice causes mtDNA damage and complex IV impairment as well as increased sensitivity to MPTP and paraquat (Norris et al., 2007). Accumulation of SNCA and decreased complex I activity were reported in mitochondria isolated from the ST and SNc of PD patients compared to healthy subjects (Chinta, Mallajosyula, Rane, & Andersen, 2010). PINK1 and parkin are important for mitochondrial quality control and turnover (Abramov et al., 2011; Clark et al., 2006; Exner et al., 2007; Gegg, Cooper, Schapira, & Taanman, 2009; Geisler et al., 2010). In healthy mitochondria PINK1 is degraded and parkin remains in the cytosol. When the mitochondria is damaged, PINK1 accumulates on the outer mitochondrial membrane and activates the E3 ligase function of parkin by phosphorylating it at Ser65(Shiba-Fukushima et al., 2012). Parkin ubiquitinates is substrates on the outer mitochondrial membrane, which results in mitochondria-associated degradation of the individual proteins by the proteasome and triggers mitophagy (Karbowski & Youle, 2011; Taylor & Rutter, 2011).

Parkin mutations are a common cause of autosomal recessive juvenile PD. Parkin knockout mice ($Park2^{-/-}$) have decreased respiratory chain function with reduced complex I activity in the ST (Exner et al., 2007; Hawong, 2014; Narendra, Tanaka, Suen, & Youle, 2008; Springer & Kahle, 2011). Pink1 knockout mice and human DA neurons have abnormalities in mitochondrial morphology, reduced membrane potential, and increased ROS generation (Wood-Kaczmar et al., 2008). Furthermore, PINK1 and $Park2^{-/-}$ mice both have synaptic dysfunction that correlates with progressive loss of mitochondrial function and increased ROS in the ST (Johri & Beal, 2012).

DJ-1 is a neuroprotective redox sensor protein (Wilson, 2011). When oxidized, DJ-1 translocates to the mitochondrial outer membrane and protects neurons by an unknown

mechanism (Lev, Ickowicz, Melamed, & Offen, 2008; Yanagida et al., 2009). Loss of DJ-1 function results in fragmentation of mitochondria in mammalian models of PD due to loss of mitochondrial membrane potential (Thomas et al., 2011). Loss of function of DJ-1 leads to decreased in mitochondrial complex I activity, whereas over-expression of DJ-1 confers protection against MPTP impairment of complex I (Kim et al., 2005). Although the function of LRRK2 in relation to the mitochondria is unclear, LRRK2 is associated with the mitochondria outer membrane (Biskup et al., 2006). HTRA2 is a serine peptidase localized to mitochondria and is released during mitochondrial membrane permeabilization during apoptosis (Suzuki et al., 2001). HTRA2 activates autophagy through the digestion of Hax-1, a Bcl-2 family related protein (B. Li et al., 2010). Mutations in HTRA2 make cells more susceptible to oxidative stress (Moisoi et al., 2009).

Abnormal DA Metabolism

In PD, axon terminal degeneration is thought to occur prior to NSDA neuronal loss (Cheng et al., 2010). In the synthesis and catabolism of DA, many of the metabolites and the catabolic enzymatic processes themselves can be toxic to the neuron. ROS generating enzymes such as TH and flavin-containing enzymes like MAO-B produce hydrogen peroxide as a byproduct of their activity (Haavik, Almas, & Flatmark, 1997; Zang & Misra, 1993). Because of this, abnormal DA homeostasis is a suspect mechanism of PD pathology.

DA is an organic base that consists of a catechol with one amine group (**Figure 1-7**). In the acidic pH of the vesicle, DA is protonated and stable. At more basic pH, as in the cytosol, DA rapidly becomes deprotonated. Deprotonated DA is easily auto-oxidized to form DA quinones that are highly reactive and toxic (He et al., 2011; Sulzer et al., 2000).

Cellular protein function can be irreversibly altered through the formation of 5-cysteinyl-catechols on proteins. For example, the formation of DA quinones can lead to the covalent modification and inactivation of tyrosine hydroxylase (Di Giovanni, Pessia, & Di Maio, 2012). DA quinones are potent cellular toxins, and there is evidence that these may contribute to NSDA neuronal loss that occurs in PD (Miyazaki & Asanuma, 2008). Due to the damage that DA quinones can inflict, it is crucial that the amount of cytoplasmic DA be tightly controlled within the DA neuron. There are several mechanisms in place that prevent the accumulation of cytosolic DA: (1) transport of DA into synaptic vesicles by VMAT2, (2) enzymatic metabolism, and (3) polymerization of DA, its toxic quinones to form neuromelanin.

Cytoplasmic DA is packaged into the synaptic vesicles by VMAT2. Disruption of VMAT2 function prevents uptake of cytosolic DA into the vesicles and leads to auto-oxidation, ROS formation, and axon terminal damage (Riddle, Fleckenstein, & Hanson, 2006). In addition, Pifl et al. (Pifl et al., 2014) demonstrated that after correcting for DA nerve terminal loss, vesicular uptake of DA per VMAT2 in the ST is reduced by 53-55% in PD patients compared to normal patients. Mice with elevated VMAT2 are protected from PD related neurotoxic insult (Lohr et al., 2016).

Cytoplasmic DA levels are maintained by metabolic enzymes such as MAO-B on the outer mitochondrial membrane. The mitochondria associated enzyme MAO-B deaminates excess Amounts of cytosolic DA to form the toxic metabolite DOPAL. DOPAL is a toxic aldehyde that can form protein adducts and cause the opening of the mitochondrial transition pore complex, which leads to programmed cell death (Kristal et al., 2001). Because DOPAL is a toxic aldehyde, it must be quickly metabolized to a less toxic product. As described earlier, DOPAL is oxidized

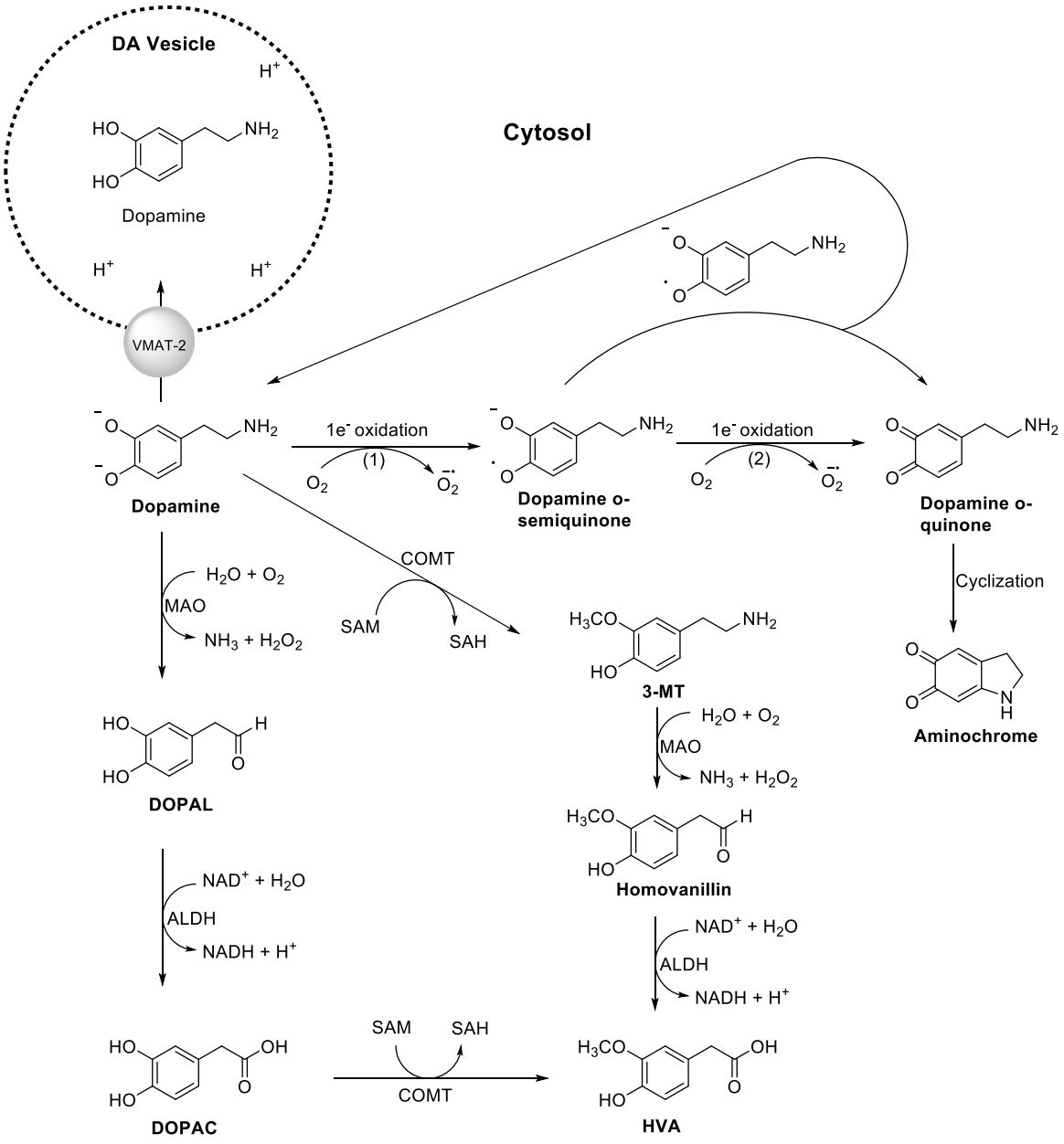

Figure 1-7 Metabolism or oxidation of cytosolic DA. At physiologic pH DA is deprotonated and if not metabolized will oxidize to its semiquinone radical. Two DA semiquinone radicals will generate one molecule of DA and one molecule of its quinone. Alternatively, the semiquinone radical can undergo a one-electron oxidation to the quinone. The quinone immediately cyclizes to form aminochrome, which is only stable at acidic pH. To prevent the formation of toxic DA quinones, cytosolic DA is deaminated by MAO-B to produce DOPAL which is oxidized by ALDH to produce DOPAC. DOPAC is methylated by COMT to produce water soluble HVA, which is excreted. Extracellular DA can either be taken back up into the DA neuron via DAT or methylated by COMT to form 3-MT which is deaminated and hydroxylated to produce HVA. Figure based off of (Munoz, Paris, Sanders, Greenamyre, & Segura-Aguilar, 2012).

by ALDH to its acid (3,4-dihydroxyphenylacetic acid, DOPAC) and DOPAC is further methylated by COMT to form HVA which is excreted after sulfation.

Both glia and DA neurons oxidize DA via the mitochondria associated enzyme MAO to form the toxic metabolite, DOPAL, with hydrogen peroxide as a byproduct. During MAO activity, the amount of hydrogen peroxide produced in the cell can increase approximately 100-fold (Bao et al., 2009). The SNc is an iron rich environment (Snyder & Connor, 2009). In the presence of this transition metal, the Fenton reaction allows hydrogen peroxide to generate hydroxyl radicals, which are highly reactive. The resulting hydroxyl radicals cause DNA, lipid and protein damage resulting in degeneration of the neuron.

To prevent free radical damage to the neuron, hydrogen peroxide must be neutralized. Hydrogen peroxide can be detoxified via the endogenous antioxidant glutathione (GSH) system. Reduced GSH reacts with hydrogen peroxide to produce water and GSSG via glutathione peroxidase activity. GSSG is recycled back to GSH by the action of GSH reductase using NADPH as a cofactor. GSSG is toxic and reacts with free cysteinyl residues of proteins to form disulfide bonds and results in loss of protein function. Enzymes involved in the citric acid cycle or the electron transport chain are sensitive to this reaction and damage here can lead to mitochondrial dysfunction (Delcambre, Nonnenmacher, & Hiller, 2016).

NSDA neurons are degenerated in PD and, because of this, PD is associated with a higher DA turnover due to the lower number of neurons compensating for the decreased DA innervation (Zigmond, Hastings, & Perez, 2002). The compensatory increase in DA synthesis results in increased MAO activity and thus an increase in MAO associated toxicity. Increased DA turnover also promotes an increase in cytosolic DA, the increased production of toxic DA metabolites and auto-oxidation of DA.

Cytosolic DA can be removed by packaging it into DA vesicles, or metabolism of DA into less toxic metabolites. The remaining cytosolic DA metabolites and oxidation products can be polymerized and sequestered in neuromelanin. Neuromelanin is a dark pigment found in NSDA neurons of the SN (Zecca, Zucca, Wilms, & Sulzer, 2003). It is comprised of proteins and lipids and polymerized oxidation products of DA (Zecca, Zucca, Costi, et al., 2003). Neuromelanin granules are enclosed by a lipid bilayer and can undergo autophagy (Munoz, Huenchuguala, Paris, & Segura-Aguilar, 2012). Neuromelanin production depends on the cytosolic availability of DA and may serve a neuroprotective function. Treatment of DA synthesizing PC-12 cells with levodopa causes increased production of neuromelanin while overexpression of VMAT2, which removes DA from the cytosol, inhibits neuromelanin synthesis (Meiser, Weindl, & Hiller, 2013).

In addition to sequestering cytoplasmic DA and its metabolites, neuromelanin also chelates a large amount of metal ions, such as iron, as well as neuronal toxicants such as paraquat and MPP^+ (D'Amato, Lipman, & Snyder, 1986; Lindquist, Larsson, & Lyden-Sokolowski, 1988). Sequestration of these toxic products acts to prevent iron-catalyzed oxidation of DA and metal ion or toxicant mediated neurotoxicity. The iron bound to neuromelanin is mainly in the ferric state and tightly bound to high affinity sites to remain inactive, thus preventing oxidative reactions (Zecca et al., 1996). Under conditions of excess iron, the high affinity sites are saturated and the excess iron binds to the remaining low affinity binding sites (Zecca et al., 2001). These low affinity sites are not as effective at sequestering the iron. Therefore, the iron remains redox-active and can promote oxidative reactions (Faucheux et al., 2003).

Following the death of NSDA neurons, neuromelanin is released into the extracellular space where it induces microglial activation. Several pro-inflammatory molecules are released from the microglia, such as NO, resulting in an inflammatory process (W. Zhang et al., 2011). Due to its composition, neuromelanin is not soluble and is only slowly degraded (Zecca, Zucca, Wilms, et al., 2003). Metal ions and toxicants, which were previously bound to neuromelanin are released in the extracellular space which acts as another source of ROS production (Zecca, Zucca, Wilms, et al., 2003). As more neurons and glial cells die, a cycle of oxidative stress leading to more neuronal death and more neuronal death creating more ROS occurs.

Abnormal Protein Aggregation

Many neurodegenerative diseases are precipitated by oxidative modifications of proteins that result in protein misfolding and subsequent aggregation (Bourdenx et al., 2017; A. B. Singleton et al., 2003; Takalo, Salminen, Soininen, Hiltunen, & Haapasalo, 2013). To prevent damage, post mitotic neurons rely upon tightly controlled cellular protein degradation pathways to remove damaged and unneeded proteins. Damaged or modified proteins can often lead to neuronal dysfunction and neurodegeneration because of a toxic gain of function that creates aberrant cellular pathways, and ultimately, cell death (Winklhofer, Tatzelt, & Haass, 2008). In understanding these neurodegenerative diseases, it is important to understand the cellular pathways that regulate the presence of toxic protein species (**Figure 1-8**). In PD the synaptic protein α-synuclein oligomerizes, fibrilizes and forms aggregates that are sequestered into cytoplasmic inclusions called Lewy bodies and Lewy neurites (Wan & Chung, 2012). Although the mechanisms by which α-synuclein aggregates and causes neurodegeneration are not fully known, strong genetic and molecular evidence implicates a crucial role for this protein in PD.

Molecular chaperones are the first line of defense against misfolded proteins (Lopez-Otin, Blasco, Partridge, Serrano, & Kroemer, 2013). Chaperone proteins act to stabilize, or refold, aggregate prone misfolded proteins. Under pathological conditions chaperone proteins can become depleted allowing the aggregate prone proteins to overwhelm the capacity of the chaperone system. When this occurs, the proteins that do not get refolded can be eliminated via one of two proteolytic systems that are in place. The two major proteolytic systems are the autophagy-lysosomal pathway (ALP) and the UPS.

The ALP is thought of as an evolutionarily conserved adaptive response to nutrient deprivation (Levine & Kroemer, 2008). However, the pathway can also be induced by hypoxia and decreased energy supply (Bellot et al., 2009). Under these conditions, intracellular components, including long-lived proteins and cellular organelles, are degraded and the resulting amino acids are recycled back into the cytoplasm to be used in essential biosynthetic pathways. There is increasing evidence that autophagy is important for neuronal survival and that dysregulation of autophagy results in neurodegeneration. Studies on the PD associated transmembrane lysosomal P5-type ATPase (ATP13A2) indicate that loss of function of this

protein is correlated with impaired lysosomal acidification and decreased degradation of lysosomal substrates (Dehay et al., 2012). Both PINK1 and parkin control the process of mitophagy, the autophagic removal of damaged mitochondria (R. F. Roberts, Tang, Fon, & Durcan, 2016)(Lee et al, 2012). Impaired removal of defective mitochondria results in increased oxidative damage, protein modification, protein misfolding and further impairment to protein degradation pathways.

There are three main types of autophagy that are defined by the pathways in which the cargo is delivered to the lysosome. These types are macroautophagy, microautophagy, and

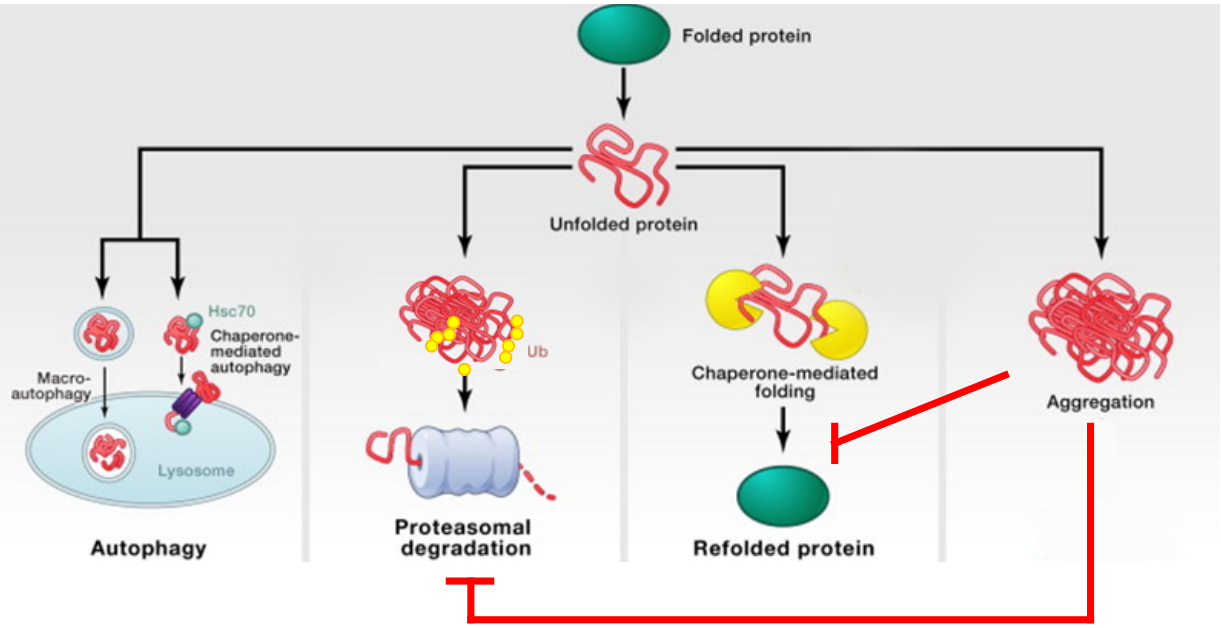

Figure 1-8 Cellular pathways that regulate the presence of misfolded proteins. Molecular chaperones are the first line of defense against misfolded proteins. Chaperone proteins act to stabilize, or refold, aggregate prone misfolded proteins. Under pathological conditions chaperone proteins can become depleted allowing the aggregate prone proteins to overwhelm the capacity of the chaperone system. When this occurs, the proteins that do not get refolded can be eliminated via one of two proteolytic systems that are in place. The two major proteolytic systems are the autophagy-lysosomal pathway (ALP) which includes both macroautophagy and chaperone mediated autophagy and the ubiquitin proteasome system (UPS). Adapted from (Lopez-Otin et al., 2013).

chaperone-mediated autophagy (CMA). In macroautophagy some of the cytoplasm, including its organelles is non-selectively degraded (Kanki, 2009; Shintani, Huang, Stromhaug, & Klionsky, 2002). The cytoplasmic contents are engulfed by a double membrane phagophore. This phagophore expands into a cytosolic vesicle called an autophagosome which is targeted to the lysosome (Klionsky, 2005). The outer membrane of the autophagosome fuses with the lysosomal membrane so that hydrolases can enter the inner autophagosome and hydrolyze the contents so that the cellular components that are captured inside the autophagosome can be recycled.

Microautophagy is like macroautophagy except, in microautophagy, the cargo is directly sequestered into the lysosome instead of first being captured in the autophagosome. In this process, a portion of the lysosome invaginates so that it can directly take up cytoplasmic materials at the lysosome surface. After vesicles containing the cytosolic substrates pinch off into the lumen of the lysosome, the contents are rapidly degraded (Kunz, Schwarz, & Mayer, 2004) and the products are recycled.

CMA mediates the selective lysosomal degradation of soluble cytosolic proteins. Chaperone protein, Hsc70, mediates this process by binding to specific cytosolic substrates and delivering them to the lysosomal receptor LAMP-2A (Cuervo & Wong, 2014). The substrate protein is then unfolded and translocated across the lysosomal membrane to be degraded by lysosomal proteases. CMA is crucial to the elimination of certain misfolded proteins, but this system is also subject to the toxic effects of these pathogenic proteins (Cuervo, Stefanis, Fredenburg, Lansbury, & Sulzer, 2004; Martinez-Vicente et al., 2008; Orenstein et al., 2013). For example, both α-synuclein and LRRK2 are degraded by CMA. However, aberrant forms of these proteins bind to and block LAMP-2A, leading to a blockade of the degradation of CMA substrates (Cuervo & Wong, 2014).

The UPS is the major pathway that mediates the degradation of short-lived soluble proteins within the cell (**Figure 1-9**)(Fuertes, Villarroya, & Knecht, 2003). To be degraded via the UPS, substrates must first be tagged with a polyubiquitin chain (Scheffner, Nuber, & Huibregtse, 1995). A chain of activated ubiquitin monomers is covalently linked to lysine residues of the substrate protein in an ATP-dependent manner. Ubiquitination is carried out in three steps: activation, conjugation and ligation. In the first step, ubiquitin is activated by an E1 ubiquitin activating enzyme which is dependent upon ATP. This results in a thioester linkage between the C-terminal carboxyl group of ubiquitin and the E1 cysteine sulfhydryl group. Once activated E2 ubiquitin conjugating enzymes catalyze the transfer of ubiquitin from E1 to the active site cysteine of the D2 via a trans(thio)esterification reaction. Last, E3 ubiquitin ligase create an isopeptide bond between a lysine of the target protein and the C-terminal glycine of ubiquitin. This step requires the activity of one of the several hundred substrate specific E3 ubiquitin ligases. This process is carried out multiple times until a chain of at least four ubiquitin subunits is produced.

The polyubiquitin chain serves as a recognition signal for the 26S proteasome (Grice & Nathan, 2016). The 26S proteasome is a barrel shaped protein complex (**Figure 1-10**), that consist of a catalytic core (the 20S core proteasome) and regulatory subunits that form two 19S "caps" on either end of the 20S core particle (K. Tanaka, 2009). The 19S caps regulate the entry of protein substrates and modulate the catalytic activities that occur within the barrel of the 20S core particle (Ehlinger & Walters, 2013). The catalytic core is formed by 28 subunits organized in two outer α-rings and two inner β-rings (K. Tanaka, 2009). The inner β- rings contain the catalytic sites for three proteolytic activities: chymotrypsin-like (CT-L), trypsin-like (T-L) and caspase-like (Cas-L) hydrolytic activity (K. Tanaka, 2009). Proteasomal degradation of the

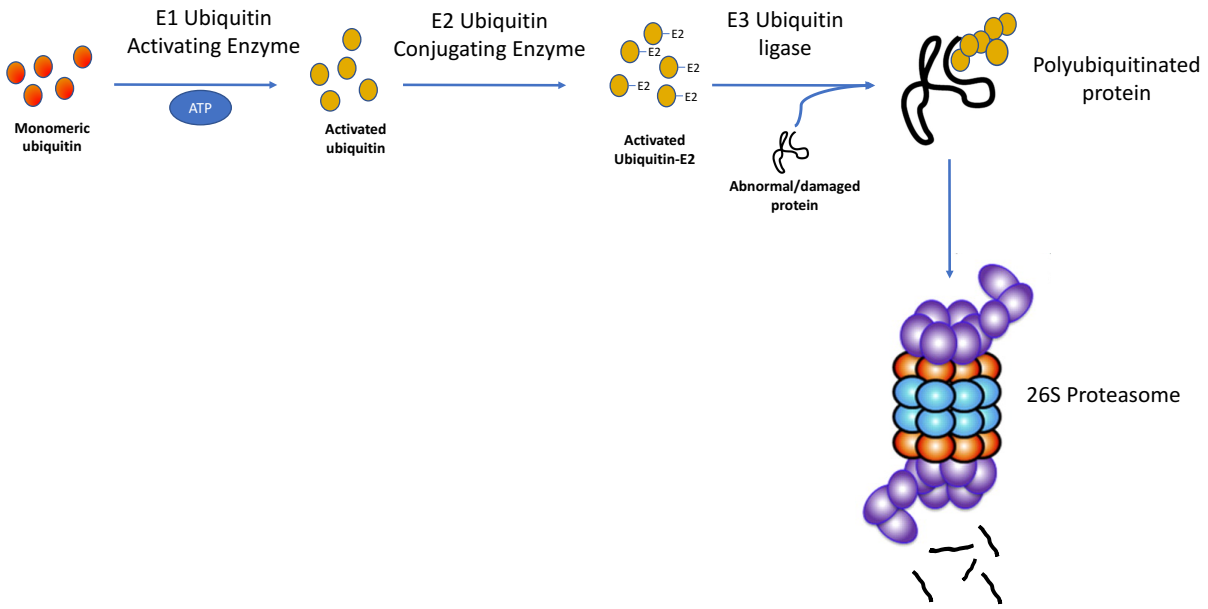

Figure 1-9 The ubiquitin proteasome system (UPS). To be degraded via the UPS, substrates must first be tagged with a polyubiquitin chain. Ubiquitination is carried out in three steps: activation, conjugation and ligation. In the first step, ubiquitin is activated by an E1 ubiquitin activating enzyme which is dependent upon ATP. This results in a thioester linkage between the C-terminal carboxyl group of ubiquitin and the E1 cysteine sulfhydryl group. Once activated E2 ubiquitin conjugating enzymes catalyze the transfer of ubiquitin from E1 to the active site cysteine of the D2 via a trans(thio)esterification reaction. E3 ubiquitin ligases create an isopeptide bond between a lysine of the target protein and the C-terminal glycine of ubiquitin. Finally, the polyubiquitin chain is recognized by the 26S proteasome and the substrate is degraded.

protein substrates results in the production of short peptide fragments that are subjected to further degradation to its individual amino acids by peptidases.

There is mounting evidence that UPS dysfunction is involved in PD. Lewy bodies are a pathological hallmark of PD (Braak & Braak, 2000). The protein aggregates that are sequestered within Lewy bodies are highly ubiquitinated (Olanow et al., 2004). In addition, several components of the UPS, including proteasome subunits, ubiquitinating and deubiquitinating enzymes and proteasome activators are present in Lewy bodies (McNaught, Shashidharan, Perl, Jenner, & Olanow, 2002). Furthermore, several of the PD related gene mutations are in genes that encode for proteins involved in the UPS. Parkin (PARK2) is an E3 ubiquitin ligase (Kitada et al., 1998) that also has the ability to bind to and enhance 26S proteasome activity (Um et al., 2010), UCH-L1 (PARK5) is a deubiqutinating enzyme (Leroy, Boyer, & Polymeropoulos, 1998) and FBX07 (PARK15) is an adaptor protein for an E3 ubiquitin ligase complex called SCF (Deng, Liang, & Jankovic, 2013; Di Fonzo et al., 2009). Post mortem brain tissue from the SN has decreased CT-L and T-L proteasome activity compared to age matched control tissue (McNaught, Belizaire, Isacson, Jenner, & Olanow, 2003; McNaught & Jenner, 2001).

While there is decreased proteasome activity in the SN of PD brain tissue, other brain regions do not appear to have decreased proteasome activity and may even have enhanced proteasome activity (McNaught, Perl, Brownell, & Olanow, 2004). This regional specific reduction in proteasome activity suggests that decreased proteasome activity may either be a precipitating event that predisposes NSDA neurons to degenerate in PD or conversely, decreased proteasome activity could be secondary to the existing pathology of PD.

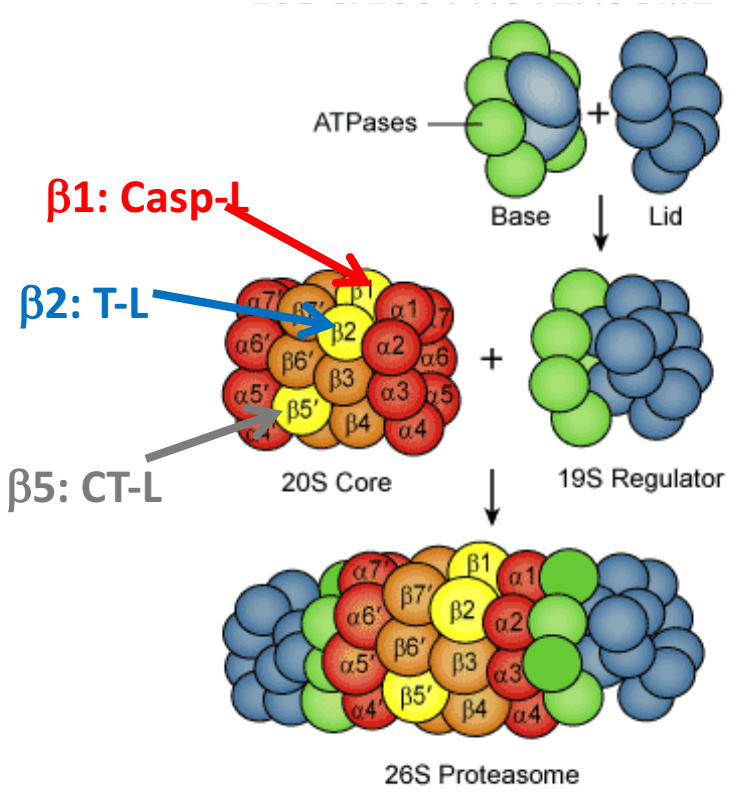

Figure 1-10 The 26S proteasome. The 26S proteasome is a barrel shaped protein complex, that consist of a catalytic core (the 20S core proteasome) and regulatory subunits that form two 19S "caps" on either end. The 20S core particle is comprised of two heptameric β-subunit rings containing the Cas-L, T-L, and CT-L catalytic subunits β1, β2, and β5 and two heptameric α-subunit rings that form a gate that prohibits entry of proteins into the catalytic chamber. Binding of the 19S regulatory caps to the 20S core opens the gate and depends upon ATP binding to the RPT subunits within the 19S base. Substrate recognition occurs via the RPN subunits of the 19S lid. Image modified from bostonbiochem.com.

MPTP is a DA Neuron Specific Neurotoxicant

The toxicity of MPTP was discovered in the early nineteen eighties after several drug users inadvertently self-injected "Super Demerol", a street narcotic related to meperidine (J.W. Langston & Palfreman, 2013). The drug was contaminated with MPTP, a side product created in the synthesis of the drug (J. W. Langston, Ballard, Tetrud, & Irwin, 1983). Upon self-injection, the users rapidly developed symptoms that closely resembled late stage PD. These patients were coined "The Frozen Addicts" because their rigid bodies appeared frozen (J.W. Langston & Palfreman, 2013).. Upon further investigation, it was found that MPTP induces many of the biochemical, pathological and clinical features of PD in primates as well as mice (Jackson-Lewis & Przedborski, 2007; J. W. Langston et al., 1983). Systemic MPTP exposure closely parallels the pattern of molecular dysfunction to that observed in PD. Despite many similarities between MPTP toxicity and PD, researchers are cautious to note that there are differences between the MPTP neurotoxicant model of PD and idiopathic PD including: disease progression, acute onset and lack of typical Lewy body formation. However, the ability of MPTP toxicity to cause similar molecular pathology within NSDA neurons makes MPTP a useful tool for studying the early molecular events that may be involved in PD.

MPTP is a pro-toxicant (**Fig 1-11**), it is not itself toxic (Dauer & Przedborski, 2003). The small lipophilic molecule is easily able to cross the blood brain barrier and lipid bilayers. MPTP is oxidized to 1-methyl-4- phenyl-2,3-dihydropyridinium ($MPDP^+$) by the MAO-B enzyme present in glial cells on the outer membrane of the mitochondria. $MPDP^+$ is unstable, and is further oxidized to form the toxic metabolite 1-methyl-4-phenylpyridinium ion, (MPP^+), which is transported out of glial cells and into the extracellular fluid by the organic cation transporter-3,

OCT-3. Because it is charged, MPP^+ cannot cross membrane lipid bilayers, however, it is actively transported specifically into DA neurons by the DAT.

MPP^+ is a Redox Cycler

MPP+ is structurally related to the redox cycling agent paraquat (**Figure 1-12**). Although through different mechanisms, both molecules are subject to redox cycling. An electron donor such as NADPH, reduces paraquat before being oxidized by an electron acceptor such as dioxygen to produce superoxide, a major ROS. At physiologic pH and without the help of available enzymes, MPP^+ is a poor redox cycler compared to paraquat (J. D. Adams, Jr., Chang, & Klaidman, 2001). However, in the presence of flavin containing enzymes, such as MAO, ALDH, complex I, or TH, MPP^+ is subject to hydride transfer from the reduced flavin, which is produced during enzymatic activity of the flavin containing enzyme (J. D. Adams, Jr. et al., 2001). This forms the unstable product,1-methyl-4-phenyl-1,4-dihydropyridine (DHP). DHP decays in a one electron step to produce the MPP radical. The MPP radical is a very strong reducing agent that can catalyze the formation of hydroxyl radical from hydrogen peroxide. The MPP radical transfers its extra electron to molecular oxygen to form MPP+ and superoxide anion.

MPP^+ Inhibits Complex I of the Mitochondria

Once within the DA axon terminal, MPP+ is concentrated in the mitochondria where it inhibits Complex I of the mitochondrial electron transport chain(Nicklas, Youngster, Kindt, & Heikkila, 1987). The complexes of the electron transport chain use energy from the oxidation of NADH to transport protons across the inner membrane from the matrix to the

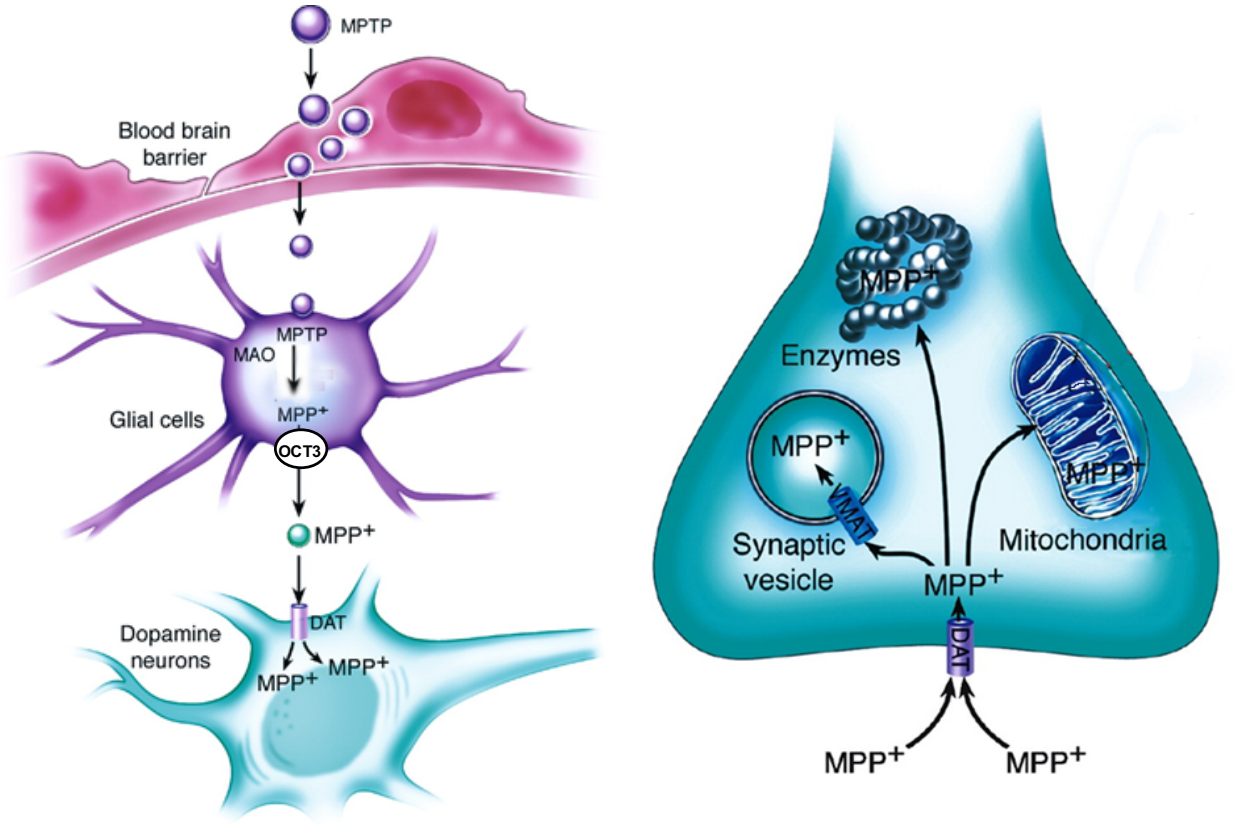

Figure 1-11 Bioactivation of MPTP and the effects of MPP^+ on DA neurons. MPTP crosses the blood brain barrier and is converted to MPP^+ by MAO in glial cells. MPP^+ is transported out of the glial cell by OCT3 and is specifically taken up into DA neurons by DAT. Once in the DA neuron MPP^+ incites its toxicity by accumulating in the mitochondria where it inhibits complex I of the electron transport chain, displacing DA from DA vesicles and directly inhibiting enzymatic activity via the production of the MPP radical. Abbreviations: MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; MAO, monoamine oxidase; MPP^+, 1-methyl-4-phenylpyridinium ion; OCT3, Organic Cation Transporter 3; DAT, dopamine transporter. Figure adapted from (Dauer & Przedborski, 2003).

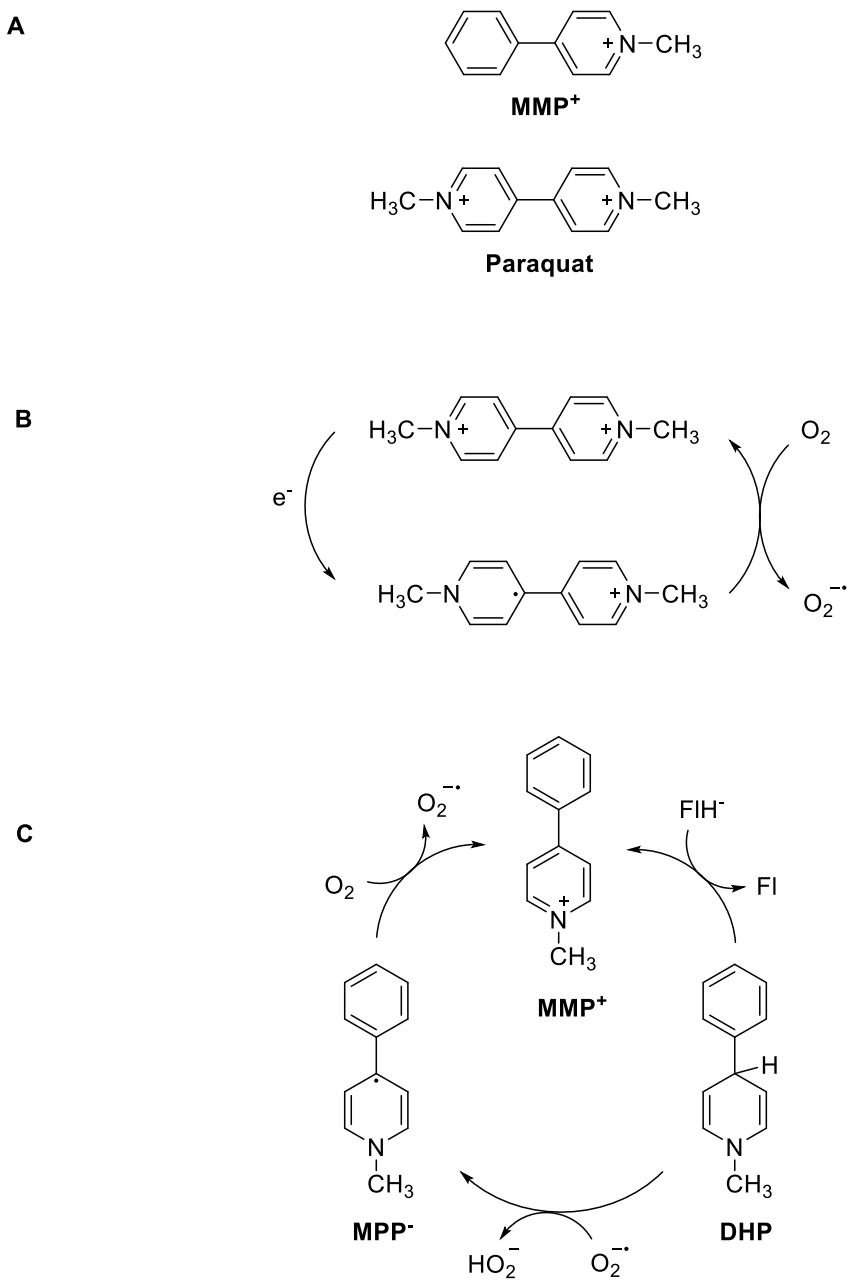

Figure 1-12 MPP^+ is structurally related to paraquat and is also subject to redox cycling. **A**, Structures of MPP^+ and paraquat, **B**, Paraquat is subject to a one electron reduction to give rise to paraquat radical. The paraquat radical transfers the extra electron to molecular oxygen to form superoxide anion. **C**, MPP^+ is subject to hydride transfer from reduced Flavin produced during MAO activity. Anionic reduced Flavin binds to MPP^+ and reduces it to DHP, which is then oxidized to produce MPP radical. The MPP radical transfers the extra electron to molecular oxygen to form superoxide anion.

intermembrane space, which forms the proton gradient that is needed to drive ATP synthase activity. MPP^+ accumulates in the mitochondria because it is passively transported down the gradient into the mitochondria (Hoppel et al., 1987). Once in the mitochondria, MPP^+ binds to and inhibits complex I of the electron transport chain (Nicklas et al., 1987). Inhibition of complex I can cause cell death due to decreased ATP production (Przedborski et al., 2000), and generation of free radicals and ROS (Cleeter, Cooper, & Schapira, 1992). However, the mitochondrial toxicity of MPP^+ is self-limiting. As MPP^+ binds to and inhibits complex I, the membrane potential falls and less ATP is produced (Nicklas et al., 1987). With the decrease in membrane potential, MPP^+ is no longer able to concentrate within the mitochondria and is released back into the cytosol. Although contributory, it is thought that mitochondrial Complex I inhibition is not the primary mechanism of NSDA neurodegeneration. MPTP exposure results in toxicity independent of any action that occurs in the mitochondria (J. D. Adams, Yang, & Klaidman, 2006).

MPP^+ and DA Toxicity

Once inside the DA axon terminal, MPP^+ predominantly localizes within the DA vesicles. This occurs via high affinity uptake into the DA vesicle by VMAT2 (Liu, Roghani, & Edwards, 1992). Sequestration of MPP^+ into the DA vesicles appears to be protective as VMAT2 over-expressing cells are resistant to MPP^+ toxicity and VMAT2 knockout mice are more susceptible to MPTP toxicity (Lohr et al., 2016).

Sequestration of MPP^+ into the DA vesicle has a consequence. When MPP^+ enters the vesicle, DA is displaced from within the vesicle into the cytoplasm. While DA is protonated and stable within the acidic confines of the DA vesicle, once it is displaced into the cytosol it

becomes rapidly deprotonated. The deprotonated DA is easily auto-oxidized to form DA quinones and free radicals (He et al., 2011; Sulzer et al., 2000). MPP^+ mediated displacement of DA from vesicles not only leads to cytoplasmic DA oxidation and generation of free radicals, but also leads to DAT mediated reverse transport of DA into the extracellular space and to extracellular oxidation of DA (Lotharius & O'Malley, 2000).

MPP^+ is a Competitive Inhibitor of MAO-B

Under normal conditions cytosolic DA is metabolized to DOPAL by MAO-B. As a substrate of MAO-B, MPTP acts as a competitive inhibitor (Chiba, Trevor, & Castagnoli, 1984; Salach, Singer, Castagnoli, & Trevor, 1984). Competitive inhibition of MAO-B results in reduced ability of the neuron to metabolize cytosolic DA and thus causes increased formation of toxic DA metabolites and oxidation products. Consequently, inhibition of MAO-B results in inhibition of protoxicant conversion to MPP^+ and protection against MPP^+ toxicity (Wu, Chen, & Chiueh, 2000).

MPP^+ and UPS Dysfunction

MPP^+ directly inhibits the 20S proteasome *in vitro* (Caneda-Ferron et al., 2008) and mice exposed to MPTP exhibit impaired UPS function and accumulation of damaged proteins in the SNc, a characteristic that is similar to what has been observed in the SNc of post-mortem brains of PD patients (McNaught & Jenner, 2001). Marmosets treated with MPTP also have decreased proteasome activity (Zeng et al., 2006). Acute administration of MPTP results in transient impairment of the UPS, while chronic MPTP exposure results in long-term proteasome

inhibition and the formation of protein inclusions that resemble Lewy bodies (Fornai et al., 2005).

MPTP and Differential Susceptibility of Central DA Neurons

Many different cell types are affected in PD however; central DA neurons are severely affected (Braak & Braak, 2000; Sulzer, 2007). Not all DA neuronal populations are affected in PD. While NSDA neurons degenerate, TIDA neurons retain their DA stores, do not form inclusions, and do not degenerate (Ahlskog, 2005; Braak et al., 2003; J. W. Langston & Forno, 1978; Matzuk & Saper, 1985). Mechanisms responsible for TIDA resistance to neurodegeneration are not understood. Characterization of the responses of TIDA neurons compared to NSDA neurons that degenerate in PD could shed light onto mechanisms that may provide protection.

Cell bodies of TIDA neurons are located in the ARC in the mediobasal hypothalamus and their axons terminate in the ME where they release DA into the hypophyseal portal blood (Ben-Jonathan, Oliver, Weiner, Mical, & Porter, 1977). DA is carried by the portal blood to the anterior pituitary where it tonically inhibits prolactin secretion (Fitzgerald & Dinan, 2008). Reuptake of DA occurs mostly by high volume low affinity transporters, and to a smaller extent by low volume high affinity DAT (Annunziato, Leblanc, Kordon, & Weiner, 1980; Demarest & Moore, 1979; Revay, Vaughan, Grant, & Kuhar, 1996) . The process of DA synthesis, storage, release, and metabolism in TIDA neurons are much the same as in NSDA neurons. The similarities between the NSDA and TIDA neuronal populations provides an interesting platform in which to compare the differential responses of TIDA neurons with neurons that degenerate in PD.

Similar to the differential susceptibility observed in PD DA neuronal populations, neurotoxicant treated animals also present differentially susceptible NSDA and TIDA neurons. Following neurotoxicant exposure, NSDA axon terminals are depleted of DA and exhibit loss of TH-ir cells, while the TIDA neurons are spared (Behrouz, Drolet, Sayed, Lookingland, & Goudreau, 2007; Mogi, Harada, Kojima, Kiuchi, & Nagatsu, 1988; G. L. Willis & Donnan, 1987).

Toxicant distribution, bio-activation, and uptake into the terminal, are not responsible for TIDA neuron resistance to MPTP exposure. The possibilities that there is reduced bioactivation of MPTP by astrocytes in the mediobasal hypothalamus or that there are differences in drug distribution has been suggested as reasons that TIDA neurons are not susceptible to MPTP toxicity (Hirsch, 1992; Hirsch, Faucheux, Damier, Mouatt-Prigent, & Agid, 1997; Uhl, 1998). Differential sensitivity of TIDA and NSDA neurons to MPP^+ is not due differential drug distribution to the DA neuronal populations. In fact, at 4 h post MPTP exposure (when MPP^+ is at its maximal concentration in both the ME and ST) the amount of MPP^+ in the ME is approximately 4 times higher than in the ST (M. Benskey et al., 2012). In addition, primary DA neurons from the mediobasal hypothalamus were not affected by direct exposure to MPP^+, while the same concentration of MPP^+ markedly increased apoptosis of primary DA neurons from the midbrain (Behrouz et al., 2007). The fact that TIDA neurons are not directly affected by MPP^+ implies that the resistance to MPTP is not due to differences in the bio-activation of MPTP to MPP^+ by astrocytes in the mediobasal hypothalamus. Resistance of TIDA neurons to MPTP does not appear to be due to lower expression of DAT, which might decrease MPP^+ uptake into the TIDA neurons. TIDA neurons are also resistant to rotenone, a toxicant that readily crosses membranes and does not require DAT to enter the neurons (Betarbet et al., 2000; Bywood &

Johnson, 2003; Le et al., 1999). TIDA axon terminals are initially depleted of DA 4 h following MPTP exposure. Following the initial depletion, DA is replenished 16 h following MPTP exposure (Behrouz et al., 2007). The time course of DA depletion and subsequent recovery indicates that MPP^+ within TIDA terminals is sufficient to initially incite some toxicity before the neuron can adapt and recover.

The recovery of TIDA neurons from MPTP toxicity is dependent upon protein synthesis and more specifically the increased expression of the PD related protein, parkin. This is interesting because mutations in the parkin gene result in autosomal recessive familial PD (Kitada et al., 1998). Following MPTP exposure, TIDA neurons up-regulate the expression of parkin mRNA and protein, while NSDA neurons do not (M. Benskey et al., 2012). Furthermore, AAV mediated knockdown of parkin in the ARC prevents the recovery of TIDA neurons following MPTP exposure (M. J. Benskey, Manfredsson, Lookingland, & Goudreau, 2015). Taken together, these data indicate that the recovery of TIDA neurons following MPTP exposure, a neurotoxicant that closely mimics PD pathology, is dependent upon the upregulation of parkin.

Parkin

Mutations in the parkin gene are the most common cause of familial PD (von Coelln, Dawson, & Dawson, 2004). Autosomal recessive juvenile PD (AR-JP) is associated with large homozygous exon deletions within the long arm of chromosome 6. The association of this locus with AR-JP, led to the discovery of PARK2, the parkin gene (Kitada et al., 1998). Mutations in PARK2 have been identified in familial as well as in sporadic PD patients and these mutations are the most common cause of early onset PD (von Coelln et al., 2004). Many types of parkin

mutations appear to cause AR-JP including: missense and nonsense point mutations, multiplications of entire exons, deletions of entire exons and frameshifts (von Coelln et al., 2004). With the caveat that many published studies are restricted to those with young-onset disease (Grunewald, Kasten, Ziegler, & Klein, 2013), the detection frequency of all types of pathogenic PARK2 variants is 80-90% in familial cases (with onset before 20 years of age) and lower than 10% in sporadic case with onset around 40 years (Periquet, Corti, Jacquier, & Brice, 2005).

PARK2 encodes a protein of 465 amino acids that is specifically expressed in tissue with high energy requirements including: the brain, heart, liver, skeletal muscle, kidney and testis (Kitada 2000). The parkin protein consists of an N-terminal ubiquitin like domain (UBL), a linker region, and a C-terminal region containing two **R**eally **I**nteresting **N**ew **G**ene (RING) domains separated by an in-between RING (IBR) domain. Most PD associated parkin mutations have been localized to the UBL, RING1 or RING2 domains (Marder et al., 2010). It is notable that S-nitrosylation of parkin results in loss of function and this modification has been observed in the brains of patients with idiopathic PD (Chung et al., 2004).

Parkin is an E3 ubiquitin ligase that mediates K48 linked polyqubiquitination, K63 linked ubiquitination, and monoubiquitination. K48 linked polyubiquitination results in 26S Proteasome recognition and degradation of the substrate while K63 linked polyubiquitination results in autophagic degradation of the substrate (Lim, Dawson, & Dawson, 2006). Most parkin mutations that cause AR-JP are located within the UBL, RING1 or RING2 domain and result in the loss of function (Fiesel et al., 2015; Henn, Gostner, Lackner, Tatzelt, & Winklhofer, 2005; Sriram et al., 2005). Most if these mutations inhibit the E3 ubiquitin ligase activity of parkin and because of this, it is thought that parkin loss of function results in decreased ubiquitination of its specific

substrates and corresponding deficits in the degradation of specific proteins and deficits in the mitochondrial quality control pathways that parkin is so integrally involved in. In parkin knockout mice, several parkin substrates including: aminoacyl-tRNA synthase-interacting multifunctional protein type 2 (AIPM2), fuse-binding protein 1 (FBP1) and Paris accumulate, which suggests that parkin mediated polyubiquitination of these proteins is required for their targeting to the 26S proteasome for degradation (Ko, Kim, Sriram, Dawson, & Dawson, 2006; Shin et al., 2011).

Native parkin exists in an auto-inhibited state in which the UBL domain is folded over the RING1 domain where E2 ubiquitin binding would take place. Parkin is recruited to damaged mitochondria by PINK1 which phosphorylates parkin at ser65 of the UBL domain (N. Matsuda & Tanaka, 2010; Narendra et al., 2008). Phosphorylation activates parkin activity by releasing the UBL domain from its folded state resulting in a structure that allows E2 ubiquitin binding (Chaugule et al., 2011; Trempe et al., 2013; Wauer & Komander, 2013; Wauer, Simicek, Schubert, & Komander, 2015). Once in the activated state, parkin ubiquitinates numerous mitochondrial membrane as well as cytosolic proteins (N. C. Chan & Chan, 2011; Geisler et al., 2010; A. Tanaka, 2010; Yoshii, Kishi, Ishihara, & Mizushima, 2011; Ziviani & Whitworth, 2010). Turnover of these proteins requires both parkin and 26S proteasome function. While the proteasome ubiquitin receptors RPN10 and RPN13 bind ubiquitinated proteins, the UBL domain of parkin also binds to these ubiquitin receptors (Aguileta et al., 2015; Dachsel et al., 2005; Sakata et al., 2003). In this manner, binding of parkin to the RPN subunits facilitates 26S proteasome degradation of its ubiquitinated substrates (Um et al., 2010). Upon knockdown of RPN13, parkin is unable to bind to the proteasome causing a significant delay in the clearance of

mitochondrial proteins (TIM23, TIM44, TM20), K48 polyubiquitinated parkin and its ubiquitinated substrates (Aguileta et al., 2015).

Goal of Dissertation and Experimental Paradigm

The overall goals of this dissertation are to characterize parkin-dependent proteasome modulation in central DA neuronal populations and determine if this process contributes to the differential susceptibility of these neurons to neurotoxicant exposure. MPTP is a protoxicant that crosses the blood brain barrier and is oxidized to the DA neuronal specific toxicant, MPP^+. MPTP exposure causes many of the biochemical, pathological and clinical features of PD.

Following MPTP exposure, parkin mRNA and protein expression is increased in resistant TIDA neurons, while parkin mRNA expression is unchanged in susceptible NSDA neurons (M. Benskey et al., 2012). Parkin is an E3 ubiquitin ligase that contains an UBL domain that has been reported to bind to RPN13 of the proteasome 19S regulatory complex and enhance the activity of the 26S proteasome (Um et al., 2010). Parkin dependent proteasome modulation in central DA neuronal populations will be examined by manipulating levels of parkin expression in mice and measuring proteasome activity in tissue lysates or synaptosome extracts prepared from tissue lysates. In several of the studies presented herein, proteasome activity in WT mice was compared to either parkin knockout mice or mice over-expressing parkin. The exon 3 deletion mutation is one of the most common mutations in AR-JP and results in the absence of parkin protein. To this effect, parkin knockout mice were generated (Jackson Labs) by introducing a frame shift in exon three which results in a premature stop codon in exon 4 that prevents the translation of the parkin protein. Over-expression of parkin was achieved using AAV mediated gene transfer of F-parkin (M. J. Benskey et al., 2015).

The effects of MPTP toxicity vary in intensity with the dose, timing, and route of administration. There are many studies in the literature that utilize sub-acute and chronic dosing paradigms. Chronic administration of MPTP to mice results in degeneration of NSDA neurons and long-lasting inhibition of the UPS (Fornai et al., 2005). Although chronic administration of MPTP does not result in the formation of classic Lewy bodies in mice, it does result in the formation of a-synuclein positive aggregates that also contain ubiquitin (Forno, DeLanney, Irwin, & Langston, 1993; Meredith & Rademacher, 2011). The studies described here utilize a single acute MPTP dosing paradigm which allows the comparison of early events that occur following toxicant exposure that might play a role in the protection of TIDA axon terminals. This dosing paradigm results in decreases in striatal DA and TH immunoreactivity (TH-ir), ROS formation, and early ATP depletion and recovery (P. Chan, DeLanney, Irwin, Langston, & Di Monte, 1991; Chiba et al., 1984; Jackson-Lewis, Jakowec, Burke, & Przedborski, 1995; Lotharius & O'Malley, 2000). Observation of the events that occur following a low dose and short time course of a single injection is crucial to understanding the mechanism that renders some populations of DA neurons vulnerable to MPTP toxicity while others are resistant.

The low exposure dose and short time course following the single acute MPTP exposure paradigm results in initial dysfunction, such as loss of TH and displacement of vesicular DA, in both NSDA and TIDA axon terminals. However, TIDA neurons adapt and recover while NSDA neurons do not (Behrouz et al., 2007; Jackson-Lewis et al., 1995). In both mice and monkeys as well as PD post mortem brains, the loss of TH in NSDA neurons consistently precedes degeneration (Jackson-Lewis et al., 1995; Kastner et al., 1994; Kastner, Hirsch, Herrero, Javoy-Agid, & Agid, 1993). Therefore, axon terminal TH and DA concentrations serve as indices of impending neuronal degeneration. Examination of the early changes in these DA phenotypic

markers, that predict DA neuronal susceptibility, and comparison of these events to those in resistant TIDA neurons, will allow the characterization of the effects of differential parkin expression and the resulting effect on proteasome activity within these neuronal populations.

Summary

PD is a neurodegenerative disease with debilitating motor symptoms that are the consequence of the progressive loss of NSDA neurons. While there is severe loss of NSDA neurons, TIDA neurons of the mediobasal hypothalamus are not affected. A similar pattern of NSDA susceptibility and TIDA resistance occurs in a MPTP model of DA neuronal toxicity. Corresponding with the time frame of recovery of DA stores in TIDA neurons following MPTP exposure, expression of parkin mRNA and protein increases in a dose and time dependent manner in the Arc. However, parkin mRNA and protein expression remains unchanged in the SN. AAV mediated knockdown of parkin expression attenuates the ability of TIDA neurons to recover from this neurotoxic insult. Parkin is an E3 ubiquitin ligase that acts to covalently tag proteins with ubiquitin so that they may be recognized and degraded by the proteasome. Parkin contains a C-terminal RING-IBR-RING motif that is responsible for E3 ligase activity and a UBL domain necessary to bind to the 19S regulatory complex and enhance the activity of the 26S proteasome. Increased parkin expression within TIDA neurons following neurotoxic insult suggests that these DA neurons may have the ability to maintain proteostasis through the up-regulation of parkin expression. The overall goals of this dissertation are to characterize parkin-dependent proteasome modulation in central DA neuronal populations and determine if this process contributes to the differential susceptibility of these neurons to neurotoxicant exposure.

Thesis Objective

The studies described in this dissertation were developed to test the central hypothesis that increased parkin expression in TIDA neurons confers protection from oxidative insult by increasing proteasome activity and that this process contributes to the differential susceptibility of these neurons to neurotoxicant exposure. To this end, the following specific aims were designed to test this hypothesis:

1) Characterization differential proteasome activity in the ST and ME of vehicle and MPTP treated mice.

Hypothesis: Following MPTP exposure, proteasome activity is impaired in brain regions containing the axon terminals of NSDA neurons, but is sustained in regions containing TIDA neurons.

Null Hypothesis: Following MPTP exposure, proteasome activity is not different in regions containing NSDA or TIDA terminals.

2) Determine if parkin expression is necessary to maintain proteasome activity in TIDA neurons following MPTP exposure.

Hypothesis: Experimental alterations in parkin expression will produce corresponding changes in proteasome activity and susceptibility of TIDA neurons in response to MPTP exposure.

Null Hypothesis: Experimental alterations in parkin expression do not produce a change in proteasome activity.

3) *Determine if over-expression of hParkin in NSDA perikarya in the SN confers resistance to NSDA axon terminals in the ST after MPTP exposure*.

Hypothesis: hParkin over-expression in the SN is sufficient to maintain proteasome activity and protect NSDA axon terminals from neurotoxicant insult.

Null Hypothesis: hParkin over-expression in the SN is not sufficient to maintain proteasome activity and protect NSDA axon terminals from neurotoxicant insult.

The following chapters describe the research performed to address the above specific aims of this thesis. **Chapter 2** contains details for the Materials and Methods used for all of the studies described. **Chapters 3-5** describes findings as they relate to the Specific Aims of the thesis. **Chapter 6** provides a General Discussion of this research as it relates to previous findings and the information from the literature.

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Chapter 2. Material and Methods

Animals

All experiments were conducted in 8 to12 week old, male, C57BL/6J mice (Jackson Laboratories, Bar Harbor, MA) or in house homozygous parkin ($Park2^{tm1Shn}$) knockout (KO) mice (Goldberg et al., 2003). Purchased animals were acclimated for one week in our animal care facility before experiments were conducted. Mice were randomly assigned to treatment groups, and housed two to five animals per Optimice® cage (Animal Care Systems, Centennial, CO), maintained in a light-controlled (12 h light/dark cycle; lights on 0600 h) temperature-controlled (22 ±1°C) room, with standard lab chow and tap water provided *ad libitum*. The Michigan State University Institutional Animal Care and Use Committee approved all experiments using live animals (AUF 10/14 183-00).

Drugs and Administration

Unless otherwise stated, all drugs were purchased from Sigma-Aldrich (St. Louis, MO). All drug injections were administered at 0.01 mL/g of body weight.

Single Acute MPTP

1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) purchased from Santa Cruz Biotechnology (Dallas, Texas) was dissolved in 0.9% sterile saline in a crimped amber vial at a freebase concentration of 2 mg /mL. The solution was vigorously vortexed and placed on ice. Mice received a single injection of either saline (0.01 mL/g; s.c.) or MPTP (20 mg/kg; s.c.) and the experiment was terminated at 2, 4, 6, 8, 12, 16 or 24 h after the MPTP injection. Saline

injected control mice were sacrificed 2 h post injection and used as zero time controls for time course studies. In all other cases, all mice were sacrificed either 2 or 24 h after injection depending on the endpoint of the experiment.

NSD-1015

3-hydroxybenzylhydrazine (NSD-1015) was dissolved in 0.9% sterile saline in a crimped amber vial at a freebase concentration of 10 mg /mL. The solution was vigorously vortexed and placed on ice. Mice were treated with NSD-1015 (100 mg/kg; i.p.) 30 min prior to sacrifice.

Levodopa/Benserazide

3,4-dihydroxy-L-phenylalanine (Levodopa) was dissolved in 0.9% sterile saline in a crimped amber vial at a freebase concentration of 2.5 mg/mL with the peripheral DOPA decarboxylase inhibitor, benserazide, at a freebase concentration of 1.25 mg/mL. The solution was vigorously vortexed and placed on ice. Levodopa (25 mg/kg; i.p.) and benserazide (12.5 mg/kg; i.p.) were co-administered 1 h prior to the start of the treadmill session for each mouse.

Ketamine/Xylazine

Ketamine (100 mg/mL Ketathesia^{TM,} Butler Schein Animal Health, Dublin, OH) was diluted to 12 mg/mL and xylazine was diluted to 1.8 mg/mL in 0.9% sterile saline. For all surgical procedures mice were anesthetized using an i.p. injection of 120 mg/kg ketamine and 18 mg/kg xylazine.

Tissue Preparation

For biochemical analyses, mice were sacrificed by decapitation and the brains were quickly removed and placed on an ice cooled glass stage. Under a dissecting microscope, the ME was collected using forceps and iridectomy scissors. The location of the ME on the ventral side of the brain is demonstrated in **Figure 2-2A**. The remaining brain was placed on dry ice on its rostral-caudal axis so that the olfactory bulb pointed upward. Frozen coronal sections (500 μm) containing the DA cell body and terminal regions of interest were collected using a cryostat set at -10°C (CTD-Model Harris, International Equipment Co., Needham, MA). Each section was thaw mounted onto glass slides and refrozen on dry ice. The regions of interest were micro-dissected using a modification of the method previously described (Palkovits, 1973, 1983). **Figures 2-1** and **2-2** demonstrate the locations in the brain were the tissue was collected. Bilateral tissue samples were collected from the ST (**Figure 2-1A**) with a round 18g micropunch and from the SN (**Figure 2-1B**) with an oval 21g micropunch. Samples of the ARC were collected from two consecutive sections (Figure 2-2B) using a round 18g micropunch. The tissue samples that were collected were used for all biochemical assays and were processed for each type of analysis as described below.

For immunohistochemical (IHC) analysis, mice were anesthetized with ketamine/xylazine (26.6 mg/kg ketamine and 4 mg/kg xylazine; i.p.). Once anesthetized, each mouse was transcardially perfused with 0.9% saline followed by 4% paraformaldehyde. The brains were post-fixed in 4% paraformaldehyde for 24 h and then cryoprotected in 30% sucrose for no less than 48 h. Coronal sections (20 μm) were prepared with a cryostat (-19°C) and sections were free floated in 0.05 M phosphate buffer containing 0.01% sodium azide.

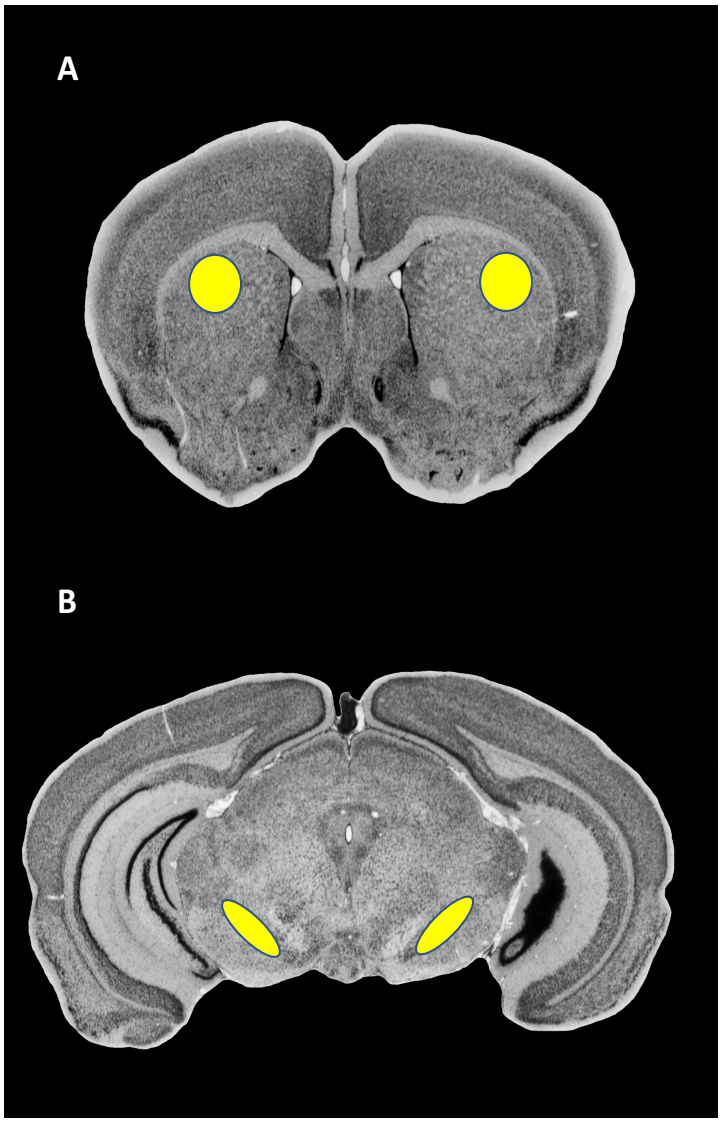

Figure 2-1 Coronal brain sections showing the localization of the micropunches used to dissect the ST and SN. The yellow circles and ovals represent the location of the micropunches. The ST was dissected from a 500 µm section about 1.0 mm rostral to Bregma using an 18g round micropunch. The SN was dissected from a 500 µm section about 3.1 mm caudal to Bregma using a 21g oval micropunch. Modified from the Mouse Brain Library Atlas (http://www.mbl.org).

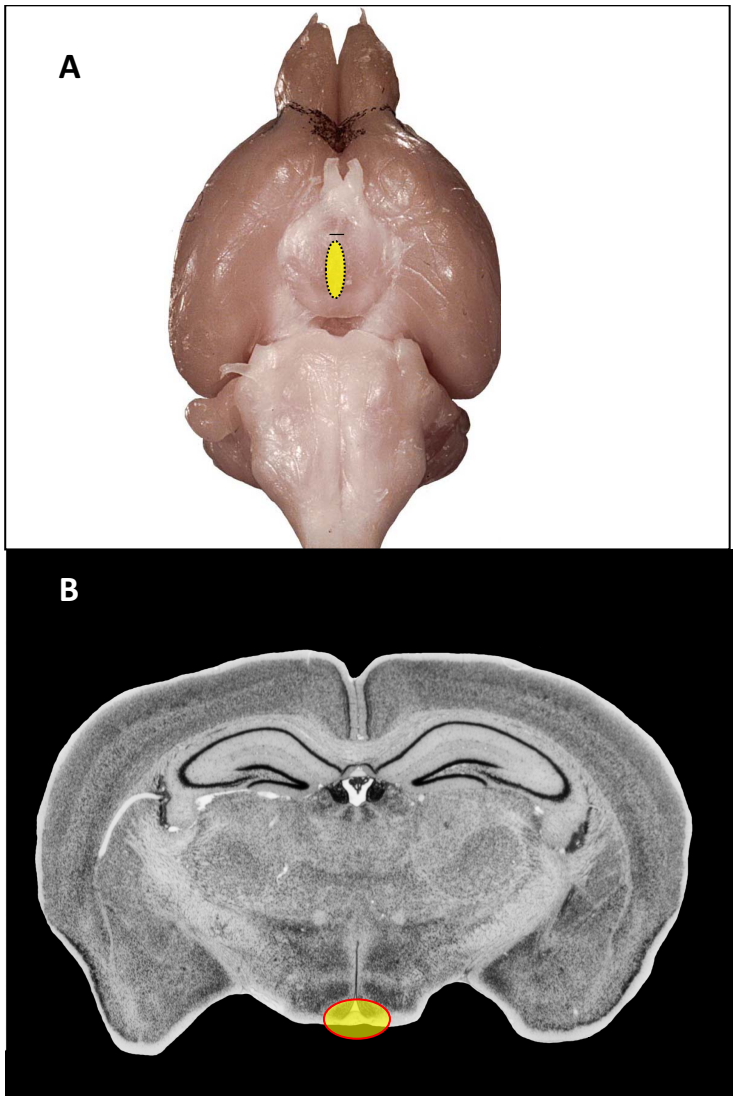

Figure 2-2 Coronal brain sections showing the localization of the ME dissection and ARC micropunch. The yellow ovals represent the location of the microdissection (ME) or micropunch (ARC). The ME was dissected from the ventral side of the brain. The ARC was dissected from two consecutive 500 μm sections at 1.58 mm and 2.06 mm caudal to Bregma. Modified from the Mouse Brain Library Atlas (http://www.mbl.org).

Neurochemical Analyses

Microdissected brain tissue samples were placed into cold tissue buffer (0.1M phosphate-citrate buffer, pH 2.5) and sonicated with three 1 sec bursts (Heat Systems Ultrasonics, Plainview, NY). The samples were subjected to centrifugation at 12,000 X g (Beckman Coulter Microfuge, Palo Alto, CA) for 1 min. The content of DA, DOPAC, DOPA, HVA, 3-MT and DOPA in the supernatants was determined with high pressure liquid chromatography coupled with electrochemical detection (HPLC-ED) using a Waters 515 HPLC pump (Waters Corporation, Milford, MA) and an ESA Coulochem 5100A electrochemical detector with an oxidation potential of +0.4V. Neurochemical standards and experimental samples were injected onto a C18 reverse phase analytical column (Bioanalytical Systems, West Lafayette, IN). The HPLC mobile phase (0.5M sodium phosphate, 0.03M citrate, 0.1mM EDTA, 0.03% sodium octylsulfate, 10-15% methanol, pH 2.65) was adjusted by altering concentrations of SOS, methanol and the pH to optimize neurotransmitter peak resolution. Neurochemical content was quantified by comparing the peak heights of each sample to the peak heights of standards of each neurochemical measured.

Tissue pellets were resuspended in 1N NaOH and assayed for protein using the bicinchoninic acid (BCA) protein assay (Walker, 1994). To account for differences in sample size, the neurochemical content was normalized to the amount of protein in each sample and expressed as a concentration of neurochemical in ng per mg protein.

Synaptosome Lysate Preparation

Mice were decapitated 24 h following treatment and the brain was immediately removed. The region containing the ST was blocked using a razor blade in an ice cold stainless steel

matrix; rostrally at the olfactory bulb, and caudally at the optic chiasm. The thick coronal section was then placed on an ice chilled glass stage and the cortical layer was peeled away, leaving only the ST. The ventral ST was cut away just above the anterior commissure. The dorsal ST was placed in a pre-chilled 2 mL Dounce homogenizer containing ice cold isolation buffer (IB; 300 mM sucrose, 10 mM HEPES, 1 mM EGTA, pH 7.4 at 4 ˚C). The tissue was homogenized using 10 even strokes with a 1 min rest after the fifth stroke (one stroke equals one up and down movement with each stroke taking about 5 sec). The homogenate was transferred to pre-chilled 2 mL Eppendorf tubes. The homogenate was centrifuged at 1000 X g for 5 min at 4 ˚C. The supernatant was collected (S1) and further centrifuged for 10 min at 21,000 X g at 4 ˚C. The loose synaptosome pellet was diluted in proteasome assay buffer (10 mM Tris-HCL, pH 8.0 containing 2 mM ATP-Mg^{2+}, 1 mM DTT, and 0.1% Triton X-100) and sonicated for 20 sec to lyse the synaptosomes. The lysate was subjected to centrifugation at 14,000 x g for 10 min at 4 ˚C to remove any debris. The supernatant was assayed for protein using the BCA assay and then used to measure proteasome activity or for Western blot.

Proteasome Peptidase Activity Assays

Microdissected brain tissue samples were placed into cold proteasome assay buffer (10 mM Tris-HCL, pH 8.0 containing 2 mM ATP-Mg^{2+}, 1 mM DTT, and 0.1% Triton X-100) and sonicated for 20 s in an iced water bath. The samples were subjected to centrifugation at 14,000 x g (Beckman Coulter Microfuge, Palo Alto, CA) for 10 min to remove debris. The supernatant was assayed for protein using the BCA assay.

The fluorogenic substrates (2 μL, 100 μM) Suc-LLVY-AMC, Z-LLE-AMC and Z-ARR-AMC (Boston Biochem, Cambridge, MA) were used to measure the chymotrypsin-like (CT-L),

caspase-like (Cas-L) and Trypsin-like (T-L) activity within the tissue lysate. A total of 1 μg of protein (2 μL of diluted extract in proteasome assay buffer) was added to 16 μl of assay buffer containing 100 μM substrate in a black 384-well plate. Each peptidase activity was determined by monitoring the fluorescence caused by the release of aminomethylcoumarin using a Tecan Infinite M1000 Pro multi-well plate reader (Tecan, Männedorf, Switzerland) at an excitation wavelength of 380 nm and emission wavelength of 460 nm, with the bandwidth set at 5 nm. Fluorescence was measured at 37 ˚C every min over a period of 30 min and the maximum increase in fluorescence per second (FU/sec) was used to calculate total peptidase activities of each sample over the linear portion of the curve. Two measurements from each sample were made in triplicate: one with the addition of 100 nM of the specific proteasome inhibitor, bortezomib, and one with vehicle. Specific proteasome activity was calculated by subtracting the values obtained from the assays with inhibitor (non-proteasome related background activity) from the total activity (no inhibitor). Proteasome activity across different extracts was normalized to the amount of protein present in the assay well.

Native Gel Electrophoresis, in Gel Proteasome Assay and Corresponding Western Blot

Microdissected brain tissue samples were placed into cold proteasome assay buffer (10 mM Tris-HCL, pH 8.0 containing 2 mM ATP-Mg^{2+}, 1 mM DTT, and 0.1% Triton X-100) and sonicated for 20 s in an iced water bath. The samples were subjected to centrifugation at 14,000 x g (Beckman Coulter Microfuge, Palo Alto, CA) for 10 min to remove debris. The supernatant was assayed for protein using the BCA assay.

Protein extract (50 μg protein) in 1X loading dye (80 mM Tris-Cl pH 8.3, 45mM boric acid, 1mM EDTA, 20% glycerol, 0.04% bromophenol blue) were electrophoresed in a 4% native

polyacrylamide gel containing 2 mM ATP-Mg^{2+} in an ice water bath at 20 mA (c) until the loading dye ran off the gel. The running buffer was ice cold 1X TBE (80 mM Tris-Cl pH 8.3, 45 mM boric acid, 1mM EDTA).

The gel was equilibrated in 10 mM Tris-Cl, pH 8.0 and then incubated in 10 mM Tris-Cl, pH 8.0 containing 2 mM ATP- Mg^{2+} and 100 μM Suc-LLVY-AMC. The gel was incubated at 37° C for 30 min and liberated AMC was visualized with a handheld UV lamp (215 nm) and photographed using. Relative activity of the 26S and 20S bands was determined using ImageStudio software. The density of each band was determined by measuring the signal to area of each band.

To determine the relative amount of 26S and 20S protein complexes, the gel was transferred to PVDF membrane (Millipore, Pittsburgh, MA) using a semidry transfer apparatus at 15 V for 30 min. The membrane was air dried for 1 h to allow the proteins to bind to the membrane. The membrane was stained with Ponceau S and the stained proteins were used as the native loading control. The membrane was de-stained with 0.1 N NaOH and washed three times in Tris buffered saline (TBS) for 5 min before it was blocked in 5% nonfat milk in TBS for 1 h. The membrane was incubated in TBST (Tris buffered saline, 0.1% Tween 20) containing 1:2000 rabbit anti-PSMB5 antibody overnight at 4° C.

Following primary antibody incubation, the membrane was washed three times in TBST and incubated for 1 h at room temperature in TBST containing 5% milk and 1:5000 dilution of horseradish peroxidase (HRP) conjugated goat anti-rabbit antibody. The membrane wash washed four times in TBST and then rinsed in water before adding luminol substrate (Super Signal West Femto Maximum Sensitivity Substrate, Thermo Scientific) to the blot and imaging with the Odyssey-FC imager (LiCor Biosciences). The signal to area of each band was measured using

Image Studio software. The relative amounts of 26S and 20S complex was normalized using the density of the corresponding Ponceau S stained lane.

Denaturing Western Blots

Microdissected brain tissue samples were placed into cold RIPA buffer (10 mM Tris-Cl pH 8.0, 1 mM EDTA, 0.5 mM EGTA, 1% Triton X-100, 0.1% sodium deoxycholate, 0.1% SDS, 140 mM NaCl, 1 mM PMSF, 1X Halt protease phosphatase inhibitor cocktail) and sonicated for 20 s in an iced water bath. The samples were subjected to centrifugation at 14,000 x g (Beckman Coulter Microfuge, Palo Alto, CA) for 10 min at 4° C to remove debris. The supernatant was assayed for protein using the BCA assay. Protein (10 μg) from each sample was diluted in 1X Laemmli sample buffer (62.5 mM sodium phosphate pH 7, 100 mM DTT, 10% glycerol, 2 % SDS, 0.01% bromophenol blue) and boiled for 5 min before loading onto a 4-20% Tris-TGX polyacrylamide gel (BioRad, Hercules, CA). The gel was run in 1X Tris-Glycine running buffer (25 mM Tris, 192 mM glycine, 0.1% SDS, pH8.3) at 150 V until the dye ran off the bottom of the gel.

The gel was equilibrated in Towbin transfer buffer (25 mM Tris, 192 mM glycine, pH8.3, 20% methanol) and transferred to PVDF membrane (Millipore, Pittsburgh, MA) using a semidry transfer apparatus at 15 V for 30 min. The membrane was air dried for 1 h to allow the proteins to bind to the membrane before it was rewet in methanol, rinsed in TBST and blocked in 5% nonfat milk in TBST for 1 h. The membrane was then incubated in primary antibody in 5% BSA/TBST overnight at 4° C. The source, dilution and company supplying each primary antibody used are shown in **Table 2-1**.

Primary Antibody	Dilution	Source	Company
Dinitrophenol, DNP	1:10000	Rabbit	Cell Signaling (14681)
GAPDH	1:5000	Mouse	Sigma (G8795)
GFP	1:1000	Rabbit	Life Technologies (A11122)
K48 Ubiquitin	1:500	Rabbit	Cell Signaling (4289)
K63 Ubiquitin	1:500	Rabbit	Cell Signaling (12930)
Ubiquitin	1:500	Rabbit	Cell Signaling (3933)
Nitrotyrosine	1:1000	Rabbit	Millipore (06-284)
Parkin	1:1000	Rabbit	Cell Signaling (2132)
PSMB5	1:1000	Rabbit	Cell Signaling (12919)
RPN10	1:1000	Rabbit	Cell Signaling (3846)
TH	1:2000	Rabbit	Millipore (AB152)
TH	1:2000	Sheep	Millipore (AB1542)
pSer19-TH	1:1000	Rabbit	Sigma (P2247)
pSer40-TH	1:1000	Rabbit	Cell Signaling (2791)

Table 2-1 Description of primary antibodies. Antibody list describes the standard dilution used for immunoblotting or immunohistochemistry, the animal the antibody was derived from (source) as well as the company and catalog number from which the antibody was purchased. Abbreviations: DNP, dinitrophenol; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GFP, green fluorescent protein; K48, lysine 48 branched chains; K63, lysine 63 branched chains; PSMB5, proteasome subunit beta 5, RPN10, regulatory particle number 10; TH, tyrosine hydroxylase.

Secondary Antibody	Dilution	Source	Company
Anti-Mouse IgG-HRP	1:5000	Horse	Cell Signaling (7076)
Anti-Rabbit IgG-HRP	1:5000	Goat	Cell Signaling (7074)
Anti-Sheep IgG-HRP	1:10000	Donkey	Sigma (AB324P)
Cy3 conjugated Anti-Rabbit	1:500	Goat	Jackson Immunoresearch (111-165-003
Alexa-488 Anti-Sheep	1:500	Donkey	Life Technologies (A11055)

Table 2-2 Description of secondary antibodies. Antibody list describes the standard dilution used for immunoblotting or immunohistochemistry, the animal the antibody was derived from as well as the company and catalog number describing where the antibody was purchased.

Following primary antibody incubation, the membrane was washed three times in TBST and incubated for 1 h at room temperature in TBST containing 5% milk and 1:5000 dilution of HRP conjugated secondary antibody. The source, dilution and company supplying each secondary antibody used are shown in **Table 2-2**. The membrane was washed four times in TBST and then rinsed in water before adding luminol substrate (Super Signal West Femto Maximum Sensitivity Substrate, Thermo Scientific) to the blot and imaging with the Odyssey-FC imager (LiCor Biosciences). The signal to area of each band was measured using Image Studio software. The relative amount of each band was normalized to the amount of GAPDH in each lane.

Immunoprecipitation

Microdissected brain tissue samples were placed into cold RIPA buffer (10 mM Tris-Cl pH 8.0, 1 mM EDTA, 0.5 mM EGTA, 1% Triton X-100, 0.1% sodium deoxycholate, 0.1% SDS, 140 mM NaCl, 1 mM PMSF, 1X Halt protease phosphatase inhibitor cocktail) and sonicated for 20 s in an iced water bath. The samples were subjected to centrifugation at 14,000 x g (Beckman Coulter Microfuge, Palo Alto, CA) for 10 min at 4° C to remove debris. The supernatant was assayed for protein using the BCA assay. Tissue lysate (1 mg) was precleared with 30 μL ProteinA agarose bead slurry for 30 min at 4°C. Antibody was added to the precleared lysate (1 μg antibody per mg protein) and the solution was rotated at 4°C overnight. The solution was washed 4 times in 500 μL RIPA buffer by centrifugation at 14,000 x g for 30 s each wash. The final antigen-antibody-bead conjugate was diluted in 20 μL sample buffer and 15 μL was run on a 4-20% denaturing Tris-TGX gel. The gel was transferred to PVDF and was subjected to Western blot for TH, K48 ubiquitin, K63 ubiquitin and nitrotyrosine.

Immunohistochemistry

Fluorescent immunohistochemistry was used to visualize the location and spread of viral vector mediated expression. Free-floating sections were washed in 0.05 M phosphate buffer containing 0.1% Triton X-100 (PB-TX) and incubated overnight in primary antibody. The source, dilution, and company supplying each primary antibody are shown in **Table 2-1**. The sections were washed in PB-TX three times for 5 min each and incubated in secondary antibody for 1 h at room temperature in the dark. The source, dilution, and company supplying each secondary antibody are shown in **Table 2-2**. Following secondary incubation, the sections were washed in 0.05 M phosphate buffer, mounted onto slides and cover-slipped using ProLong Gold antifade reagent (Molecular Probes, Eugene, OR). Sections were viewed on a Nikon TE-2000-U-Fluorescence Microscope using Metamorph Software (Melville, NY).

Stereotaxic Surgery

Mice received a unilateral injection to the right, ipsilateral, SN of rAAV2/5-F-Parkin (3 X 10^{12} vg/mL) and a unilateral injection to the left, contralateral SN, of rAAV2/5-GFP. Viral vector was engineered by a previous laboratory member, Dr. Matthew Benskey, and packaged by Dr. Frederic Manfredsson (Benskey, Manfredsson, Lookingland, & Goudreau, 2015). Analgesic (ketoprofen, 5 mg/kg, s.c.) was administered prior to surgery. The mice were anesthetized using 120 mg/kg ketamine and 18 mg/kg xylazine and the surgical site was shaved. The animals were placed in a stereotaxic frame and the surgical site was scrubbed three times with alcohol and then betadine. A single incision was made along the rostrocaudal axis of the skull and the tissue overlying the skull was retracted to expose the skull surface. A Hamilton syringe with a 30 gauge blunt tip needle was fitted with parafilm, and then a siliconized pulled borosilicate glass

micropipette with an opening of 60-80 μm was placed over the parafilm. The parafilmed needle was heated to melt the parafilm and form a seal. Unilateral SN injections of 500 nL of rAAV2/5–F-parkin or rAAV2/5-GFP were performed using the following coordinates from Bregma: R/C -3.0 mm, L/M -1.6 mm and D/V -4.6 mm from the skull. **Figure 2-3** depicts the locations of the rAAV2/5-GFP and rAAV2/5-F-Parkin injections in the SN. The volume was injected at a rate of 125 nL/min using an automated micropump (UltraMicroPump, World Precision Instruments). The needle was left in place for an additional 5 min to prevent reflux and then slowly withdrawn. The hole in the skull was filled with sterile bone wax and the skin was closed using surgical staples. For identification, ears were marked using an ear punch. Mice were kept on a heating pad until recovery from anesthesia and then returned to their home cages. Mice were given ketoprofen, for pain, 24 h following surgery and checked daily for signs of infection or distress. Staples were removed 10-14 d following surgery. rAAV-treated mice were housed for 4 weeks prior to experiments.

Behavioral Analyses

Gait dynamics for adult 10-16 week old male C57BL/6J and $Park2^{tm1Shn}$ homozygous parkin KO mice ($Park2^{-/-}$) mice were assessed using the DigiGait treadmill and software (Mouse Specifics). Mice were acclimated to the static treadmill, the enclosed acrylic compartment, and lights for 10 min prior to gait analysis. Ventral plane videography captured the gait of each mouse through a transparent treadmill belt over approximately 6 s (Amende et al., 2005; Kale, Amende, Meyer, Crabbe, & Hampton, 2004). Digital images of the paws of each mouse were taken at 150 frames/s as mice ran at a speed of 25 cm/sec with no incline or decline. Each mouse was tested individually on the treadmill, in an enclosed acrylic compartment (5 cm in width, 25

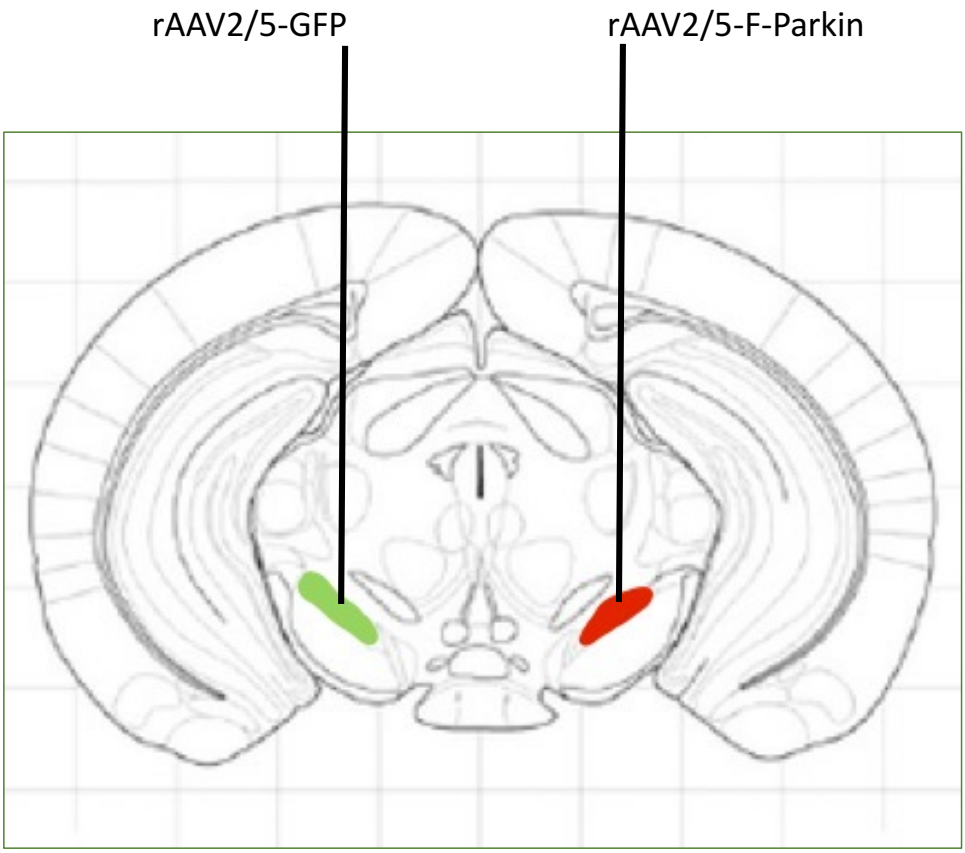

Figure 2-3 Localization of ipsilateral and contralateral stereotaxic injections into the SN. Mice received 500 nL unilateral injection to the right, ipsilateral SN of 3.4 X 10^{12} vg rAAV2/5-F-Parkin (red) and a unilateral injection to the left, contralateral SN of 1 X 10^{12} vg rAAV2/5-GFP (green) at the following coordinates from Bregma: R/C -3.0, M/L -1.6 or +1.6, D/V: -4.6. Modified from Paxinos and Watson (2003).

cm in length). Temporal and spatial measurements were indicated by the area of the paw relative to the treadmill belt at each frame. The following gait parameters were measured: swing time, % swing stride, brake time, % brake stride, propel time , % propel stride, stance, % stance stride, stride time, % brake stance, % propel stance, stance/swing, stride length, stride frequency, paw angle, absolute paw angle, paw angle variability, stance width, step angle, stride length variability, step angle variability, stride length CV, stance width CV, step angle CV, swing duration CV, paw area at peak stance, paw area variability at peak stance, hind limb shared stance time, % shared stance, stance factor, gait symmetry, maximal dA/dT, minimal dA/dT, Tau propulsion, overlap distance, paw placement positioning, ataxia coefficient, midline distance, axis distance, and paw drag.

Statistical Analyses

SigmaPlot was used to conduct power analyses to determine the sample size required for each experiment. The analyses were based on an α of 0.05 for all planned comparisons and the expected standard error of measurement for each endpoint. For proteasome activity, a sample size of 10 was required to detect a 15% difference with >80% power between the saline and MPTP treated groups. For neurochemical endpoint, a sample size of 8 per group was sufficient to obtain power > 80% and detect a difference of 15% between saline and MPTP treated groups. Mice were assigned random numbers during treatment allowing the experimenter to remain blind to experimental conditions during data collection and analysis.

GraphPad software was used to determine significant differences between groups. If all statistical assumptions were met, a one-way analysis of variance (ANOVA) was used to detect statistical significance between two or more groups with a single independent variable. Two-way

ANOVA was used to detect significant differences between two or more groups when there were two independent variables in the study. In experiments were there was an internal control a repeated measures ANOVA was used to detect significant differences. Statistical differences were considered significant when the p value was less than or equal to 0.05. When the ANOVA revealed a significant interaction, a post-hoc Holm-Sidak analysis was used to determine which groups were different.

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Chapter 3. TIDA and NSDA axon terminals are differentially susceptible to a single acute MPTP exposure

Introduction

The motor symptoms of PD are primarily caused by the chronic and progressive degeneration of NSDA neurons of the SNc. Loss of these neurons results in decreased DA levels within the ST (Ehringer and Hornykiewicz 1960) and deregulation of the basal ganglia circuitry, which normally allows the execution of voluntary movement. While there is severe loss of NSDA neurons, not all dopaminergic neurons are susceptible in PD. Another population of DA neurons, the TIDA neurons in the mediobasal hypothalamus are not affected in this disease (Langston and Forno 1978). The molecular basis of this differential susceptibility remains unclear. An increased understanding of the molecular pathways involved in the differential susceptibility between these neuronal populations could lead to the identification of new targets that could be exploited for the development of neuroprotective strategies and the treatment of PD.

The exact cause of PD is unknown. However, in both familial and sporadic disease, oxidative stress appears to be an underlying mechanism that leads to NSDA neurodegeneration. PD is associated with increased DA turnover (de la Fuente-Fernandez et al. 2001; de la Fuente-Fernandez et al. 2004; Sossi et al. 2006), mitochondrial dysfunction (Mizuno et al. 1989; Schapira 1993), neuroinflammation (McGeer et al. 1988; Bartels et al. 2010; Gerhard et al. 2006), increased iron (Dexter et al. 1989; Sofic et al. 1991; Riederer et al. 1992; Double et al. 2000), and impairment of the UPS (McNaught et al. 2002; Olanow and McNaught 2006; McNaught et al. 2006; Bukhatwa et al. 2010; Chang et al. 2016; Martins-Branco et al. 2012), all

of which favor the increased production of ROS and their resulting damage.

Oxidative modification of proteins can result in conformational changes and misfolding that render the protein inactive and promote the formation of toxic protein aggregates (Mirzaei and Regnier 2008). In this respect, cellular homeostasis is dependent upon the removal of oxidatively damaged proteins. The UPS plays a pivotal role in the selective recognition and degradation of these oxidized proteins (Chung, Dawson, and Dawson 2001; Ross and Pickart 2004; Ciechanover 2005; Lehman 2009). Dysfunction of the UPS can result in the accumulation and aggregation of misfolded proteins, which can further impair catalytic activity within the neuron. Proteasome activity is reduced in susceptible brain regions in PD patients (McNaught et al. 2002; Bukhatwa et al. 2010; McNaught and Jenner 2001; Martins-Branco et al. 2012) and restoration of proteasome activity could be an important therapeutic avenue in the treatment of neurodegenerative diseases.

The neurotoxicant MPTP produces oxidative stress in DA neurons and can replicate the pattern of DA neuronal susceptibility that is seen in PD (Behrouz et al. 2007; Benskey et al. 2012; Benskey et al. 2013; Melamed et al. 1985; Mogi et al. 1988). While both neuronal populations initially respond to the neurotoxicant, TIDA neurons recover from the initial insult, while NSDA neurons do not (Benskey et al. 2012). Recovery of TIDA neurons following MPTP exposure is dependent upon increased expression of parkin mRNA and protein (Benskey et al. 2012). Furthermore, rAAV mediated shRNA knockdown of parkin in the ARC containing cell bodies of TIDA neurons results in susceptibility of these neurons to MPTP (Benskey, Kuhn, et al. 2015). Taken together, it appears that parkin expression is necessary to protect TIDA neurons from neurotoxicity induced by exposure to MPTP. This is important because NSDA neurons rapidly degenerate in patients with loss of parkin function due to homozygous mutations (Kitada

et al. 1998; von Coelln, Dawson, and Dawson 2004) and in patients with sporadic PD, the ventral midbrain, which contains NSDA neurons, has reduced parkin expression (Kitada et al. 1998).

Parkin is an E3 ubiquitin ligase that promotes the degradation of its specific protein substrates via the UPS (Shimura et al. 2000; Olzmann and Chin 2008) and autophagy (Olzmann and Chin 2008; Narendra et al. 2008). In addition to its E3 ligase function, the N-terminal UBL domain of parkin directly interacts with proteasome subunits Rpn10 (Sakata et al. 2003), RPN13 (Aguileta et al. 2015), Rpt6 (Tsai et al. 2003), and α4 (Dachsel et al. 2005) and directly enhances proteasome activity in cell culture (Um et al. 2010; Hyun et al. 2002; Dachsel et al. 2005). **The studies presented here are designed to test the hypothesis that following MPTP exposure, proteasome activity is impaired in brain regions containing the terminals of NSDA neurons where there is loss of parkin protein, and is sustained in regions containing TIDA terminals where parkin expression is increased.** In order to test this hypothesis mice were injected with a single acute dose of MPTP (20 mg/kg, s.c.) or saline (10 mL/kg, s.c.) and were decapitated 24 h post injection. Regional differences in DA, TH, parkin expression, protein carbonylation, proteasome activity, and consequences of proteasome impairment were characterized in the ME and ST.

Results

Differential Responses of Phenotypic Markers in the ST and ME Following a Single Injection of MPTP

A single acute exposure to MPTP results in sustained depletion of DA vesicular storage pools in NSDA axon terminals, while vesicular DA is recovered in TIDA axon terminals

(Benskey et al. 2012; Benskey et al. 2013). DOPAC and DA concentrations were measured in the ST (location of NSDA axon terminals) and ME (location of TIDA axon terminals) 24 h following a single acute MPTP exposure (**Figure 3-1**). The amount of DA in the ST of MPTP treated animals was decreased compared to the saline controls, while the amount of DA in the ME was not different compared to the saline treated controls 24 h post MPTP (**Figure 3-1A**). The unchanged DA in the ME following acute neurotoxicant injury reflects the recovery of terminal DA stores, consistent with prior reported findings (Benskey et al. 2012; Benskey et al. 2013). Striatal DOPAC concentrations decreased in MPTP treated animals compared to saline treated controls, as did DOPAC concentrations in the ME (**Figure 3-1B**). A single acute exposure to MPTP increased the DOPAC to DA ratio in the ST (**Figure 3-1C**), which reflects an increased rate of release, reuptake and metabolism of DA relative to vesicular storage in axon terminals of NSDA neurons (Lookingland and Moore 2005). Conversely, the DOPAC to DA ratio was decreased in the ME, which is consistent with decreased TIDA neuronal activity following MPTP exposure.

Loss of TH in the soma and terminals of NSDA neurons occurs after MPTP exposure and may be an early indicator of the inability of these neurons to recover (Muroyama, Kobayashi, and Mitsumoto 2011) (Benskey et al. 2012; Ara et al. 1998; Jackson-Lewis et al. 1995; Kastner et al. 1994). To determine if there is differential expression of TH in the ST and ME 24 h following MPTP exposure, TH was measured by Western blot. **Figure 3-2A** demonstrates that 24 h following a single acute MPTP injection, TH protein is decreased in the ST and unchanged in the ME. Since the amount of TH was decreased in the ST and maintained in the ME, the relative change in TH activity was measured 24 h post MPTP exposure and 30 min following the administration of the AADC inhibitor, NSD-1015.

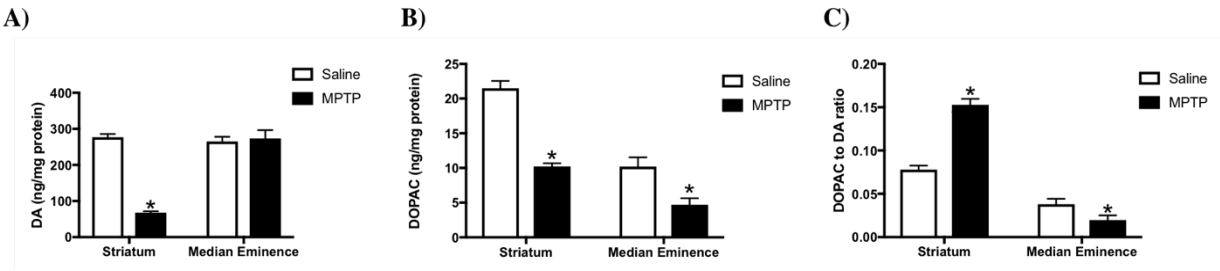


Figure 3-1 Effect of MPTP on DA and DOPAC concentrations in the ST and ME. C57BL/6J mice were treated with 20 mg/kg MPTP. Twenty-four h later, the ST and ME were collected and analyzed for DA, and DOPAC by HPLC-ED. The bars represent the mean concentration of (**A**) DA, (**B**) DOPAC or (**C**)the DOPAC to DA ratio. Error bars represent + 1 SEM. * indicates the treatment was significantly different ($P < 0.05$, n=8) from the corresponding vehicle control.

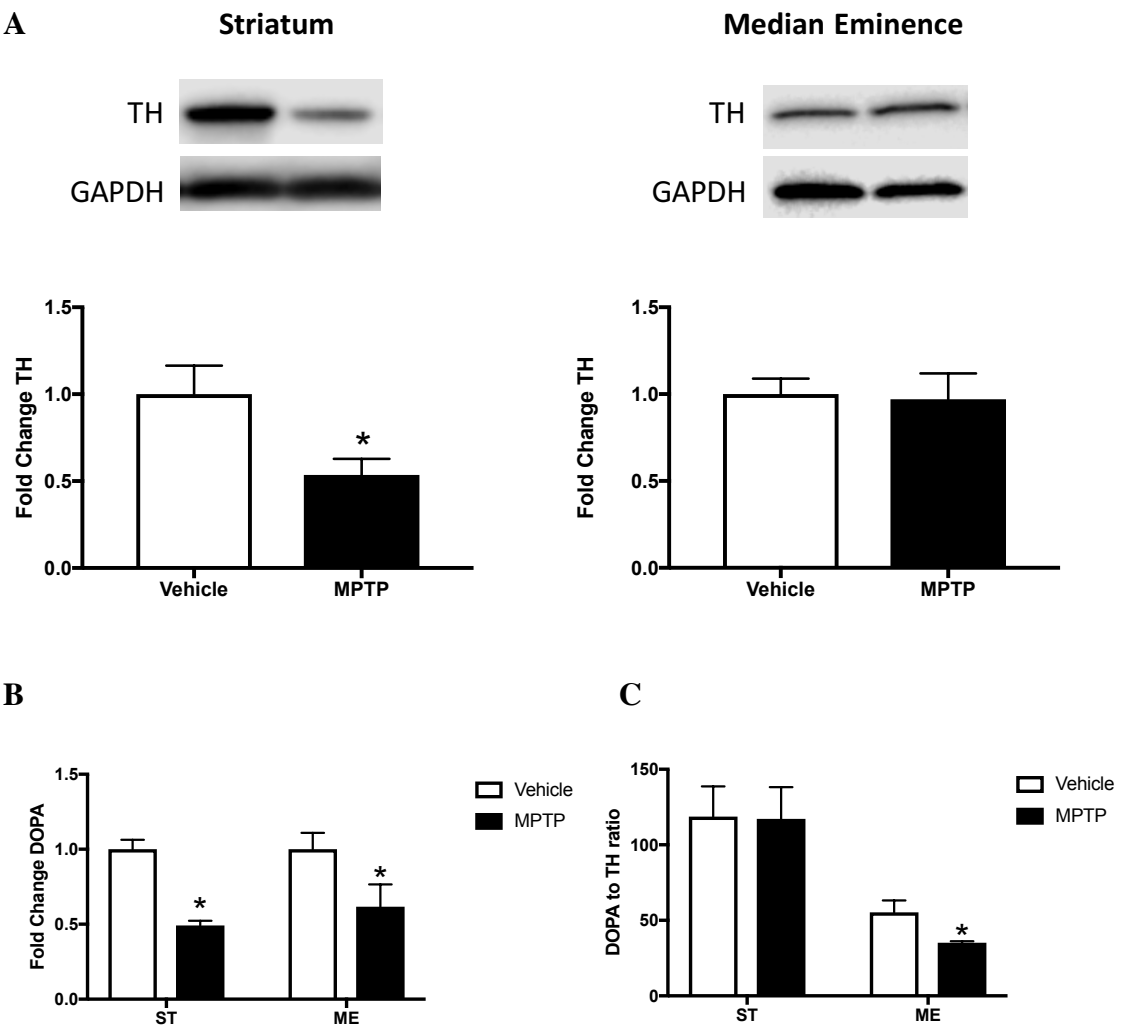
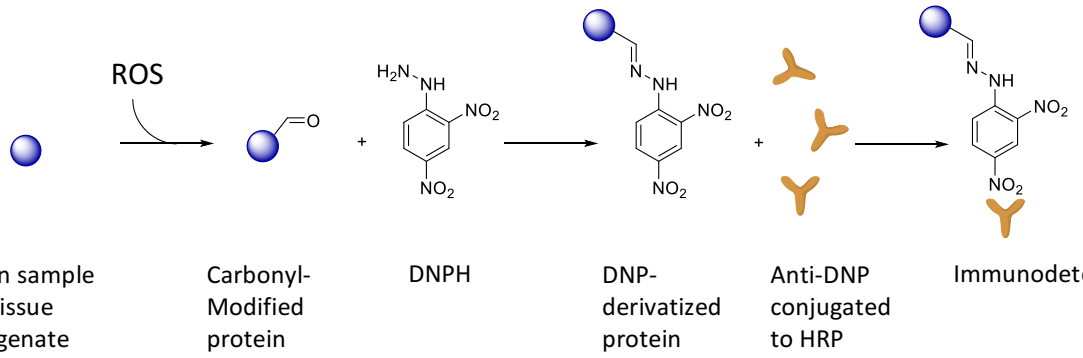


Figure 3-2 Effect of MPTP on the amount and activity of TH in the ST and ME. A, C57BL/6J mice were treated with 20 mg/kg MPTP. Twenty-four h later, the ST (left panel) and ME (right panel) were collected and analyzed for TH and GAPDH by Western blot. The bars represent the mean fold change of protein (normalized to GAPDH). Error bars represent + 1 SEM. * indicates the treatment was significantly different ($P < 0.05$, n=8) from the corresponding vehicle control. **B**, C57BL/6J mice were treated with 20 mg/kg MPTP or saline for 24 h. All animals were treated with NSD-1015 30 min prior to decapitation. Bars represent the mean fold change. Error bars represent + 1 SEM. Saline control values were 24.92 ± 1.50 ng/mg protein in the ST and 16.61 ± 1.81 **C**, The DOPA to TH ratios. Bars represent the mean DOPA to TH ratio. Error bars represent +1 SEM. * indicates the treatment was significantly different ($P < 0.05$, n=8) from the corresponding vehicle control.

Following treatment with MPTP, DOPA accumulation was significantly decreased in both the ST and ME (**Figure 3-2b**). However, the relative change in DOPA accumulation in the ST and ME in the MPTP treated group compared to the saline treated group was not different. When normalized for the amount of TH in each region, it appears that DOPA accumulation per TH (TH activity) was maintained in the ST and decreased in the ME. This change is also reflected in the change in the DOPAC to DA ratios, which indicate that neuronal activity was increased in the ST and decreased in the ME following MPTP exposure.

Differing antioxidant capacities of the ST and ME could cause differential susceptibility to the effects of MPTP. In order to determine if one region sustains more oxidative damage than the other following MPTP exposure, a protein carbonylation assay was used. Protein carbonylation is a type of protein oxidation that forms reactive ketones or aldehydes on side chains of methionine, histidine, and tyrosine to form cysteine disulfide bonds (Davies and Delsignore 1987; Davies, Delsignore, and Lin 1987) (Davies et al, 1987). As a consequence of oxidative modification, carbonyl groups are introduced to protein side chains (Levine 2002) and can be used as a biomarker for oxidative stress. These protein carbonyl groups can be reacted with 2,4-dinitrophenylhydrazine and derivatized to 2,4-dintrophenylhydrozone (DNP-hydrozone), which is detected using an antibody directed to the DNP group of the proteins (**Figure 3-3A**). Twenty-four h following MPTP exposure, protein carbonylation was increased in both the ST and the ME (**Figure 3-3B**). Furthermore, there was no difference in the amount of carbonylated protein in the ST and ME suggesting that both regions have similar capacities for ROS production 24 h following MPTP exposure.

A



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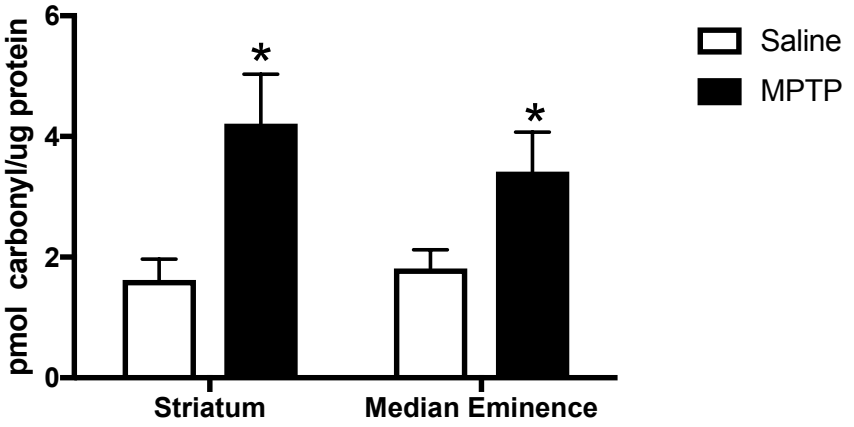


Figure 3-3 Effect of MPTP on protein carbonylation in the ST and ME. A, Protein carbonyl derivatization and detection. Reactive oxygen species can modify the side chains of methionine, histidine, and tyrosine to form cysteine disulfide bonds (Davies, Delsignore, and Lin 1987). As a consequence of oxidative modification, carbonyl groups are introduced into protein side chains. These carbonyl groups can be derivatized to 2,4-dinitrophenyldyrazone (DNP-hydrozone). These derivatized proteins are then measured using antibody directed at the DNP group of these proteins. **B,** C57BL/6J mice were treated with saline or 20 mg/kg MPTP (s.c.). Twenty-four h later, the ST and ME were collected and protein carbonylation was measured by dot-blot. The bars represent the mean pmol carbonyls/ug protein. Error bars represent + 1 SEM * indicates significant difference from the saline control ($p<0.05$, $n=8$).

Susceptibility to the effects of acute neurotoxicant exposure on phenotypic markers of DA axon terminal neurochemical activity is dependent upon protein synthesis and upregulation of the UPS linked protein parkin (Benskey et al. 2012; Benskey et al. 2013; Benskey, Manfredsson, et al. 2015). Previous measurements of parkin were made in the cell body regions of these two neuronal populations. In agreement with these findings, parkin protein decreased in the ST and increased in the ME 24 h following MPTP exposure, (**Fig. 3-4**).

Direct Effects of MPTP and MPP^+ on Proteasome Activity

The N-terminal UBL domain of parkin is important for binding to the 19S regulatory cap of the 26S proteasome and positively modulates 26S catalytic activity *in vitro* (Um et al. 2010; Dachsel et al. 2005). Based on this, it is reasonable to question if differential parkin expression in the ST and ME following MPTP exposure will result in differential proteasome activity in these two regions.

MPTP exposure will be used to induce differential parkin expression in the ST and ME. Therefore, it is important to determine if MPTP or its bioactive metabolite have an effect on proteasome activity. To determine if either MPTP or MPP^+ had any direct effect on proteasome activity, purified 26S proteasome was tested for CT-L proteasome activity in the presence of MPTP, MPP^+ or the proteasome inhibitor bortezomib (**Figure 3-5**). Proteasome activity was not inhibited by the parent molecule MPTP, but was inhibited by the toxic metabolite MPP^+. MPTP exposure also results in the displacement of DA from the DA vesicle and the formation of reactive metabolites that could form toxic adducts with the proteasome.

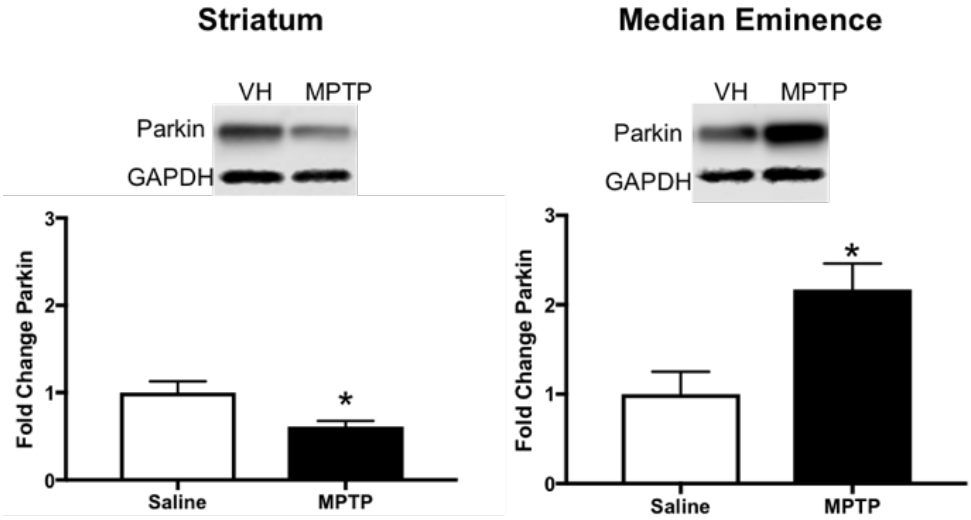


Figure 3-4 Effect of MPTP on parkin protein expression in the ST and ME. C57BL/6J mice were treated with 20 mg/kg MPTP (s.c.). Twenty-four h later, the ST and ME were collected and parkin protein was measured by Western blot. The bars represent the mean fold change in Parkin normalized to GAPDH. Error bars represent + 1 SEM. * indicates significant difference from the vehicle (VH) control ($p<0.05$, $n=10$).

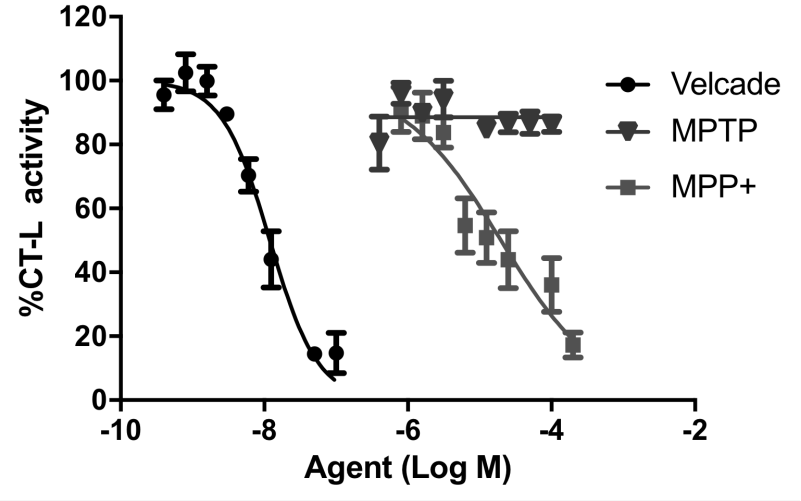


Figure 3-5 Effect of MPTP and MPP$^+$ on proteasome activity *in vitro*. Purified 26S proteasome was treated with bortezomib, MPTP or MPP$^+$ and CT-L activity was measured by measuring the rate of hydrolysis of Suc-LLVY-AMC. The bars represent the mean fluorescent units (FU) released per second per μg protein. Error bars represent +1 SEM. * indicates significant difference from saline treated control animals ($p<0.05$, $n=4$).

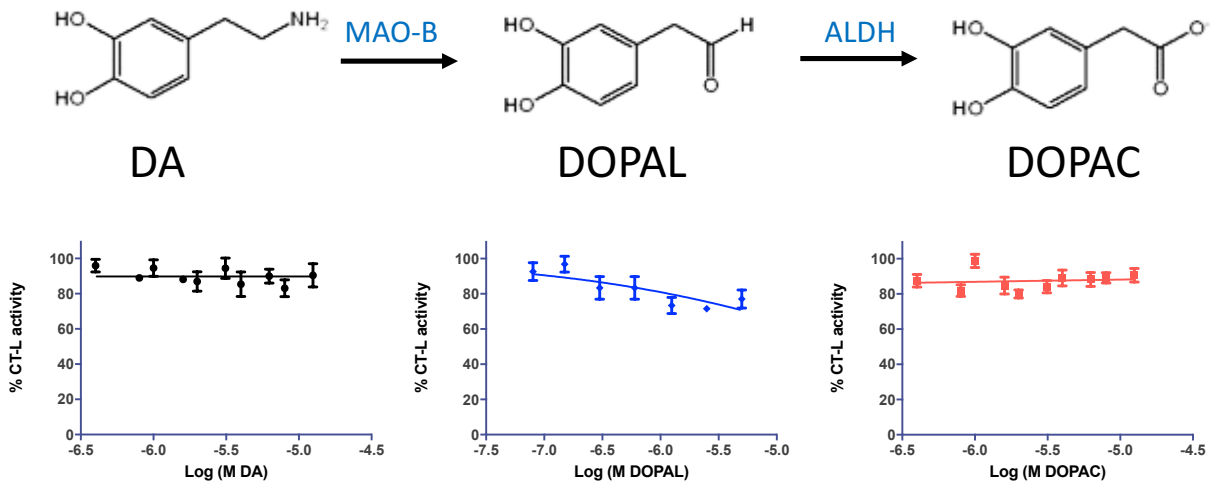


Figure 3-6 Effect of DA and its metabolites on proteasome activity *in vitro*. Purified 26S proteasome was treated with either **A**, DA **B**, DOPAL or **C**, DOPAC and CT-L activity was measured by measuring the rate of hydrolysis of Suc-LLVY-AMC. The bars represent the mean fluorescent units (FU) released per second per µg protein and is expressed as % CT-L activity relative to the saline treated control. Error bars represent +1 SEM. * indicates significant difference from saline treated control animals ($p<0.05$, $n=4$).

Therefore, it was also important to determine if there were any direct effects of DA, or its metabolites DOPAL and DOPAC on proteasome activity. None of these metabolites had any effect on proteasome activity (**Figure 3-6**).

Differential Effects of MPTP on Proteasome Activity

To determine if there are regional differences in proteasome activity, CT-L activity of the proteasome was measured in tissue lysates collected from regions containing the axon terminals and cell bodies of NSDA neurons (ST and SN), and regions containing the axon terminals and cell bodies of the TIDA neurons (ME and ARC) of mice treated with either vehicle or MPTP for 24 h (**Figure 3-7**). CT-L proteasome activity decreased in the ST of MPTP treated mice compared to the saline treated controls. However, MPTP exposure did not change proteasome activity in the SN. This indicates that in NSDA neurons, proteasome activity is only affected in the terminal regions where MPP^+ gains entry into the DA neuron. CT-L activity in tissue collected from the ME of mice treated with MPTP was not different from the saline treated controls and activity was increased in the ARC.

Characterization of Decreased Proteasome Activity in the ST Following MPTP Exposure

Proteasome activity was decreased in the ST, which contains the terminals of the susceptible NSDA neurons. Tissue in the ST contains multiple cell types that may or may not be affected by MPTP exposure. To determine if proteasome activity was affected specifically in the axon terminals of the ST, differential centrifugation was used (**Figure 3-8A**) to separate fractions containing nuclei and cell debris (fraction P1), and synaptosomes (fraction P2). Synaptosomes are pinched off presynaptic terminals that contain all the functional components for

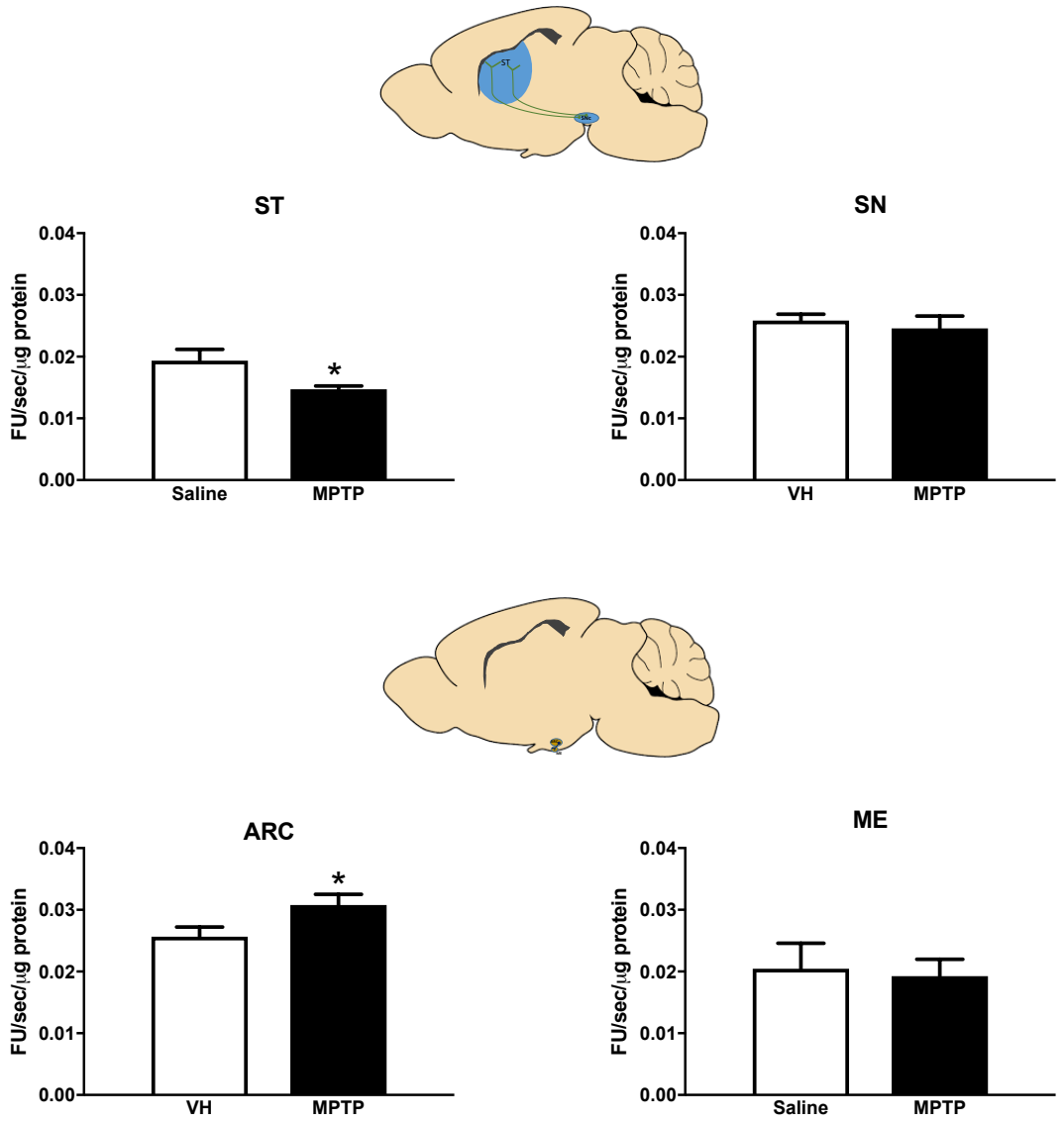


Figure 3-7 Regional effects of MPTP on proteasome activity in the ST, SN, ARC and ME. C57BL/6J mice were treated with either saline or 20 mg/kg MPTP (s.c.). Twenty-four h later, the ST, SN, ARC and ME were collected and CT-L activity of the proteasome was measured using Suc-LLVY-AMC substrate. Bars represent the mean CT-L activity represented as FU/sec/mg protein. Error bars represent +1 SEM. ** indicates significant difference from the respective saline injected control ($p<0.05$, $n=10$).

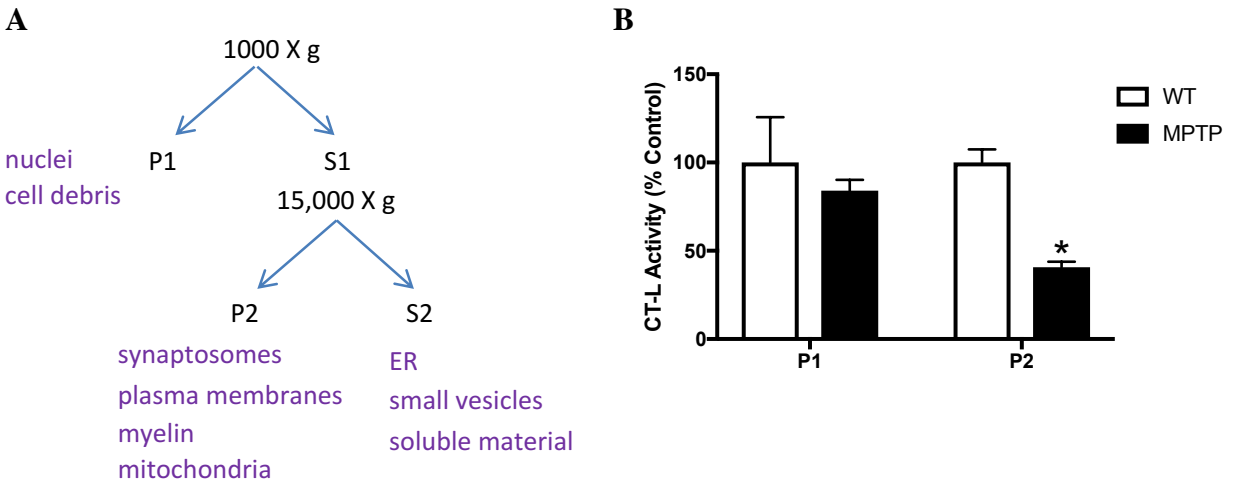


Figure 3-8 Effect of MPTP on proteasome activity in the P1 and P2 fractions of the ST homogenate. C57BL/6J mice were treated with either saline or 20 mg/kg MPTP (sc) and were decapitated after 24 h. The entire ST was removed from each side of the brain. The tissue was homogenized and subjected to differential centrifugation. The pellets were resuspended in proteasome assay buffer and CT-L proteasome activity was determined for each pelleted fraction. **A**, Scheme for differential centrifugation. **B**, CT-L activity of P1 and P2 fractions. * indicates that the group is significantly different from the respective C57BL/6J control ($p < 0.05$, n=6).

neurotransmitter release, reuptake and vesicular storage, along with the postsynaptic membrane and the postsynaptic density. **Figure 3-8B** demonstrates that in mice treated with MPTP, proteasome activity was reduced in the P2 fraction of the ST homogenate This indicates that MPTP impairs proteasome activity in the axon terminals within the ST.

To further characterize the decreased proteasome activity in the ST following neurotoxicant injury, all three catalytic activities of the proteasome were compared in tissue from the ST of mice treated with MPTP or saline for 24 h (**Figure 3-9**). Both CT-L (**Figure 3-9A**) and Casp-L (**Figure 3-9B**) catalytic activities were modestly impaired in the ST of MPTP treated mice compared to saline treated controls, while T-L (**Figure 3-9C**) catalytic activity was not affected.

The decrease in proteasome activity was modest. This small effect on proteasome activity might not be biologically relevant. To determine if the decrease was biologically significant and was sufficient to cause accumulation of ubiquitinated proteins total ubiquitinated proteins were measured by Western blot. Indeed, mice treated with MPTP had increased amount of ubiquitinated proteins in the ST compared to the saline treated controls (**Figure 3-10**).

Decreased expression of proteasome catalytic subunits in yeast results in reduced proteasome assembly and activity (Wang, Luo, and Reiser, 2008). CT-L activity of the proteasome is due to the rate limiting catalytic activity of the proteasome subunit β5 (Coux, Tanaka, and Goldberg 1996). Accordingly, it is possible that the MPTP-induced decrease in CT-L activity in the ST might be due to a reduction in β5 expression. To evaluate this possible mechanism, the amount of β5 (**Figure 3-11**) was determined in the ST of vehicle and MPTP treated mice. Twenty-four h following MPTP exposure, the amount of β5 protein expression in the ST was increased. Another possible mechanism of proteasome impairment could be the

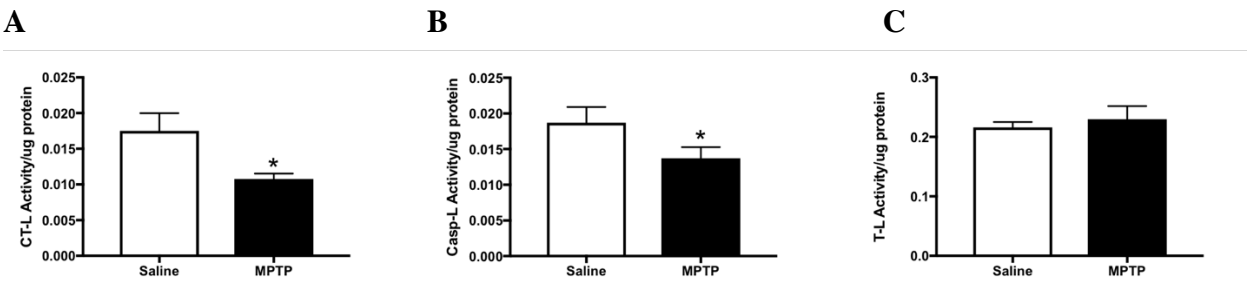


Figure 3-9 Effect of MPTP on CT-L, Casp-L, and T-L proteasome activity in the ST. C57BL/6J mice (N=10) were treated with saline or 20 mg/kg MPTP (s.c.). Twenty-four h later, the ST was collected All three catalytic activities were measured in the ST (**A**) CT-L, (**B**) Casp-L activity, (**C**) T-L activity. The bars represent the mean fluorescent units (FU) released per second per μg protein + 1 SEM. * indicates significant difference from saline treated control animals ($p<0.05$, n=10).

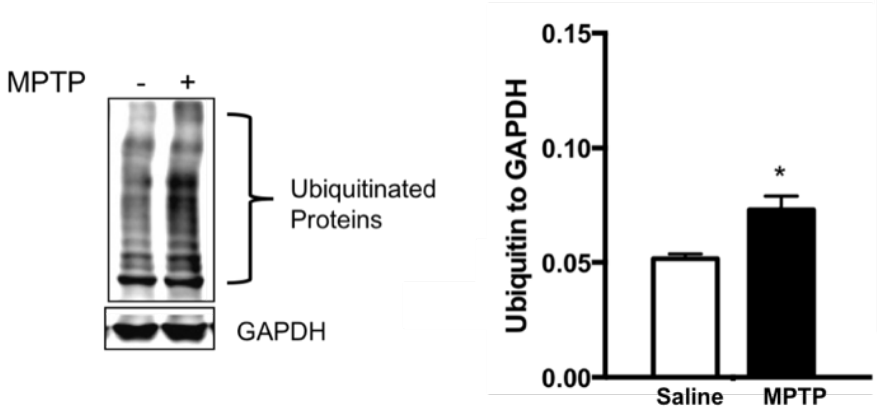


Figure 3-10 Effect of MPTP on the accumulation of ubiquitinated proteins in the ST. C57BL/6J mice (N=10) were treated with 20 mg/kg MPTP (s.c.). Twenty-four h later, the ST was collected and total ubiquitinated proteins was measured by Western blot. The bars represent the mean ratio of ubiquitin to GAPDH error bars represent +1 SEM.* indicates significant difference from saline treated control animals ($p<0.05$, n=10).

disassembly of the 26S proteasome. The amount of assembled 26S and 20S proteasome was measured using native Western blot (**Figure 3-12**). The amount of 26S proteasome complex in the ST of mice treated with MPTP was not statistically different from saline treated controls. In addition, the amount of 20S proteasome was significantly increased. When the ratio of 26S to 20S proteasome complex was compared, there was no significant difference in the proportion of one complex over the other. These data indicate that MPTP causes compensatory increase in *de novo* proteasome subunit synthesis and assembly of new proteasome complexes.

Proteasome activity is decreased in the ST and this impairment is sufficient to induce the accumulation of ubiquitinated proteins and cause the increase in expression of the 20S proteasome catalytic subunit β5. Nrf1 is a transcription factor that responds to the loss of proteasome subunits. Nrf1 is located in the endoplasmic reticulum (ER) lumen and under conditions of oxidative stress is retrotranslocated to the cytosol while still being bound by its N-terminus to the ER membrane. Under normal conditions, retrotranslocated Nrf1 is rapidly degraded by the proteasome. When the proteasome is inhibited, Nrf1 is stabilized, cleaved by Proteases and translocated to the nucleus where it binds to its antioxidant response element and increases the expression of proteasome subunits (Steffen et al. 2010; Radhakrishnan, den Besten, and Deshaies 2014). To test if proteasome inhibition in the ST was sufficient to stabilize Nrf1 in the SN, Nrf1 products were measured by Western blot. Nrf1 can produce three possible bands on a Western blot. The band at 120 kD represents Nrf1 that is proteases and translocated to the nucleus to up-regulate proteasome subunit genes (Steffen et al. 2010; Radhakrishnan, den Besten, and Deshaies 2014) To test if proteasome inhibition in the ST sequestered in the ER. The 110 kD band is partially processed Nrf1, while the band at 95 kD represents Nrf1 that has been cleaved from the ER membrane and translocated to the nucleus (Chepelev et al. 2013). In

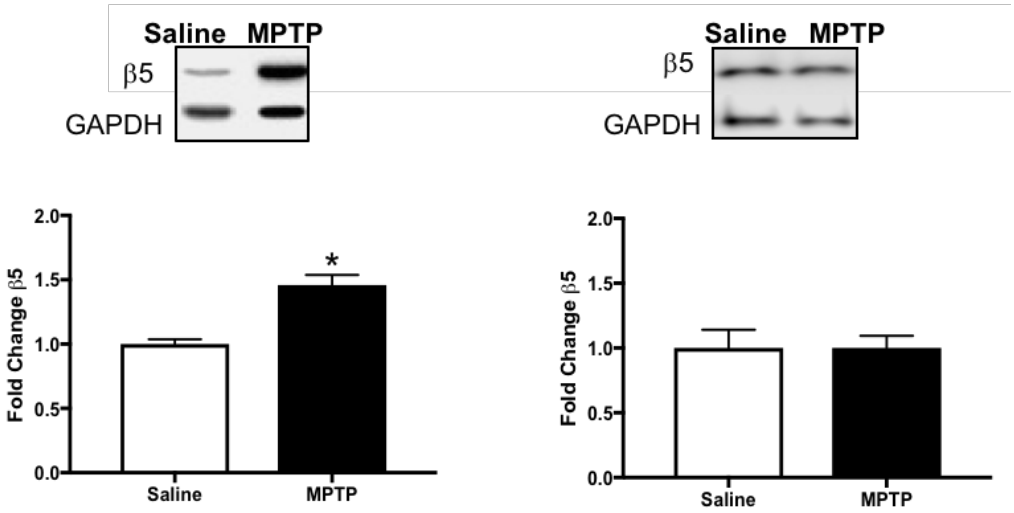
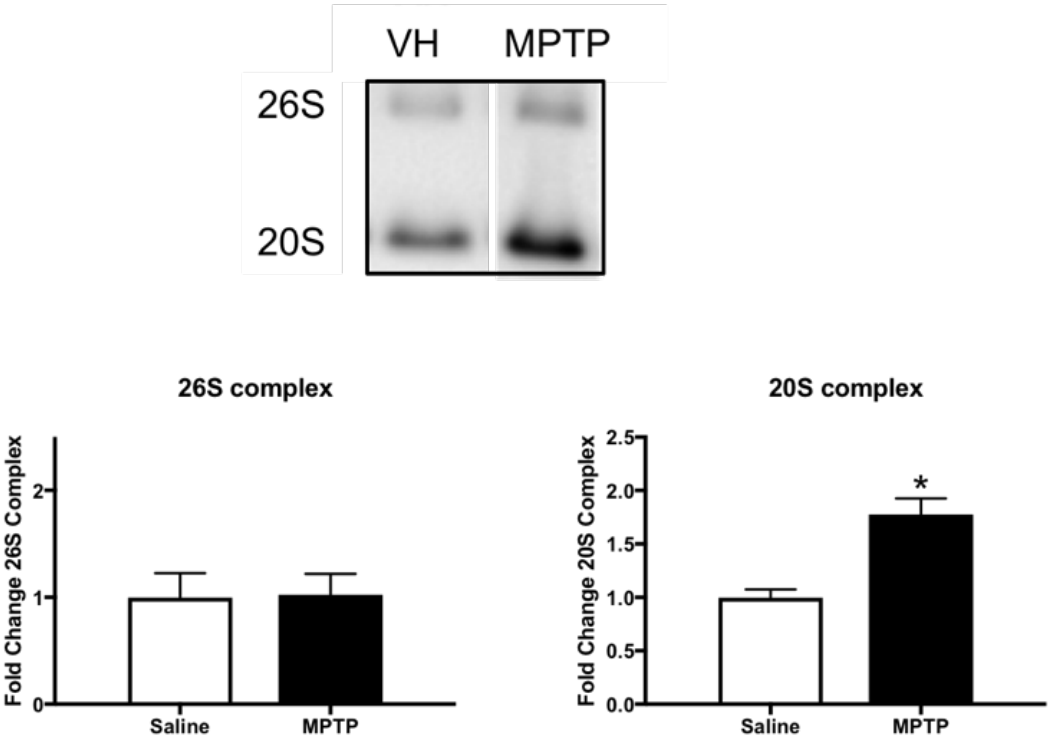


Figure 3-11 Effect of MPTP on CT-L catalytic subunit β5 expression in the ST and ME. C57BL/6J mice (N=10) were treated with 20 mg/kg MPTP (s.c.). Twenty-four h later, the tissue from the ST (left panel) and ME (right panel) was collected and β5 subunit of the proteasome was measured by Western blot and normalized to GAPDH. Bars represent the mean fold change of β5 compared to the saline controls. Error bars represent +1 SEM. * indicates significant difference from the saline control ($p<0.05$, N=10).

A



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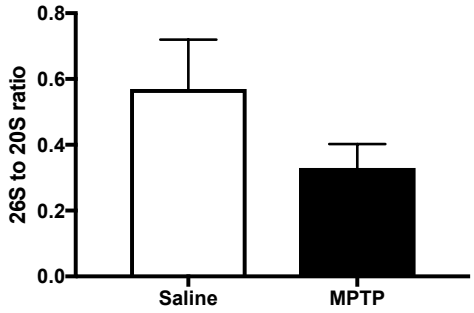

Figure 3-12 The effect of MPTP on the amount of assembled 26S and 20S proteasome in the ST. C57BL/6J mice were treated with either saline or MPTP (20 mg/kg; s.c.). Twenty-four h later, the ST was collected and the lysate was subjected to native gel electrophoresis. 20S proteasome complex was detected with an antibody directed toward the β5 catalytic subunit and the 26S proteasome was detected with an antibody directed toward the 19S regulatory subunit RPN10. Bars represent the fold change of complex compared to the saline treated controls. Saline control values were 118±8 for 20S and 67 ±17 for 26S complexes. Error bars represent +1 SEM. **B**, ratio of 26S to 20S complexes error bars represent the propagated SEM. * indicates significant difference from the saline controls ($p<0.05$, $n=8$).

the SN of MPTP treated mice, there was no change in the amount of any of the Nrf1 products (**Figure 3-13A**). This did not come as a surprise because proteasome activity in the SN was not inhibited following MPTP exposure. To determine if MPP^+ inhibition of the proteasome could result in stabilization of Nrf1, differentiated MN9D cells were treated with saline, bortezomib or MPP^+. Two h following treatment, cell lysates were used to measure Nrf1 by Western blot (**Figure 3-13B**). Treatment of MN9D cells with bortezomib, a known proteasome inhibitor, results in the stabilization and translocation of Nrf1 to the nucleus. Treatment of MN9D cells with MPP^+ results in retrotranslocation of Nrf1 from the ER lumen and its stabilization, but there is no presence of the active transcription factor.

Discussion

Most neurodegenerative diseases involve the loss of a specific subpopulation of neurons. In PD, NSDA neurons are lost while TIDA neurons are resistant to degeneration (Langston and Forno 1978). An understanding of the ability of the resistant population of neurons to recover may be beneficial in the identification of novel treatments for PD in which the patient could benefit from the protection of healthy NSDA neurons. The DA neuron specific neurotoxicant MPTP recapitulates the specific neuronal population susceptibility that is seen in PD (Langston and Forno 1978; Benskey et al. 2013). In mice, both NSDA and TIDA neuronal populations initially respond to MPTP, however, TIDA neurons recover from the initial neurotoxic insult, while NSDA neuronal toxicity persists (Benskey et al. 2012; Benskey et al. 2013).

The recovery of vesicular DA in the ME following MPTP exposure is dependent upon the expression of parkin in TIDA neurons (Benskey, Manfredsson, et al. 2015). Differential

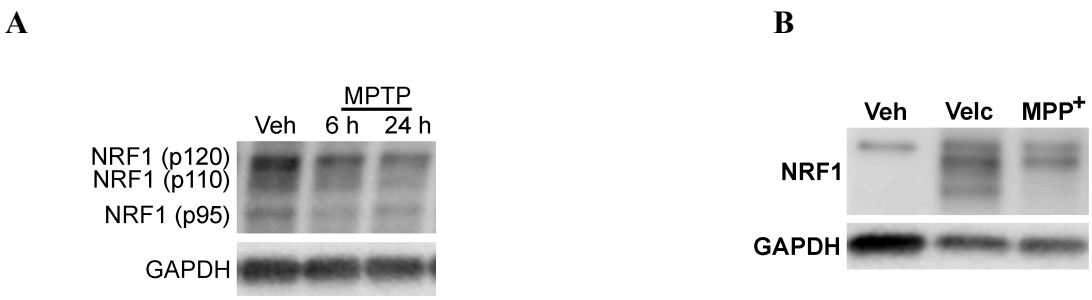

Figure 3-13 Effect of MPTP on Nrf1 stabilization in the SN. **A**, Nrf1 in the SN of MPTP treated mice. C57BL/6J mice were treated with either saline or MPTP (20 mg/kg; s.c.). Six and 24 h later, the SN was collected and the lysate was subjected to Western blot to measure Nrf1 (p120, p110, and p95). **B**, Nrf1 in MN9D cells**.** MN9D cells were grown in DMEM and differentiated with sodium butyrate for 5 d. The cells were incubated in media containing 100 nM bortezomib (Velc) and 200 mM MPP^+.

parkin expression following exposure to MPTP is of interest because mutations in the Park2 gene cause autosomal recessive juvenile PD (Hattori et al. 1998; Kitada et al. 1998; Sinha et al. 2005) and oxidative modifications causing parkin loss of function in the ST are found in patients with sporadic PD (Chung et al. 2004). Parkin is an E3 ubiquitin ligase that promotes the degradation of its specific protein substrates via the UPS (Shimura et al. 2000) as well as through autophagy (Olzmann and Chin 2008; Narendra et al. 2008). In addition to its E3 ligase function, the N-terminal UBL domain of parkin interacts with proteasome regulatory subunits Rpn10 (Sakata et al. 2003), RPN13 (Aguileta et al. 2015), Rpt6 (Tsai et al. 2003), and α4 (Dachsel et al. 2005) and directly enhances proteasome activity in cell culture (Um et al. 2010; Hyun et al. 2002; Dachsel et al. 2005). The studies in this chapter characterize the differential proteasome activity, which may be associated with differential parkin expression in regions containing TIDA and NSDA axon terminals under conditions of neurotoxicant induced oxidative stress *in vivo*.

NSDA neurons have higher basal neuronal activity than TIDA neurons, which is reflected by the DOPAC to DA ratio as well as the DOPA to TH ratios observed in the NSD-1015 study. The relatively high state of activity in NSDA neurons may come with the consequence of increased ROS production and reduced antioxidant capacity. To prevent toxic DA quinone formation, intraneuronal DA is metabolized to DOPAL by MAO, a reaction that results in the two-electron reduction of oxygen and the production of superoxide and hydroxyl radical (Przedborski et al. 2000). Superoxide and hydroxyl radical production results in the depletion of glutathione, which can render these neurons susceptible to a modest increase in ROS production. Despite the fact that NSDA neurons intrinsically produce more ROS than other DA neuronal populations (Voets et al. 2012; Castro Mdel et al. 2012; Alexeyev 2009; Enochs et al.

1994), there are no regional differences in oxidative protein modification (as measured by protein carbonylation), parkin expression, or proteasome activity in untreated WT mice.

The production of ROS markedly increases in the ST and ventral midbrain following MPTP exposure (Jackson-Lewis and Przedborski 2007). The burden of MPTP-induced ROS likely overwhelms the antioxidant capacity of DA neurons. A single acute exposure of MPTP results in a compensatory increase in NSDA neuronal activity in the ST, while TIDA neuronal activity in the ME is decreased. The increase in activity of the NSDA neurons is likely mediated by loss of DA receptor mediated feedback regulation (either long-loop post-synaptic or autoregulatory presynaptic), which is absent in TIDA neurons that lack a robust presynaptic DA feedback mechanism (Lookingland and Moore 2005). TIDA neurons are instead regulated by changes in circulating prolactin, which tonically activates the TIDA neurons. MPP^+ triggers release of DA from the TIDA axon terminals to the anterior pituitary where is inhibits prolactin release from lactotrophs (Durham et al. 1997). It is feasible that the decrease in neuronal activity of TIDA neurons following MPTP is due to loss of prolactin feedback activation of TIDA neurons. Despite the differing neuronal activity states of NSDA and TIDA neurons following MPTP, protein carbonylation is elevated to the same extent in both the ST and ME 24 h following MPTP exposure, indicating that the antioxidant capacity of both regions has been overwhelmed following MPTP exposure. The observation that parkin is elevated in the ME and not the ST following MPTP exposure suggests that changes in parkin expression are not dependent upon the oxidative modification of proteins within that region.

It was hypothesized that distinct parkin expression profiles in NSDA and TIDA neurons following MPTP exposure would be associated with differential proteasome activity within the ST and ME. In fact, proteasome activity was decreased in the ST, but not the ME 24 h following

MPTP treatment. Interestingly, proteasome activity was not changed in the SN, which contains the cell bodies of NSDA neurons. This is likely because MPP^+ enters the axon terminal via DAT and causes a retrograde mechanism of toxicity in NSDA neurons. Proteasome activity was increased in the ARC, which contains the cell bodies of TIDA neurons. This could, perhaps be due to increased parkin expression in this region following MPTP exposure.

To determine if MPTP or MPP^+ causes direct inhibition of the proteasome, purified 26S proteasome was treated with either MPTP or MPP^+ and tested for CT-L activity. MPP^+ (but not its inactive precursor MPTP) inhibits purified 26S proteasome activity *in vitro* indicating a direct effect of the toxic cation on proteasome function. This observation, however, does not rule out other indirect means of proteasome inhibition *in vivo*. Treatment of mice with MPTP likely causes pronounced oxidative stress and subsequent oxidative damage to proteins including the proteasome within the neuron.

Decreased proteasome activity in the ST following MPTP exposure was further characterized *in vivo*. Proteasome impairment following MPTP exposure occurs in both the P1 fraction, which contains cell bodies and cellular debris as well as the P2 fraction, which contains synaptosomes and membrane fragments. This indicates that proteasome impairment is occurring within the axon terminals of the ST. It is important to understand that neurotransmitter heterogeneity is a limitation of synaptosomal studies. Only about 35% of the synaptosomes in a striatal synaptosome preparation can be identified as dopaminergic (Dunkley et al. 1986). However, MPP^+ is selectively transported into dopaminergic terminals and it is likely that the proteasome impairment observed in the P2 fraction comes from the dopaminergic synaptosomes.

The proteasome has three proteolytic activities; CT-L, Casp-L and T-L (Kisselev and Goldberg 2005). Of these, the CT-L activity is rate-limiting for proteasome substrate degradation

(Rock et al. 1994). Exposure to MPTP impairs both CT-L and Casp-L activities of the proteasome in the ST; the decrease in activity is sufficient to cause the accumulation of ubiquitinated proteins and induce *de novo* synthesis of new proteasome subunits that are assembled into the 20S proteasome. It should be noted that, although T-L activity was not decreased in these assays, other serine proteases that are abundant in the brain (Wang, Luo, and Reiser 2008) could contribute to non-proteasome specific T-L activity.

Although the proteasome has multiple catalytic activities, inhibition of all three is not needed to prevent protein substrate degradation. Most proteasome inhibitors that inhibit the CT-L sites are hydrophobic molecules, while inhibitors of T-L activity must contend with charged residues within the T-L catalytic binding pocket and tend to be less cell permeable (Kisselev and Goldberg 2001). Therefore, many synthetic proteasome inhibitors impair CT-L catalytic activity and have weak to no effect on the T-L catalytic site. Nevertheless, inhibition of CT-L proteasome activity causes a large reduction in the rate of protein substrate degradation (Rock et al. 1994; Chen and Hochstrasser 1996; Heinemeyer et al. 1997), while inhibition of the T-L or Casp-L catalytic activities have limited control over the rate of protein degradation (Kisselev et al. 1999; Heinemeyer et al. 1997; Arendt and Hochstrasser 1997).

MPP^+ is a mitochondrial complex I inhibitor and parkin plays a role in mitochondrial quality control (Exner et al. 2007; Narendra et al. 2008; Springer and Kahle 2011; Hawong 2014). The amount of parkin is not increased in the ST following MPTP compared to the saline treated controls and the modest decrease in parkin protein in the ST following MPTP exposure likely reflects normal turnover of existing parkin. One might suggest that complex I inhibition by MPP^+ results in decreased ATP production, which is necessary to maintain the 26S proteasome complex. While ATP is transiently decreased by about 10% shortly (1-3 h) after MPTP

exposure, ATP is not noticeably different from control mice 24 h after MPTP exposure (Cosi and Marien 1998; Chan et al. 1991; Irwin, DeLanney, and Langston 1993). Consistent with this time course of MPTP effects, no change in the amount of assembled 26S proteasome was observed in the ST 24 h after MPTP exposure. Since maintenance of assembled 26S proteasome is ATP-dependent, the unaltered levels of assembled 26S proteasome in the ST following MPTP treatment could imply that there was sufficient ATP to keep the 26S proteasome from disassembling following MPTP treatment.

There is a growing body of evidence that parkin is necessary for maintenance of central DA neurons (Benskey, Manfredsson, et al. 2015; Petrucelli et al. 2002; Narendra et al. 2008; Jiang et al. 2004) and that parkin can act to enhance proteasome activity (Tanaka et al. 2001; Sakata et al. 2003; Aguileta et al. 2015; Tsai et al. 2003; Dachsel et al. 2005; Um et al. 2010; Hyun et al. 2002). The necessity of parkin to maintain proteasome activity following neurotoxic insult will be explored in Chapter 4 of this dissertation.

Conclusion

NSDA neurons are susceptible to neurotoxicant exposure while TIDA neurons are able to recover. MPTP exposure results in similar oxidative damage to proteins in regions containing the axon terminals of NSDA and TIDA neurons yet parkin expression is increased in TIDA neurons but not in NSDA neurons. Parkin is an E3 ubiquitin ligase that can directly bind to the 26S proteasome and enhance its activity. The increase in parkin expression is neuroprotective for TIDA neurons (Benskey, Manfredsson, et al. 2015). Regional differences in proteasome activity appear to be associated with parkin expression, as proteasome activity is decreased in the ST and maintained in the ME following MPTP exposure. However, this observation is correlative and

the causal contribution of parkin expression to proteasome activity must be examined in more detail. Further characterization of proteasome activity following neurotoxicant exposure demonstrates that decreased proteasome activity occurs in the axon terminals innervating the ST and affects both CT-L and Casp-L proteasome activity, but not T-L activity. The modest decrease in proteasome activity is sufficient to induce the accumulation of ubiquitinated proteins, the production of β5 catalytic subunits, and the assembly of new 20S proteasome in the ST.

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Chapter 4. Parkin is necessary to maintain proteasome activity in the ST

Introduction

Most cellular proteins are targeted for degradation by the 26S proteasome. In fact, over 30% of newly synthesized proteins are misfolded (Schubert et al. 2000) and even if proteins are folded into their correct tertiary structures, they often undergo damage due to ROS and other environmental stresses (Sherman and Goldberg 2001). These misfolded and damaged proteins are not normally observed in healthy cells because they are rapidly degraded by the UPS. Neuronal intracellular inclusions are a common feature of many neurodegenerative diseases (Lowe et al. 2001). In fact, an important pathological hallmark of PD is the presence of Lewy bodies in the surviving neurons (Pollanen, Dickson, and Bergeron 1993). Intracellular inclusions are composed of heavily ubiquitinated protein aggregates, some of which is α-synuclein which implies a connection between failure of UPS associated protein quality control and neurodegeneration (Olanow et al. 2004; Tofaris et al. 2003).

The parkin protein provides a link between familial and sporadic PD and UPS impairment. For greater than 50% of patients with AR-JP the causative gene mutation is in the parkin gene (Kitada et al. 1998). In sporadic PD, oxidative and nitrosative stress results in S-nitrosylation of parkin and a dramatic decrease in UPS activity (Yao et al. 2004).

The neurotoxicant MPTP produces oxidative stress in DA neurons and can replicate the pattern of DA neuronal susceptibility that is seen in PD (Benskey et al. 2012; Behrouz et al. 2007; Melamed et al. 1985; Mogi et al. 1988). NSDA neurons are susceptible to neurotoxicant exposure while TIDA neurons recover. While MPTP exposure results in oxidative damage to proteins in both NSDA and TIDA axon terminal regions, parkin expression is increased only in

the TIDA neurons and not NSDA neurons. Regional differences in proteasome activity appear to be associated with parkin expression, as proteasome activity is decreased in the ST and maintained in the ME following MPTP exposure. However, this observation is correlative and the causal contribution of parkin expression to proteasome activity will be examined here in more detail.

The studies presented in this chapter are designed to test the hypothesis that parkin expression is necessary to maintain proteasome activity following MPTP exposure. In order to test this hypothesis, proteasome activity of mice that were deficient in parkin expression was compared to proteasome activity in WT mice. In addition, markers of oxidative protein damage (protein carbonylation) and changes in DA phenotypic markers (TH expression and activity) were also explored.

Results

Park2$^{-/-}$ Mice do not Express Parkin

Park2^{tm1Shn} knockout mice (Park2$^{-/-}$) model the exon 3 deletion mutation that is most common in AR-JP. In these mice, a frame shift and premature stop codon in exon 5 are introduced by replacing exon 3 with the enhanced green fluorescent protein (EGFP) gene (Goldberg et al. 2003). Western blot with an antibody directed toward parkin indicated the absence of the parkin protein in both the ST and the ME of the Park2$^{-/-}$ mice (**Figure 4-1**). Park2$^{-/-}$ mice were backcrossed to C57BL/6J inbred mice for over 20 generations before being made available by Jackson Labs and established as a breeding colony *in house* for several years. In this dissertation, all comparisons of Park2$^{-/-}$ mice were made to C57BL/6J mice, which from

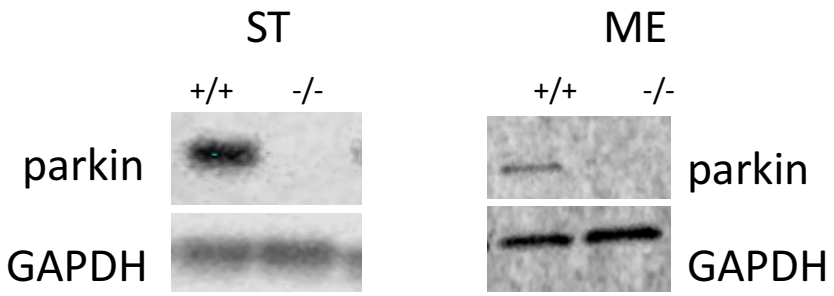

Figure 4-1 Expression of parkin in the ST and ME of C57BL/6J and Park2$^{-/-}$ mice. C57BL/6J and Park2$^{-/-}$ mice were decapitated and lysates from the ST and ME were analyzed for parkin and GAPDH by Western blot.

here on in will be referred to as WT mice. Park2$^{-/-}$ mice have increased extracellular DA in the ST, reduced synaptic excitability of ST medium spiny neurons, and behavioral deficits related to dysfunction of the NSDA neuronal pathway (Goldberg et al. 2003). For gait analysis of Park2$^{-/-}$ mice compared to WT controls data see **Appendix 1.**

Park2$^{-/-}$ Mice have Decreased Proteasome Activity in the ST but not the ME

In Chapter 3, observations were made that suggested that regional differences in proteasome activity following neurotoxicant exposure might be related to regional differences in parkin expression. Parkin protein concentrations and proteasome activity are both decreased in the ST following MPTP-induced injury. Conversely, parkin protein expression increases in the ME following MPTP treatment and this is associated with maintenance of proteasome activity in this PD resistant subset of DA neurons. Given the correlation of proteasome function and parkin expression following MPTP treatment in PD susceptible and PD resistant brain regions, it is plausible that proteasome activity may be dependent upon parkin expression.

Based on the hypothesis that parkin expression is necessary to maintain proteasome activity in the ST and ME following acute toxicant-induced injury, CT-L proteasome activity and protein carbonylation were compared in the ST and ME of WT and Park2$^{-/-}$ mice. Proteasome activity was decreased in the ST of the Park2$^{-/-}$ mice (**Fig. 4-2A**) indicating that maintenance of basal proteasome activity in the ST is dependent on parkin expression. However, proteasome activity was maintained in the ME of Park2$^{-/-}$ mice compared to WT mice which indicates that maintenance of basal proteasome activity in the ME is not dependent upon parkin expression (**Figure 4-2B**).

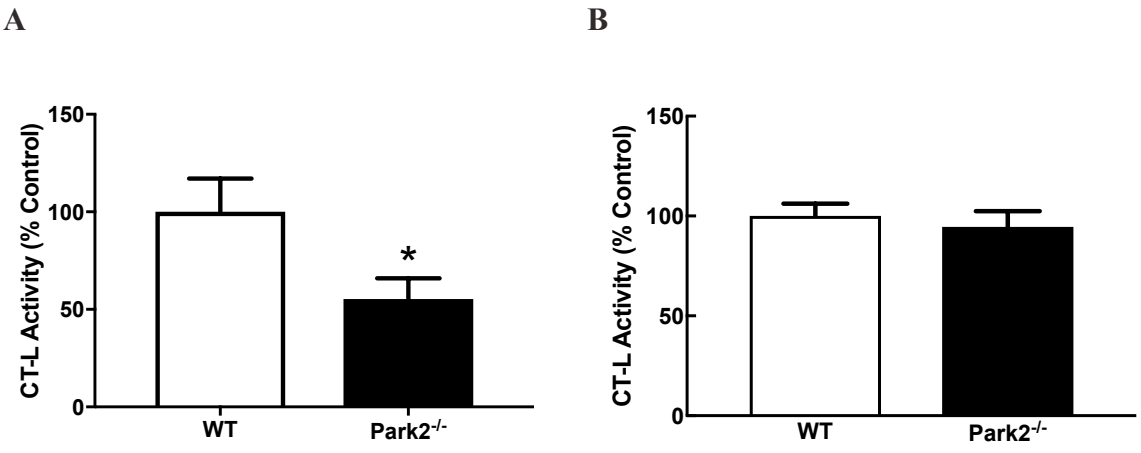

Figure 4-2 Proteasome activity in the ST and ME of C57BL/6J and Park2$^{-/-}$ mice. CT-L activity of the proteasome was measured in lysates from the ST (**A**) and ME (**B**) of C57BL/6J and Park2$^{-/-}$ mice using Suc-LLVY-AMC as the substrate. Bars represent the mean % CT-L activity compared to the WT controls. Error bars represent +1 SEM. * indicates significant difference from the WT control ($p < 0.05$, n=8).

Parkin protein is specifically expressed throughout the cytoplasm of neurons in the brain and not in glial cells (Stichel et al. 2000). To determine if decreased proteasome activity was localized to the axon terminals within the ST, CT-L proteasome activity was measured in the P1 and P2 fractions of striatal homogenates (**Figure 4-3**). Proteasome activity was decreased in both the P1 and the P2 fractions indicating that loss of parkin affects proteasome activity of both the terminals and cell bodies of neurons in the ST.

Clearance of Oxidatively Damaged Proteins in the ST is Dependent upon Parkin Expression

Damaged proteins are not normally observed in healthy cells because they are rapidly degraded by the UPS. To determine if changes in proteasome activity resulted in accumulation of damaged proteins, homogenates from the ST and ME of WT and Park2$^{-/-}$ mice were examined for protein carbonylation. Mice that were deficient of parkin were more susceptible to oxidative damage in the ST, as indicated by the increase in protein carbonylation, compared to the WT mice (**Figure 4-4A**). This indicates that clearance of oxidatively damaged proteins in the ST is dependent upon parkin expression. As expected, protein carbonyls did not accumulate in the ME of Park2$^{-/-}$ mice compared to WT mice (**Figure 4-4B**) indicating that under basal conditions, clearance of oxidatively damaged proteins is not dependent upon parkin expression.

The ME of Park2$^{-/-}$ Mice is Susceptible to Neurotoxic Insult

There are regional differences in proteasome activity in Park2$^{-/-}$ mice that are not apparent in WT mice. NSDA neurons have higher basal neuronal activity than TIDA neurons, which may come with the consequence of increased ROS production and reduced antioxidant capacity. This suggests that an added neurotoxic insult might result in loss of proteasome

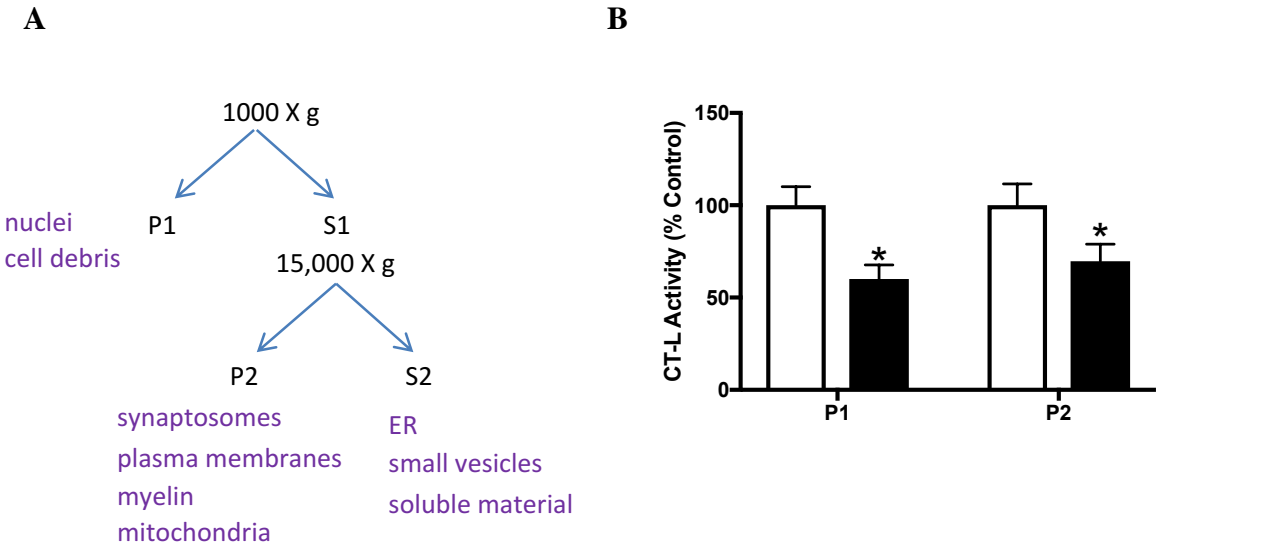

Figure 4-3 Proteasome activity in the P1 and P2 fractions of C57BL/6J and Park2$^{-/-}$ ST homogenates. C57BL/6J and Park2$^{-/-}$ mice were decapitated and the entire ST was removed from each side of the brain. The tissue was homogenized and subjected to differential centrifugation. The pellets were resuspended in proteasome assay buffer and CT-L proteasome activity was determined for each pelleted fraction. **A**, Scheme for differential centrifugation. **B**, CT-L activity of P1 and P2 fractions. * indicates that the group is significantly different from the respective C57BL/6J control. # indicates that the group is significantly different from its respective P1 control. ($p < 0.05$, n=6).

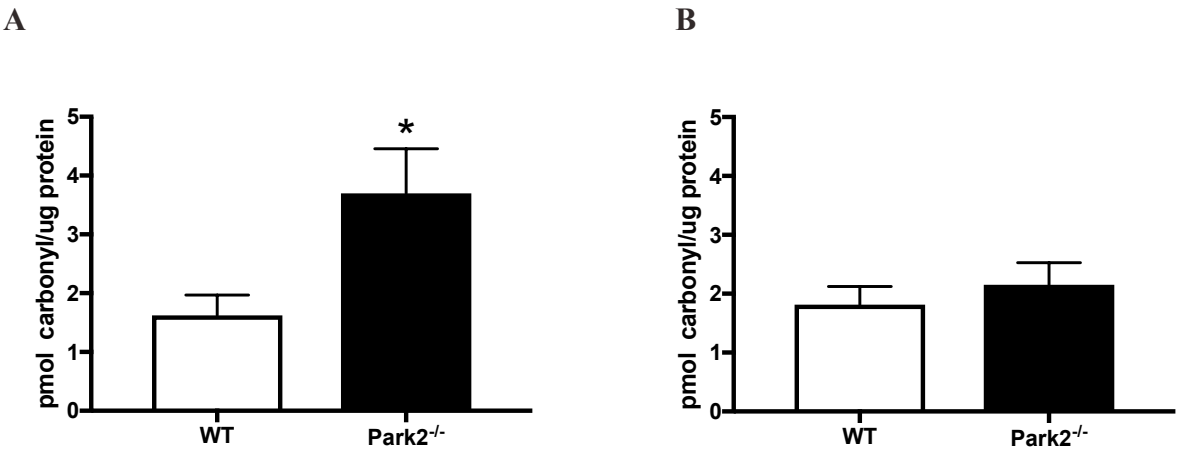

Figure 4-4 Protein carbonylation in the ST and ME of C57BL/6J and Park2$^{-/-}$ mice. The ST (**A)** and ME (**B)** were collected from C57BL/6J and Park2$^{-/-}$ mice and the amount of protein carbonyls was measured by dot-blot. The bars represent the mean pmol carbonyls/mg protein. Error bars represent +1 SEM. * indicates significant difference from the WT control ($p < 0.05$, n=8).

activity in the ME of Park2$^{-/-}$ mice but not WT mice. To this end, WT and Park2-/- mice were treated with a single acute dose of MPTP and proteasome activity was measured in the ST and ME 24 h later (**Figure 4-5**). As previously described, the ST of WT mice had impaired proteasome activity 24 h following MPTP exposure and the state of parkin deficiency resulted in decreased proteasome activity. The combination of parkin deficiency and MPTP exposure resulted in an additive loss of proteasome activity. In the ME, proteasome activity was not affected in Park2$^{-/-}$ mice, however Park2$^{-/-}$ mice treated with MPTP had decreased proteasome activity.

Protein carbonylation was measured in the ST and ME of WT and Park2$^{-/-}$ mice 24 h following a single acute exposure to MPTP (**Figure 4-6**). Interestingly, while the state of parkin deficiency results in increased oxidative damage to proteins in the ST compared to the WT, the added insult of MPTP exposure resulted in decreased oxidative damage in the ST. While proteasome activity was reduced in the ME of MPTP treated Park2$^{-/-}$ mice (**Figure 4-5**), proteins in the ME of Park2$^{-/-}$ mice did not incur oxidative damage following MPTP exposure (**Figure 4-6**).

TH Turnover in the ST of Park2$^{-/-}$ Mice

Park2$^{-/-}$ mice have reduced proteasome activity in the ST and since proteasome activity is necessary for the efficient turnover of TH (Congo Carbajosa et al. 2015; Nakashima et al. 2013; Nakashima et al. 2011), the effect of parkin deficiency on TH in the ST of Park2$^{-/-}$ mice was compared to WT mice (**Figure 4-7**). In the ST of Park2$^{-/-}$ mice, there was more phosphorylated TH than in the WT mice, which suggests that there is more active TH in the ST

A **B**

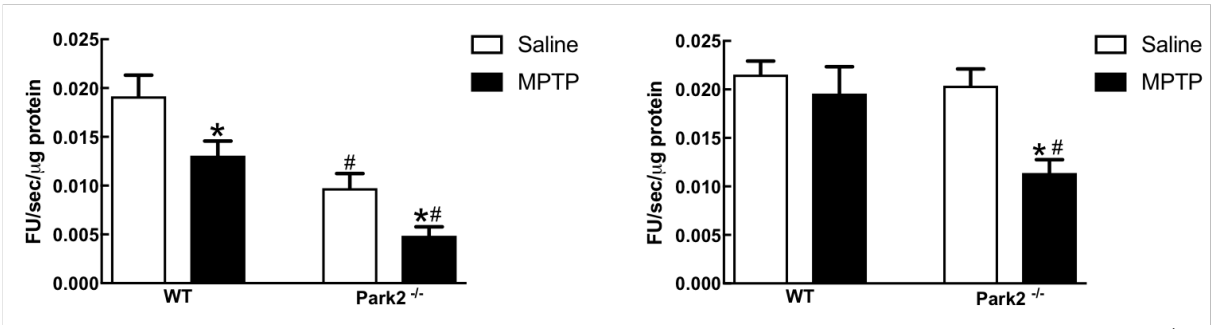

Figure 4-5 Proteasome activity in the ST and ME of MPTP treated C57BL/6J and Park2$^{-/-}$ mice. C57BL/6J and Park2$^{-/-}$ mice were treated with either saline or MPTP (20 mg/kg; s.c.). Twenty-four h later, the ST and ME were collected and CT-L activity of the proteasome was measured using Suc-LLVY-AMC substrate. **A,** CT-L proteasome activity in the ST. **B,** CT-L proteasome activity in the ME. Error bars represent +1SEM. * indicates significant difference from the corresponding saline treated control mice, # indicates significant difference between the same treatment between the strains ($p<0.05$, n=10).

A B

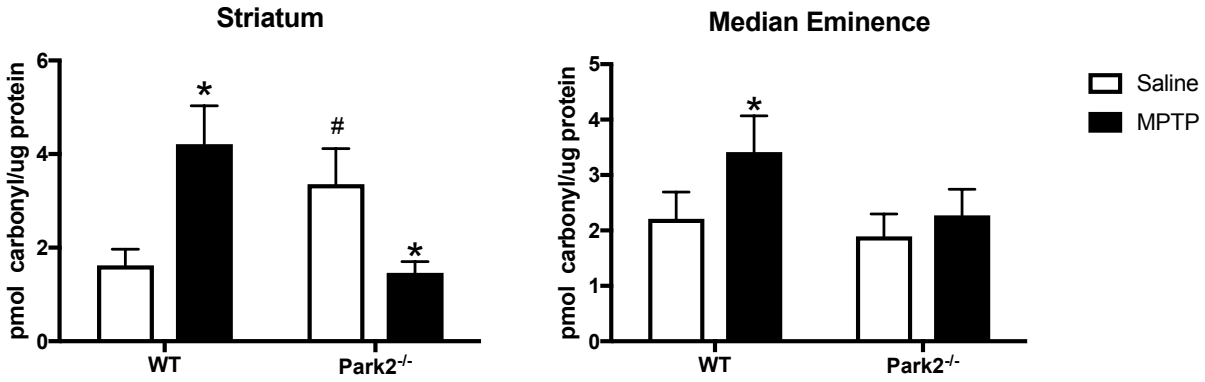

Figure 4-6 Protein carbonylation in the ST and ME of MPTP treated C57BL/6J and Park2$^{-/-}$ mice. C57BL/6J and Park2$^{-/-}$ mice were treated with either saline or MPTP (20 mg/kg; s.c.). Twenty-four h later, the **A,** ST and **B,** ME were collected and protein carbonylation was measured by dot-blot. The bars represent the mean pmol carbonyls/ug protein. * indicates significant difference from the saline control ($p<0.05$, n=8). * indicates significant difference from the corresponding saline treated control mice, # indicates significant difference between strains within the same treatment ($p<0.05$, n=10).

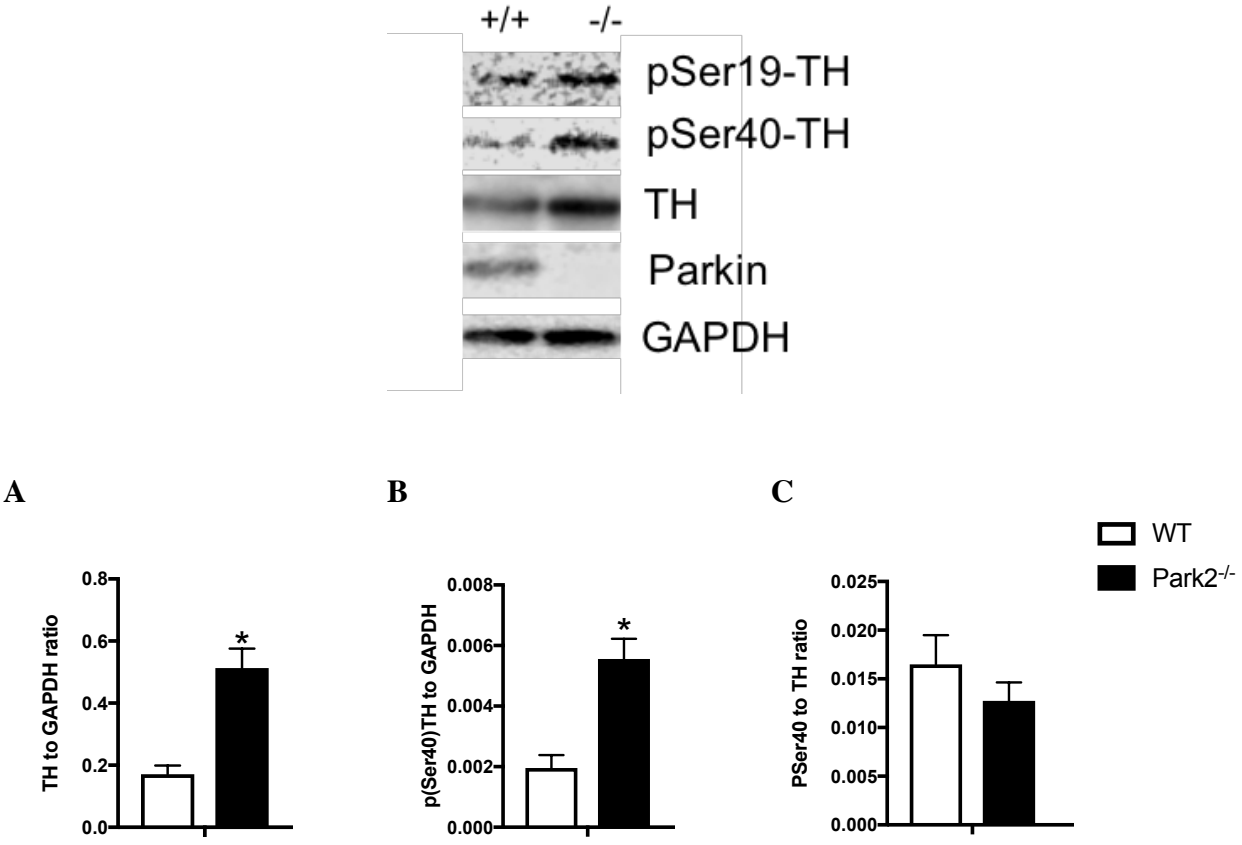

Figure 4-7 TH and pSer40-TH in the ST of C57BL/6J and Park2$^{-/-}$ mice. The ST of C57BL/6J and Park2$^{-/-}$ were analyzed for TH, pSer40-TH and pSer19-TH by Western blot. The bars represent the mean **A**, TH to GAPDH ratio **B**, pSer40-TH to GAPDH ratio and C, the mean ratio of pSer40-TH to TH ratio. Error bars represent +1 SEM. * indicates that the group was significantly different from the WT controls ($P < 0.05$, N=8).

of Park2$^{-/-}$ mice. However, DA neurochemistry (**Figure 4-8**) illustrates that there was no significant effect of parkin deficiency on DA, DOPAC or DOPAC/DA in the ST.

The measurement of DA is not a direct indication of the activity of TH. Tyrosine is first converted to DOPA by TH and then DOPA is decarboxylated by AADC to form DA. To test if Park2$^{-/-}$ mice had more TH activity, WT and Park2$^{-/-}$ mice were injected with NSD-1015, a drug that blocks the activity of AADC, the enzyme necessary for the conversion of DOPA to DA. DOPA accumulation was used as an indicator of TH activity (**Figure 4-9**). Upon NSD-1015 treatment, DOPA accumulation in the ST was not different between the Park2$^{-/-}$ and WT mice. However, when DOPA was normalized to the amount of Ser40 phosphorylated TH, it became clear that although there is not a difference in the amount of DOPA, there is more active TH available in the ST of Park2$^{-/-}$ mice.

Proteasome activity is required for the efficient turnover of TH (Nakashima et al. 2013; Nakashima et al. 2011; Congo Carbajosa et al. 2015). Since there is more phosphorylated TH in Park2$^{-/-}$ mice and TH must be phosphorylated in order to be ubiquitinated and degraded (Congo Carbajosa et al. 2015), ubiquitinated TH was measured. TH was immunoprecipitated (IP) from ST lysates of C57BL/6J and Park2$^{-/-}$ mice and ubiquitinated TH was visualized by Western blot (**Figure 4-10A**). The input lanes demonstrate that there is more ubiquitinated protein in the ST of Park2$^{-/-}$ mice compared to WT mice. The immunoprecipitation output demonstrates that there is more ubiquitinated TH present in the IPs from the Park2$^{-/-}$ mice.

Polyubiquitin chains are formed when the C-terminus of ubiquitin is linked to a lysine (K) residue on the previously added ubiquitin molecule. Polyubiquitin on defined lysines (K; primarily K48 and K63) are related to protein degradation. K48 linked polyubiquitin chains serve as a degradation signal for the 26S proteasome while K63 linked polyubiquitin chains

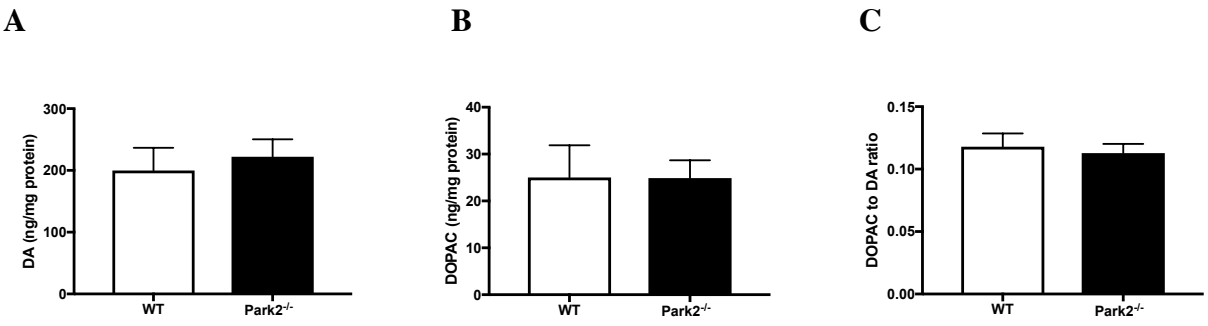

Figure 4-8 DA, DOPAC and, the DOPAC to DA ratio in the ST of C57BL/6J and Park2$^{-/-}$ mice. C57BL/6J and Park2$^{-/-}$ mice were decapitated and ST were collected and analyzed for DA and DOPAC by HPLC-ED. The bars represent the mean concentration of **A**, DA, **B**, DOPAC and **C**, DOPAC/DA ratio. Error bars represent +1 SEM. * indicates that the group is significantly different from the respective C57BL/6J control. # indicates that the group is significantly different from its respective P1 control. ($p < 0.05$, n=10).

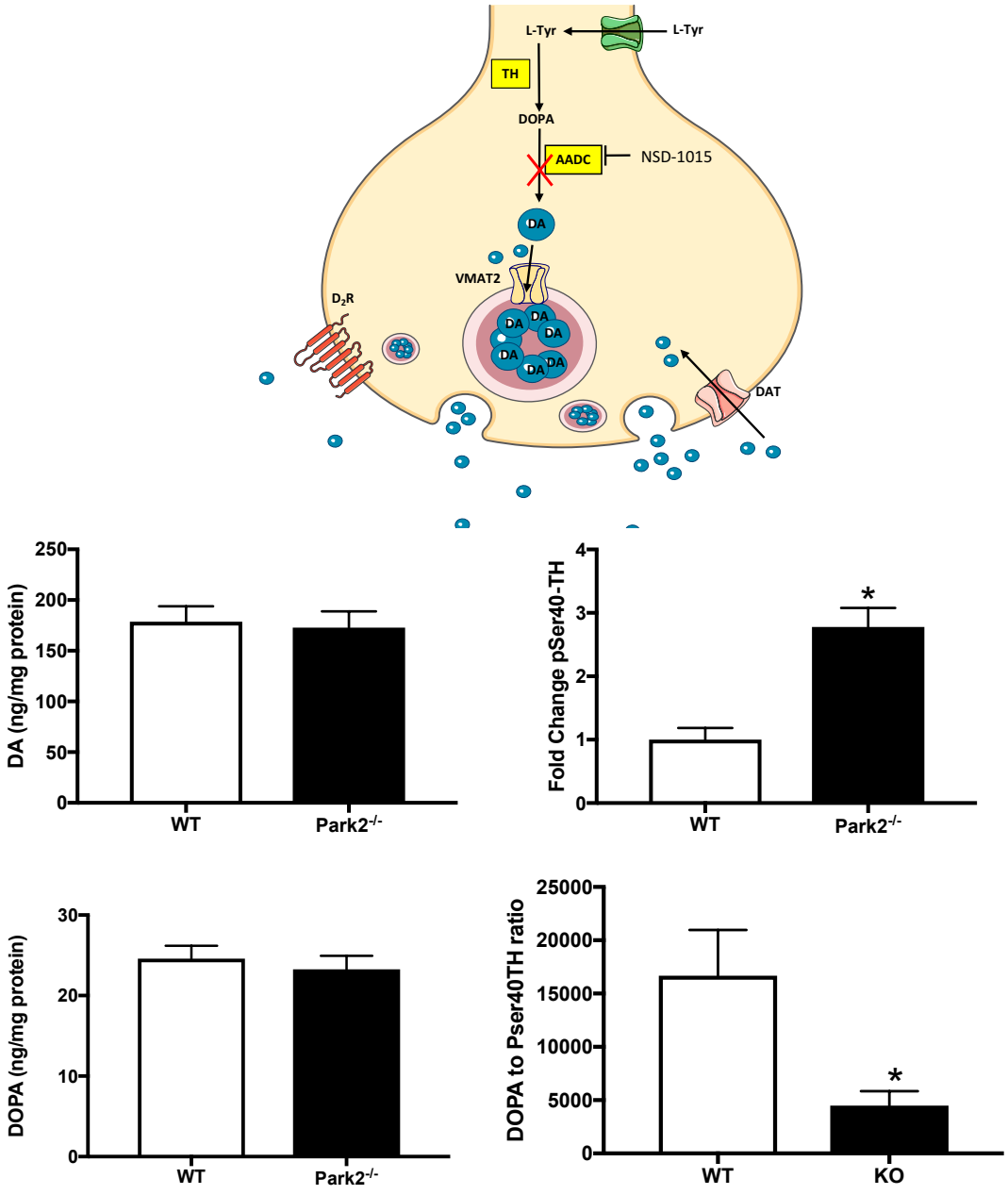

Figure 4-9 DOPA accumulation in the ST of NSD-1015 treated C57BL/6J and Park2$^{-/-}$ mice. Male C57BL/6J mice and Park2$^{-/-}$ mice were treated with 100 mg/kg NSD-1015. Thirty min following NSD-1015 injection, the mice were decapitated and the ST was analyzed for DOPA. The columns represent **A**, the mean concentration of DA **B**, the fold change of pSer40-TH **C**, the mean concentration of DOPA **D**, the mean ratio of DOPA to pSer40-TH +1 SEM. *DOPA concentrations were significantly different from the WT controls ($p < 0.05$, n=8).

A **B**

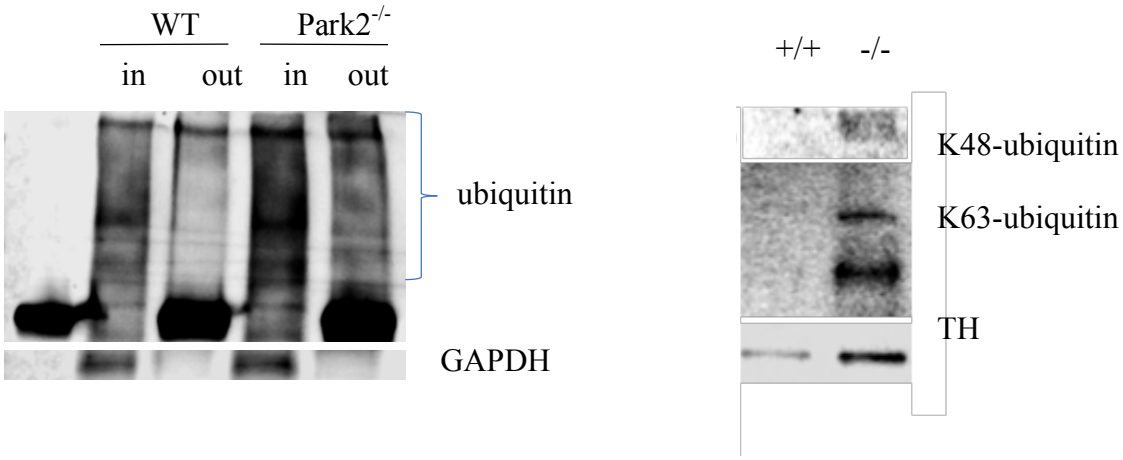

Figure 4-10 Ubiquitinated TH in the ST of C57BL/6J and Park2$^{-/-}$ mice. C57BL/6J and Park2$^{-/-}$ mice were decapitated and the ST was collected. TH was immunoprecipitated from the ST homogenates and subjected to Western blot for **A**, ubiquitin and **B**, K48-linked ubiquitin and K63-linked ubiquitin.

serve as a signal for autophagy (Komander and Rape 2012). **Figure 4-10B** demonstrates that TH from the ST of Park2$^{-/-}$ mice have accumulated both K63 linked polyubiquitinated TH and K48 linked polyubiquitinated TH.

Discussion

In mice, the recovery of vesicular DA in the ME following MPTP exposure is dependent upon the expression of parkin in TIDA neurons (Benskey et al. 2015). Twenty-four h following neurotoxicant exposure, parkin protein expression is increased in the ME and decreased in the ST. Parkin is an E3 ubiquitin ligase that promotes the degradation of its specific protein substrates via the UPS (Shimura et al. 2000) as well as through autophagy (Olzmann and Chin 2008; Narendra et al. 2008). In addition to its E3 ligase function, the N-terminal UBL domain of parkin interacts with proteasome regulatory subunits Rpn10 (Sakata et al. 2003), RPN13 (Aguileta et al. 2015), Rpt6 (Tsai et al. 2003), and α4 (Dachsel et al. 2005) and directly enhances proteasome activity (Um et al. 2010; Hyun et al. 2002; Dachsel et al. 2005). In Chapter 3, regional differences in proteasome activity correspond with changes in parkin expression following neurotoxicant exposure. Data in this chapter demonstrate that parkin is necessary to maintain basal proteasome activity in the ST but not the ME.

For experiments presented in this chapter Park2$^{-/-}$ mice were used. These mice have the exon 3 deletion mutation that is most prevalent in early onset familial PD. Park2$^{-/-}$ mice have increased extracellular DA in the ST and reduced postsynaptic excitability compared to WT mice (Goldberg et al. 2003). As a result, Park2$^{-/-}$ mice demonstrate motor deficits related to dysfunction of the NSDA pathway (Goldberg et al. 2003). While PD patients with this mutation have significant loss of NSDA neurons, the number of TH-ir neurons in the SN in Park2$^{-/-}$ mice

remain comparable to WT mice (Goldberg et al. 2003). This difference between mice and humans may be due to different lifetime environmental exposures or perhaps mice have compensatory mechanisms available that prevent the loss of these neurons.

There is a growing body of evidence that parkin is necessary for maintenance of central DA neuronal function (Benskey et al. 2015; Petrucelli et al. 2002; Narendra et al. 2008; Jiang et al. 2004) and that parkin can act to enhance proteasome activity *in vitro* (Tanaka et al. 2001; Sakata et al. 2003; Aguileta et al. 2015; Tsai et al. 2003; Dachsel et al. 2005; Um et al. 2010; Hyun et al. 2002). Proteasome activity is decreased in the ST of Park2$^{-/-}$ mice while activity is not changed in the ME. This might be due to the higher level of NSDA neuronal activity in the ST compared to TIDA neuronal activity in the ME, which may make the ST more prone to oxidative stress and damage than the ME. Along these lines, an increase in oxidative damage in the ST but not the ME of Park2$^{-/-}$ mice is observed and this corresponds to decreased proteasome activity in the ST and maintained proteasome activity in the ME. These data indicate that parkin is necessary in the ST to maintain proteasome activity and limit oxidative damage under basal conditions while it is not necessary under basal conditions in the ME.

To test the hypothesis that parkin expression is necessary to maintain proteasome activity under conditions of added oxidative insult, Park2$^{-/-}$ mice were treated with MPTP and proteasome activity was measured in the ST and ME. Maintenance of proteasome activity in the ME following this oxidative insult appears to be dependent upon parkin expression as proteasome activity was decreased in the ME of MPTP treated Park2$^{-/-}$ mice but not in WT mice. Interestingly, parkin expression does not appear to be necessary to protect proteins in the ST and ME against oxidative damage, as parkin deficiency results in reduced oxidative damage following neurotoxic insult. This is likely due adaptive mechanisms aimed at compensating for

the presence of ROS and the accumulation of damaged proteins. Periquet and co-workers (2005) identified 87 proteins that were differentially expressed between WT and Park2$^{-/-}$ mice some of these are antioxidants that might be activated by exposure to MPTP. Carboylation can influence cell-signaling pathways implicated in the antioxidant response. For example, KEAP1 (Kelch like associated protein 1) sequesters Nrf2 (nuclear factor like 2) in the cytoplasm. When KEAP1 is carbonylated, it is degraded by the proteasome and releases Nrf2. Nrf2 is then free to bind to the antioxidant response element and increases the expression of antioxidant genes (Jowsey et al, 2003).

Parkin protein is specifically and non-homogenously expressed in the cytoplasm of neurons in the brain and is not present in glial cells (Stichel et al. 2000). Differential centrifugation was used to determine if proteasome activity was decreased thoughout the ST. Proteasome activity is decreased in both the P1 and the P2 fractions from the ST homogenates of Park2$^{-/-}$ mice indicating that proteasome impairment was not enriched at the axon terminals. Measurement of proteasome activity within the S2 fraction may have added some value to this assay, unfortunately the samples were too dilute to obtain accurate measurements of activity.

Parkin has an important role in mitochondrial quality control and a reduction in mitochondrial respiration is observed in synaptosomes derived from the ST of Park2$^{-/-}$ mice (Exner et al. 2007; Narendra et al. 2008; Springer and Kahle 2011; Hawong 2014). Defective mitochondrial respiration can result in ATP depletion. The maintenance of the 26S proteasome complex is dependent upon ATP binding and it is possible that decreased proteasome activity in the ST of Park2$^{-/-}$ mice is due disassembly of the 26S proteasome secondary to ATP depletion. Although the rate of ATP synthesis is decreased in striatal synaptosomes, the decreased rate is compensated for and there is no difference in the amount of ATP in Park2$^{-/-}$ and WT mice

(Pickrell and Youle 2015). Furthermore, physiologic levels of ATP (in the mM range) have a negative effect on 26S proteasome activity. Under stress conditions, where ATP levels are lower, 26S proteasome activity actually increases from the basal level (Huang et al. 2010). Based on these reasons, it is unlikely that ATP depletion accounts for MPTP induced decrease in 26S proteasome activity.

TH is the rate limiting enzyme in DA synthesis (Levitt et al. 1965) and its activity is regulated by both short-term (allosteric regulation, tetrahydrobiopterin availability, catecholamine feedback inhibition and phosphorylation) and long-term transcriptional mechanisms (Fujisawa and Okuno 2005). In addition, TH activity is also regulated through its turnover by the UPS in the short-term and autophagy in the long-term (Congo Carbajosa et al. 2015; Nakashima et al. 2013). While TH is not a specific substrate of parkin E3 ligase function, Park2$^{-/-}$ mice had increased levels of TH phosphorylated at Ser19 and Ser40, post-translational modifications that are essential for TH activity. This data would seem to be in contrast to work by Goldberg and co-workers (2003) who demonstrated that there was no difference in the number of THir neurons in the SN or their projections in the ST of Park2$^{-/-}$ mice compared to WT mice. However, their work quantitated the number of neurons that were THir and not the actual amount of TH or amount of TH per cell.

While Park2$^{-/-}$ mice appear to have more activated TH in the ST, Park2$^{-/-}$ mice do not have different amounts of DA, or more directly DOPA, compared to WT controls. When the level of DOPA is normalized to the amount of Ser40 phosphorylated TH, the amount of DOPA per TH was decreased in the ST of Park2$^{-/-}$ mice compared to the WT controls. It appears that there is more TH in Park2$^{-/-}$ mice but it is likely that the TH is ubiquitinated and perhaps inactive. In fact, TH immunoprecipitated from the ST of Park2$^{-/-}$ mice has increased K48 linked

polyubiquitin as well as K63 linked polyubiquitin. These increases likely reflect the decreased activity of the proteasome in Park2$^{-/-}$ mice and the delayed turnover of activated TH.

Conclusion

Following neurotoxicant exposure, parkin expression is increased in TIDA neurons while it is not in NSDA neurons. The increase in parkin expression is essential for the recovery of DA in TIDA neurons. In Chapter 3, regional differences in proteasome activity corresponded with changes in parkin expression. Here it was demonstrated that parkin is necessary to maintain basal levels of proteasome activity in the ST but not in the ME. Correlating with regional differences in proteasome activity, the amount of oxidatively damaged proteins was increased in the ST but not the ME of Park2$^{-/-}$ mice compared to WT mice. Finally, the amount of TH and phosphorylated TH in the ST of Park2$^{-/-}$ mice was increased compared to the ST of WT mice while the amount of DOPA produced by the enzyme was unchanged. The increase in TH appears to be due to decreased turnover of the protein as both K48 and K63 linked polyubiquitinated TH accumulated in striatal immunoprecipitates of Park2$^{-/-}$ mice but not detectible in WT mice.

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Chapter 5. Parkin is sufficient to maintain proteasome activity in the ST following MPTP exposure

Introduction

The motor symptoms of PD are caused by the loss of NSDA neurons which results in decreased DA levels within the ST (Ehringer and Hornykiewicz 1960) and the resulting deregulation of the basal ganglia circuitry. While NSDA neurons are affected in PD, TIDA neurons in the mediobasal hypothalamus are not susceptible (Langston and Forno 1978). An increased understanding of the molecular pathways involved in the differential susceptibility between these neuronal populations could lead to the identification of pathways within the TIDA neurons that may be exploited to protect against progressive loss of NSDA neurons of a PD patient.

Although the cause of PD is unknown, cumulative evidence suggests that oxidative stress plays a key role in the progression of this debilitating disease. In brains from PD patients increased oxidative protein damage is evident in the SN (Floor and Wetzel 1998) as well as decreased levels of the reduced antioxidant glutathione (Bharath et al. 2002; Pearce et al. 1997). Oxidative modification of proteins can result in conformational changes and misfolding that render the protein inactive and promotes the formation of toxic protein aggregates (Mirzaei and Regnier 2008). In this respect, cellular homeostasis is dependent upon the removal of oxidatively damaged proteins.

The UPS plays a pivotal role in the selective recognition and degradation of oxidized proteins (Chung, Dawson, and Dawson 2001; Ross and Pickart 2004; Ciechanover 2005; Lehman 2009) and has been implicated in the pathogenesis of several neurodegenerative diseases including Alzheimer's Disease, amyotrophic lateral sclerosis and PD (Ciechanover and Brundin

2003). In fact, proteasome activity is reduced in susceptible brain regions from PD patients (McNaught et al. 2002; Bukhatwa et al. 2010; McNaught and Jenner 2001; Martins-Branco et al. 2012). This highly regulated proteolytic system affects many cellular processes through the selective degradation of an assortment of protein substrates. Dysfunction of the UPS can result in the accumulation and aggregation of misfolded proteins, which can further impair catalytic activity within the neuron.

The proteasome is a large multi-protease complex that contains multiple peptidase activities including CT-L, Casp-L and T-L activities that degrade its substrate proteins into short polypeptides and amino acids (Hershko and Ciechanover 1998). The 20S core proteasome particle is ATP-independent and can degrade small denatured or intrinsically disordered proteins. Larger ordered proteins are degraded by the 26S proteasome, an ATP-dependent complex that is composed of the 20S core particle and the 19S regulatory complex. Protein degradation by the 26S proteasome is initiated by covalent attachment of ubiquitin to the protein through the action of E1 ubiquitin activating, E2 ubiquitin conjugating, and E3 ubiquitin ligating enzymes (Scheffner, Nuber, and Huibregtse 1995). For proteins degraded via the UPS, the 19S regulatory particle is necessary to recognize polyubiquitin linked proteins and translocate the substrates into the catalytic chamber of the 20S core (Chung, Dawson, and Dawson 2001).

As demonstrated in **Figure 3-3**, the neurotoxicant MPTP is useful in producing oxidative stress in DA neurons and can replicate the pattern of DA neuronal susceptibility that is seen in PD (Behrouz et al. 2007; Benskey et al. 2012; Benskey et al. 2013; Melamed et al. 1985; Mogi et al. 1988). While both NSDA and TIDA neuronal populations initially respond to the neurotoxicant, TIDA neurons recover from the initial insult, while NSDA neurons do not (Benskey et al. 2012). Recovery of TIDA neurons following MPTP exposure is dependent upon

increased expression of parkin mRNA and protein (Benskey et al. 2012). In contrast, there is no change in parkin mRNA expression or the amount of parkin protein decreases in susceptible NSDA neurons following MPTP exposure. Furthermore, rAAV mediated shRNA knockdown of parkin in the ARC containing cell bodies of TIDA neurons results in susceptibility of these neurons to MPTP (Benskey, Kuhn, et al. 2015). Taken together, it appears that parkin expression is necessary to protect TIDA neurons from neurotoxic exposure to MPTP.

The ventral midbrain containing NSDA neurons has reduced parkin expression in sporadic PD (Kitada et al. 1998) and NSDA neurons rapidly degenerate in patients with loss of parkin function due to homozygous mutations (Kitada et al. 1998; von Coelln, Dawson, and Dawson 2004). Parkin is an E3 ubiquitin ligase that promotes the degradation of its specific protein substrates via the UPS (Shimura et al. 2000; Olzmann and Chin 2008) and autophagy (Olzmann and Chin 2008; Narendra et al. 2008). In addition to its E3 ligase function, the N-terminal UBL domain of parkin directly interacts with proteasome subunits Rpn10 (Sakata et al. 2003), RPN13 (Aguileta et al. 2015), Rpt6 (Tsai et al. 2003), and α4 (Dachsel et al. 2005) and directly enhances proteasome activity (Um et al. 2010; Hyun et al. 2002; Dachsel et al. 2005).

In Chapters 3 & 4, it was revealed that following MPTP exposure the increase in parkin expression is essential for the maintenance of proteasome activity in the ME and for the efficient turnover of the PD related UPS substrate TH which is critical to maintaining neurotransmitter levels in central DA neurons. **In the present chapter, studies are designed to test the hypothesis that increased parkin expression is sufficient to maintain proteasome activity in the ST following MPTP exposure and that maintenance of proteasome activity will result in the efficient turnover of TH.** In order to test this hypothesis, proteasome activity in the ipsilateral F-hParkin over-expressing ST was compared to the contralateral ST. In addition,

changes in TH expression and enzymatic activity were examined in these experiments to determine if F-hParkin over-expression was successful in facilitating the recovery of the NSDA neurons following neurotoxicant exposure.

Results

Parkin Enhances Proteasome Activity in Vitro

In the previous chapter, it was demonstrated that basal proteasome activity is decreased in the ST of Park2$^{-/-}$ mice compared to WT mice and that these mice are unable to maintain proteasome activity following MPTP exposure (Chapter 4). Um and co-workers (Um et al. 2010) demonstrated that parkin over-expression in HEK cells results in enhanced proteasome activity. To determine if purified parkin can increase proteasome activity in a cell free system, the activity of untreated purified 26S proteasome was measured and compared to the activity of 26S proteasome that had been treated with either bovine serum albumin (BSA) or 6His-Parkin (**Figure 5-1**). When tested *in vitro*, there was a small increase in proteasome activity in the presence of 6His-Parkin, but not BSA which indicates that parkin can directly modulate proteasome activity.

In Chapter 3, it was demonstrated that MPP^+ causes a concentration dependent decrease in proteasome activity *in vitro*. To determine if 6His-Parkin could protect the proteasome from MPP^+ impairment, dose responses to MPP^+ were measured in the presence of vehicle, BSA or 6His-Parkin (**Figure 5-2**). Treatment with 6His-Parkin results in an upward shift of the MPP^+ dose response curve, indicating that proteasome activity was enhanced compared to that of vehicle alone (**Figure 5-2**).

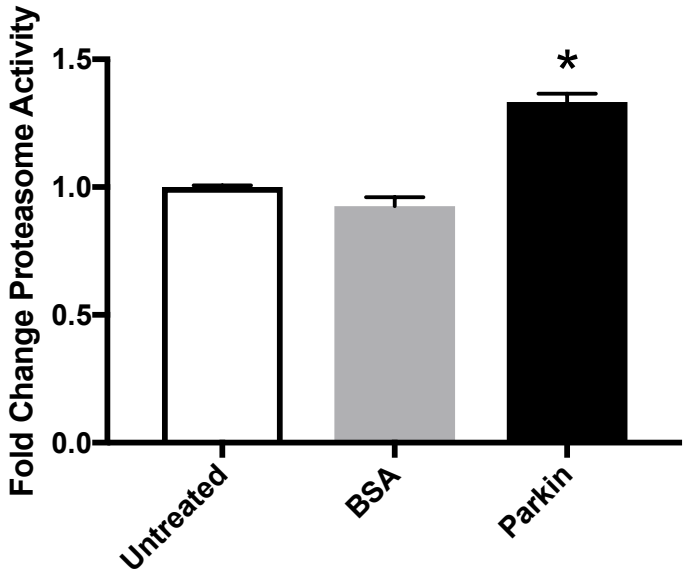


Figure 5-1 Effect of 6His-Parkin on *in vitro* proteasome activity. Purified 26S proteasome in proteasome activity buffer was left untreated or treated with 10 nM BSA or 10 nM 6His-Parkin and CT-L activity was measured using 100 μM Suc-LLVY-AMC as a substrate. Bars represent the mean fold change in CT-L proteasome activity. Error bars represent +1 SEM. * Indicates that CT-L activity was significantly different from the untreated control ($P < 0.05$, n= 4).

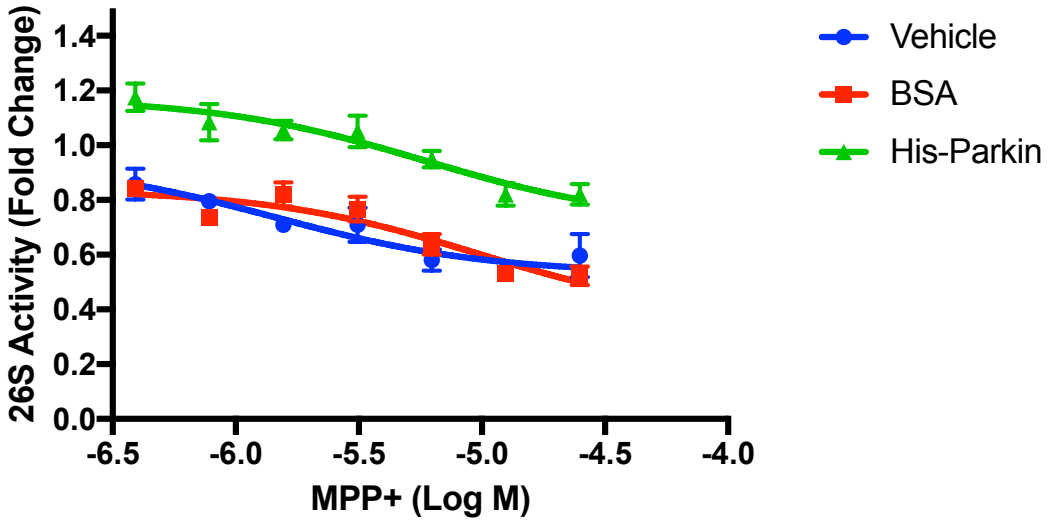


Figure 5-2 The effect of 6His-Parkin on proteasome impairment by MPP^+. Purified 26S proteasome (1 nM) was treated with either 10 nM bovine serum albumin or 10 nM 6HisParkin. The samples were then treated with various concentrations of MPP^+. Proteasome activity was measured and expressed as fluorescent units released per s. Error bars represent fold change from untreated 26S proteasome + 1 SEM. * indicates significant difference to the corresponding MPP+ sample ($P < 0.05$, n=4).

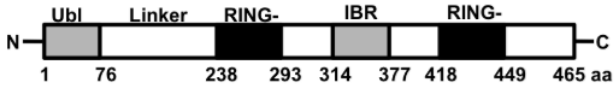
Characterization of rAAV Vector Expression in the ST and SN

Since parkin protein is necessary to maintain proteasome activity in the ME following MPTP exposure, we hypothesized that rAAV2/5 mediated over-expression of F-hParkin in the SN containing NSDA neuron cell bodies would restore proteasome activity in the ST of Park2$^{-/-}$ mice and maintain proteasome activity following MPTP exposure. Park2$^{-/-}$ mice were injected unilaterally with rAAV2/5 F-hParkin (**Figure 5-3**) and on the contralateral side with rAAV2/5 GFP. **Figure 5-4** shows immunohistochemical characterization of TH (red) and GFP (green) expressing neurons. NSDA neurons that were successfully transduced were expressing both TH and GFP (yellow). GFP expression was observed in about 15% of the NSDA soma in the SN. Four weeks following rAAV-F-hParkin injection, unilateral parkin protein expression in the ST of Park2$^{-/-}$ mice was demonstrated in 75% of the rAAV-F-hParkin injected animals by Western blot. Mice that did not demonstrate detectable amounts of parkin in the Western blot were excluded from the data analysis for this experiment.

Proteasome Activity in the ST is Enhanced with rAAV2/5 Mediated Over-expression of F-hParkin in the SN.

Four weeks following rAAV-F-hParkin injection, CT-L proteasome activity of the GFP or F-hParkin expressing ST was compared to activity in the ST of WT control mice injected with rAAV2/5 GFP (**Figure 5-5**). CT-L proteasome activity was not different between the uninjected side and the rAAV-GFP injected side of the WT control animal ST (**Figure 5-5A**). Proteasome activity was decreased in the ST of GFP injected Park2$^{-/-}$ mice compared to the GFP injected WT mice (**Figure 5-5B**). However, proteasome activity in the ST of the F-hParkin injected side of

A



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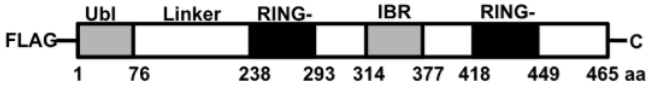

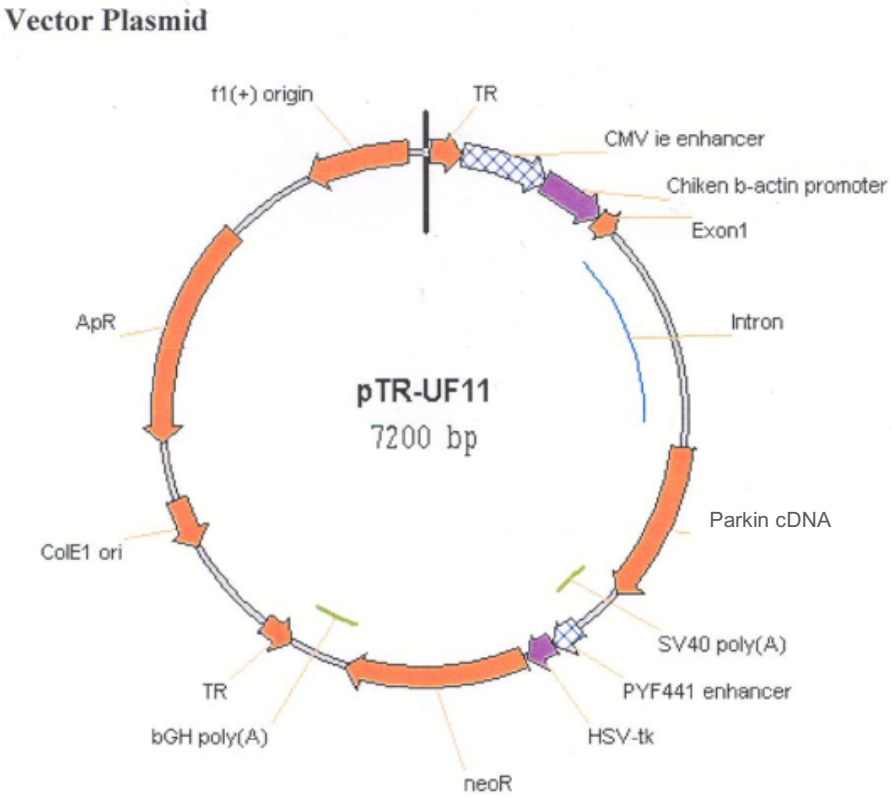

Figure 5-3 F-hParkin vector construct. A, hParkin WT containing the N-terminal UBL domain, linker region, RING-1, IBR, and RING2 domains. **B,** N-terminal Flag tag on hParkin for insertion into pTR-UF11. **C**, Map of the rAAV vector pTR UF11 containing the AAV2 inverted terminal repeats, Flag-hParkin cDNA and a CMV-chicken β-actin promoter.

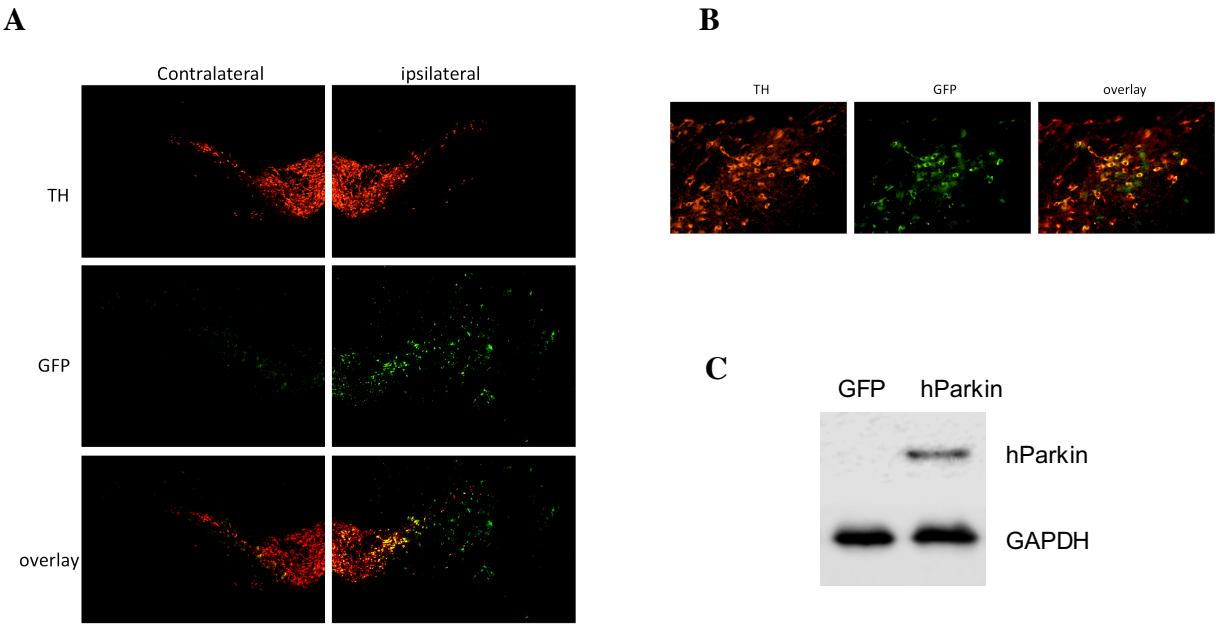

Figure 5-4 Immunohistochemical characterization of rAAV2/5 mediated gene expression in the SN. C57BL/6J and Park2$^{-/-}$ mice received unilateral 500 nL stereotaxic injections of rAAV2/5-F-parkin (3.4 X 10^{12}) into the SN. The left, contralateral SN was injected with 1 X 10^{12} rAAV2/5-GFP and used as a control. Four weeks following injection, mice were sacrificed and the brains were processed to visualize TH (red) and GFP (green). NSDA neurons are shown as TH-ir (red). NSDA neurons expressing GFP (yellow) are shown as in the overlay. **Panel A** demonstrates GFP expression in the right SN, leaving the left SN devoid of GFP expression. **Panel B** demonstrates co-localization of GFP within TH expressing neurons of the left SN. **Panel C** demonstrates that F-Parkin is expressed only in the ipsilateral ST of Park2$^{-/-}$ mice.

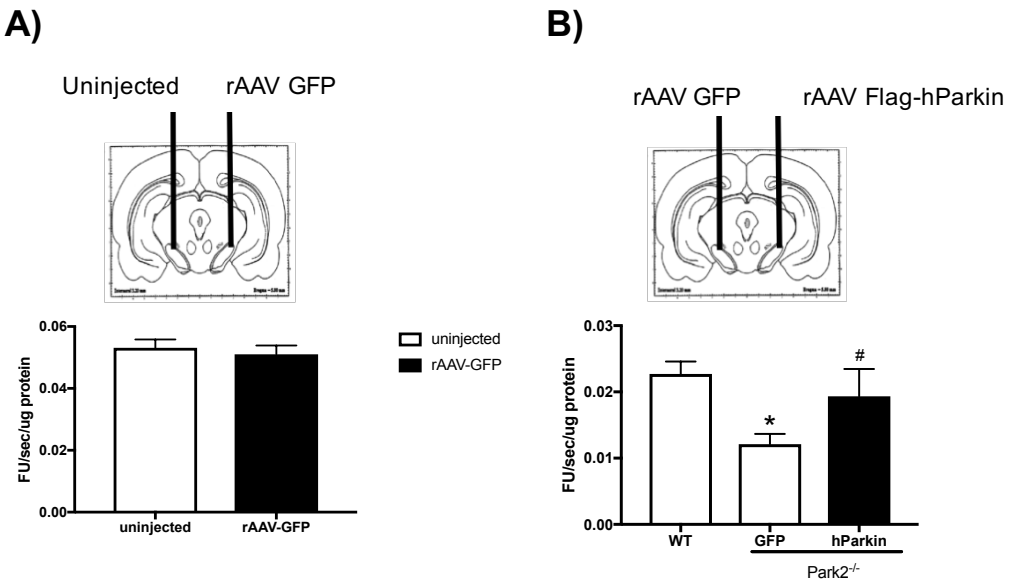

Figure 5-5 Effect of F-hParkin over-expression in the SN on proteasome activity in the ST. C57BL/6J mice were unilaterally injected with 500 nL of rAAV2/5 GFP and left uninjected on the contralateral side; Park2$^{-/-}$ mice were injected with 500 nL rAAV2/5 Flag-hParkin (500 nL; 3.4 X10^{12} vg/mL) in the SN and rAAV2/5 GFP in the contralateral SN. Four weeks following rAAV injections tissue from the ST was collected and proteasome activity was measured. **A**, Proteasome activity was not different between the rAAV2/5 GFP and the uninjected ST. **B**, Proteasome activity was decreased in the rAAV2/5 GFP injected Park2$^{-/-}$ ST compared to the WT rAAV2/5 GFP injected ST. Error bars represent +1 SEM. * indicates significant difference from the WT rAAV GFP injected control ST ($p<0.05$, n=10).

the Park2$^{-/-}$ mice was not significantly different from WT GFP treated controls. Changes in proteasome activity were further characterized by measuring changes in CT-L, Cas-L, and T-L activity (**Figure 5-6**). Over-expression of F-hParkin results in increased CT-L and T-L activity, but not Cas-L activity.

To determine if F-hParkin over-expression was sufficient to restore proteasome activity following a single acute exposure to MPTP, Park2$^{-/-}$ mice were unilaterally injected in the SN with rAAV2/5 F-hParkin and rAAV2/5 GFP contralaterally. Four weeks later, the mice were treated with either saline or MPTP and decapitated after 24 h. Proteasome activity was measured in the GFP and F-hParkin expressing ST (**Figure 5-7**). CT-L proteasome activity in the ST of the GFP expressing side of WT and Park2$^{-/-}$ mice were reduced following MPTP exposure as compared to vehicle treated animals (**Figure 5-7A**). Over-expression of hParkin in Park2$^{-/-}$ mice increased CT-L activity compared to the GFP expressing side of the ST. MPTP also caused a decrease in Casp-L activity on the GFP expressing ST, but did not have a significant effect on the hParkin over-expressing side (**Figure 5-7B**). T-L activity was increased on the hParkin over-expressing side of the ST in vehicle treated animals and T-L activity did not decrease in the ST with exogenous hParkin over-expression following MPTP treatment (**Figure 5-7C**).

Twenty-four h following MPTP exposure, the amount of 26S proteasome complex was unchanged in the ST, while the amount of 20S complex was increased compared to the saline treated controls (**Figure 3-12**). In addition, the amount of PSMβ5 in the ST was increased (**Figure 3-11**). To further evaluate observed changes in total proteasome activity following MPTP exposure, we sought to determine the changes occurred specifically in 26S or 20S proteasome activity. To this end, mice were injected unilaterally with rAAV2/5-GFP/rAAV2/5-F-hParkin and an in-gel CT-L assay was used in conjunction with a native gel Western blot to

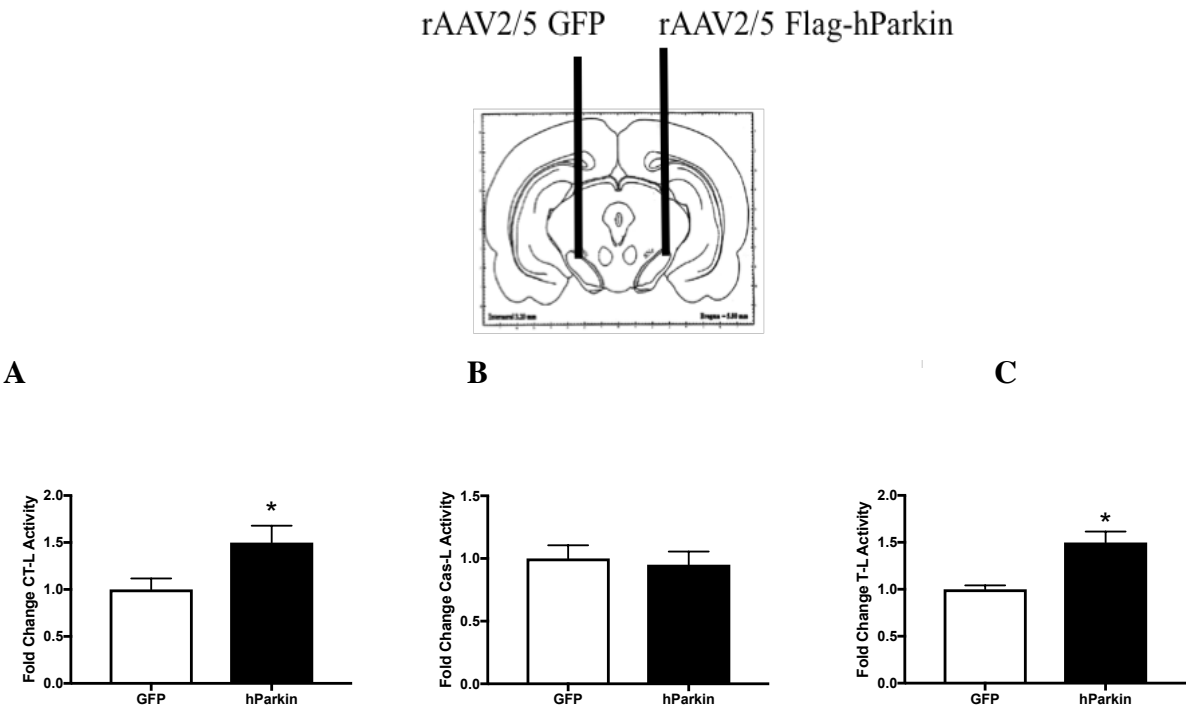

Figure 5-6 Effect of F-hParkin over-expression in the SN on CT-L, Casp-L and T-L proteasome activity in the ST. Park2$^{-/-}$ mice were injected with 500 nL rAAV2/5 Flag-hParkin (500 nL; 3.4 X10^{12} vg/mL) in the SN and rAAV2/5 GFP in the contralateral SN. Four weeks following rAAV injections tissue from the ST was collected and proteasome activity was measured. **A**, CT-L activity; **B**, Casp-L activity; **C**, T-L activity. Bars represent the mean FU/sec/μg protein. Error bars represent +1 SEM. * indicates significant difference from the WT rAAV GFP injected control ST ($p<0.05$, n=10).

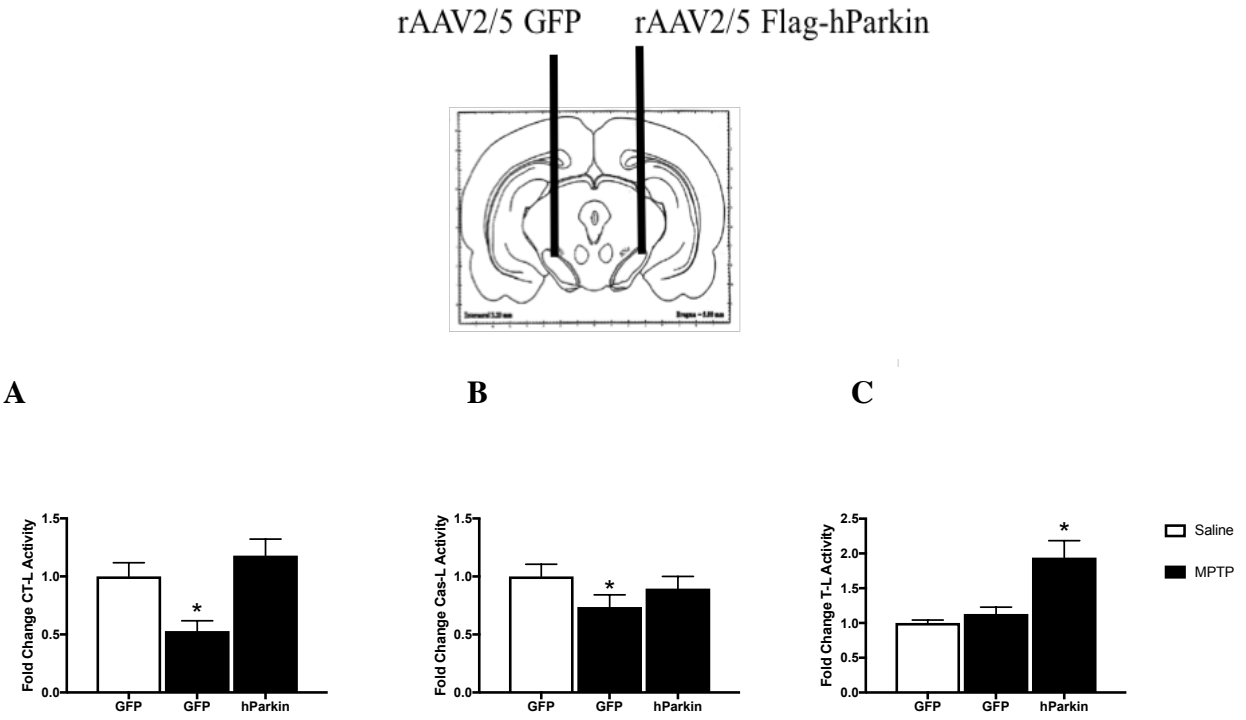

Figure 5-7 Effect of F-hParkin over-expression in the SN on CT-L, Casp-L and T-L proteasome activity in the ST of MPTP treated mice. Park2$^{-/-}$ mice were injected with 500 nL rAAV2/5 Flag-hParkin (500 nL; 3.4 X10^{12} vg/mL) in the SN and rAAV2/5 GFP in the contralateral SN. Four weeks following rAAV injections mice were injected with either saline or 20 mg/kg MPTP. Twenty-four h following injections tissue from the ST was collected and proteasome activity was measured. **A**, CT-L activity; **B**, Casp-L activity; **C**, T-L activity. Bars represent the mean FU/sec/µg protein. Error bars represent +1 SEM. * indicates significant difference from the saline injected animals ($p<0.05$, n=10).

determine both the activity and amount of the 26S and 20S components of the proteasome complex (**Figure 5-8**). In saline treated mice, F-hParkin over-expression caused an increase in 26S CT-L activity compared to the GFP over-expressing side. This increase in 26S CT-L activity on the F-hParkin over-expressing side corresponded with an increase in the amount of 26S proteasome. Twenty-four h post MPTP, 26S CT-L activity was decreased on the GFP over-expressing side, whereas 26S CT-L activity was maintained on the F-hParkin over-expressing side. The decrease in 26S proteasome activity on the GFP over-expressing side corresponded with a decrease in the amount of 26S proteasome, whereas the increase in 26S proteasome activity on the F-hParkin expressing side corresponded with maintained 26S proteasome activity.

rAAV2/5 Mediated Over-expression of F-hParkin Abrogates the Accumulation of pSer40-TH and Increases TH activity in the Park2$^{-/-}$ ST

Data presented in Chapter 4 demonstrated that although DA stores in the ST of Park2$^{-/-}$ mice are similar to that in the ST of WT mice, Park2$^{-/-}$ mice accumulate ubiquitinated TH in the ST. To determine if over-expression of F-hParkin and restoration of proteasome activity was sufficient to allow effective turnover of TH, mice were injected unilaterally with rAAV2/5-GFP/rAAV2/5-hParkin and lysates from the ST were subjected to Western blot for TH and pSer40-TH (**Figure 5-9**). As shown in **Figure 4-7**, Park2$^{-/-}$ mice have increased pSer40-TH in the ST compared to WT mice. Over-expression of F-hParkin in the ST of WT mice did not result in any change to either TH or pSer30 TH, over-expression of F-hParkin in the ST of Park2$^{-/-}$ mice resulted in decreased pSer40-TH compared to the GFP injected side. Furthermore, the

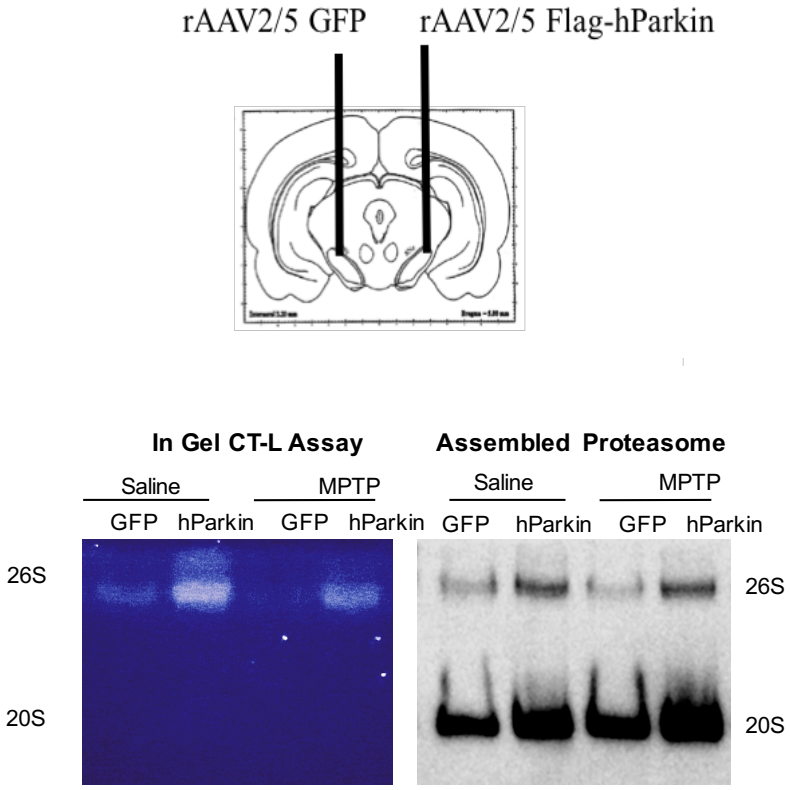

Figure 5-8 Effect of F-hParkin over-expression in the SN on proteasome assembly and activity in the ST of mice treated with MPTP. Park2$^{-/-}$ mice were injected with 500 nL rAAV2/5 Flag-hParkin (500 nL; 3.4 X10^{12} vg/mL) in the SN and rAAV2/5 GFP in the contralateral SN. Four weeks following rAAV injections striatal tissue collected and lysates were subjected to native gel electrophoresis. **A,** CT-L activity of either the 26S or 20S proteasome was measured directly in the gel. **B,** 20S proteasome complex was detected with an antibody directed toward the β5 catalytic subunit and the 26S proteasome was detected with an antibody directed toward the 19S regulatory subunit RPN10.

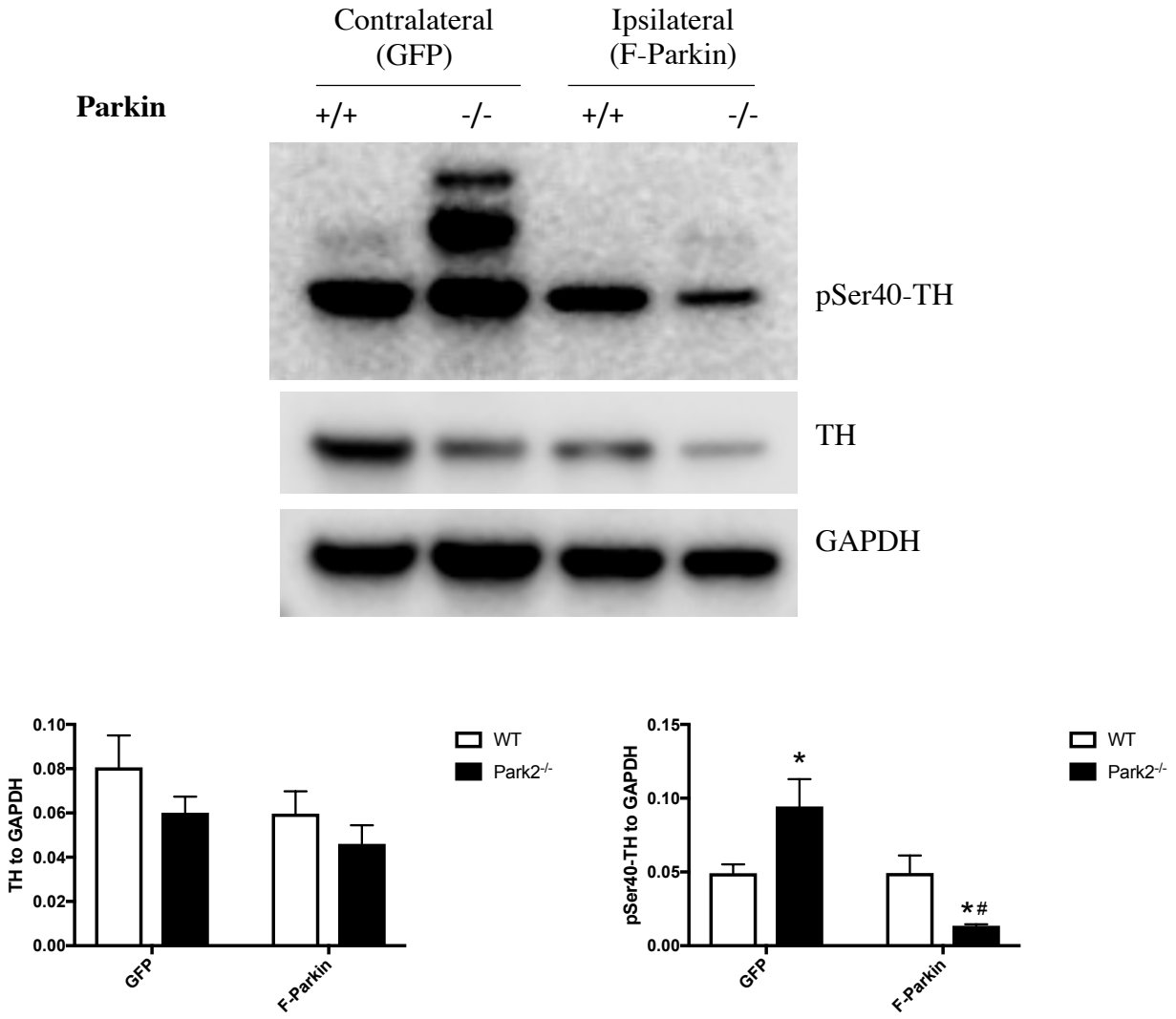

Figure 5-9 Effect of F-hParkin over-expression in the SN on accumulation of TH in the ST of C57BL/6J and Park2$^{-/-}$ mice. C57BL/6J and Park2$^{-/-}$ mice were injected with 500 nL rAAV2/5 Flag-hParkin (500 nL; 3.4 X10^{12} vg/mL) in the ipsilateral SN and rAAV GFP in the contralateral SN. Four weeks following rAAV2/5 injections tissue from the ST was collected and lysates were subjected to SDS page. TH was analyzed by Western blot using antibodies against TH and pSer40-TH. The bars represent the mean TH to GAPDH reatios. Error bars represent +1 SEM. * indicates significant difference from the GFP control ($p < 0.05$, n=10).

amount of pSer40-TH was less than that of the ST from GFP injected WT mice. Interestingly, this did not affect the amount of DA or DOPAC in the ipsilateral side of the ST (**Figure 5-10)**.

The measurement of DOPA following pharmacological blockade of conversion to DA is an index of the *in vivo* activity of TH (Demarest and Moore 1980; Demarest, Alper, and Moore 1979). Tyrosine is first converted to DOPA by TH and then DOPA is decarboxylated by AADC to form DA. To test if Park2$^{-/-}$ mice had increased TH activity, WT and Park2-/- mice were injected unilaterally with rAAV2/5-GFP/rAAV2/5-hParkin. Four weeks later, mice were injected with NSD-1015, a drug that blocks the activity of AADC, the enzyme necessary for the conversion of DOPA to DA. DOPA accumulation was used as an indicator of TH activity (**Figure 5-11**). WT mice over-expressing F-hParkin had no significant change in TH activity. However, Park2$^{-/-}$ mice that were over-expressing F-hParkin in the ST had increased TH activity compared to the GFP injected side.

Over-expression of F-hParkin does not Rescue DA Stores Following MPTP Exposure

F-hParkin over-expression increases the activity of pSer40-TH, and it is possible that this increase could facilitate the recovery of DA stores following an exposure to MPTP. Therefore, it is reasonable to question if F-hParkin over-expression will result in increased TH activity following MPTP exposure. Mice unilaterally expressing rAAV-GFP/rAAV-hParkin were treated with a single acute dose of MPTP and 24 h later treated with the AADC inhibitor, NSD-1015. DOPA accumulation and pSer40-TH were measured as indicators of TH activity. **Figure 5-12** demonstrates that pSer40-TH activity was increased on the F-hParkin expressing side of the ST in both the WT and Park2$^{-/-}$ saline treated mice. However, pSer40-TH activity remained

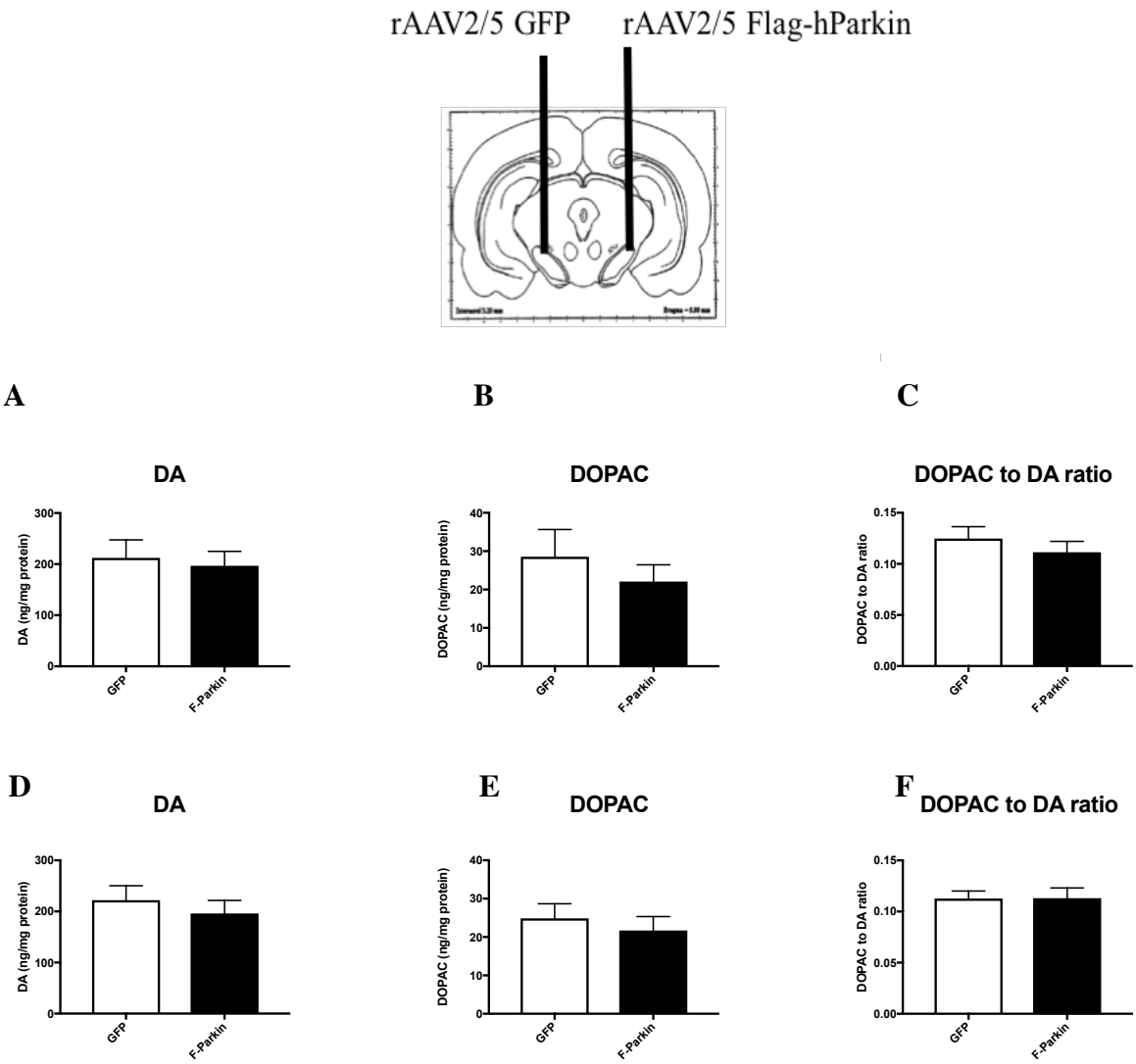

Figure 5-10 Effect of F-hParkin over-expression in the SN on DA, DOPAC, and the DOPAC to DA ratio in the ST of C57BL/6J and Park2$^{-/-}$ mice. The effects of F-hParkin on concentrations of DA, DOPAC and, the ratio of DOPAC to DA in axon terminals of NSDA neurons in the ST of C57BL/6J mice (**A-C**) and Park2$^{-/-}$ mice (**D-F**). C57BL/6J and Park2$^{-/-}$ mice received unilateral 500 nL stereotaxic injections of rAAV-F-hParkin (3.4 X 10^{12} vg/mL) into the right, SN. The left, contralateral SN was injected with 1 X 10^{12} vg/mL of rAAV-GFP and used as a control. Four weeks following injections, mice were sacrificed and the ipsilateral and contralateral ST were analyzed for DA (**A and D**), DOPAC (**B and E**), and the DOPAC to DA ratio (**C and F**). Bars represent the mean neurochemical concentration or ratio. Error bars represent +1 SEM. * Indicates significant difference ($P < 0.05$, n=10) from the saline treated controls.

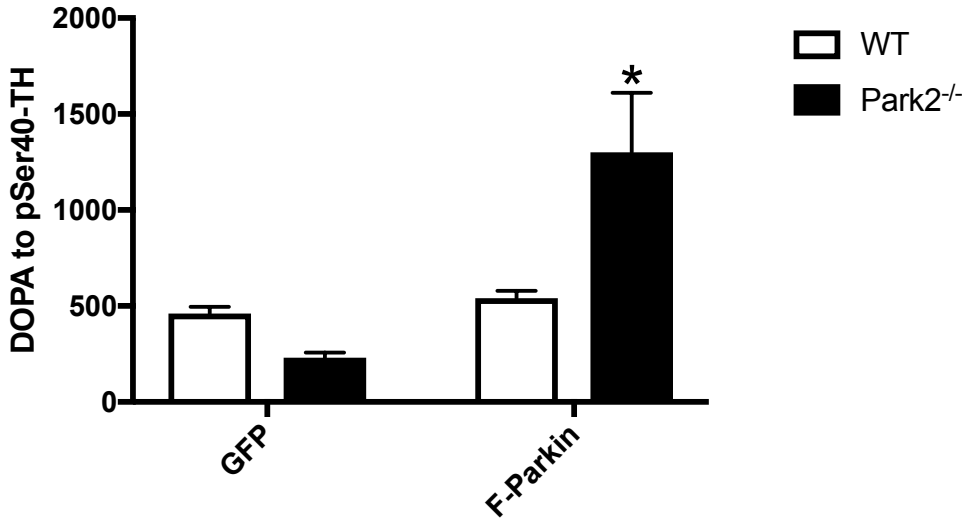

Figure 5-11 Effect of F-hParkin over-expression in the SN on TH activity in the ST of C57BL/6J and Park2$^{-/-}$ mice. C57BL/6J and Park2$^{-/-}$ mice (n=15) received unilateral 500 nL stereotaxic injections of rAAV-F-Parkin (3.4 X 10^{12}) into the SN. The contralateral SN was injected with 3.4 X 10^{12} rAAV-GFP and was used as an internal control. Four weeks following injection the mice were injected with 100 mg/kg NSD-1015. Thirty min following injection, mice were decapitated and the ST was analyzed for DOPA. The bars represent mean ratio of DOPA to pSer40-TH. Error bars represent +1 SEM. *DOPA to TH ratios were significantly different ($p < 0.05$) from the contralateral GFP controls.

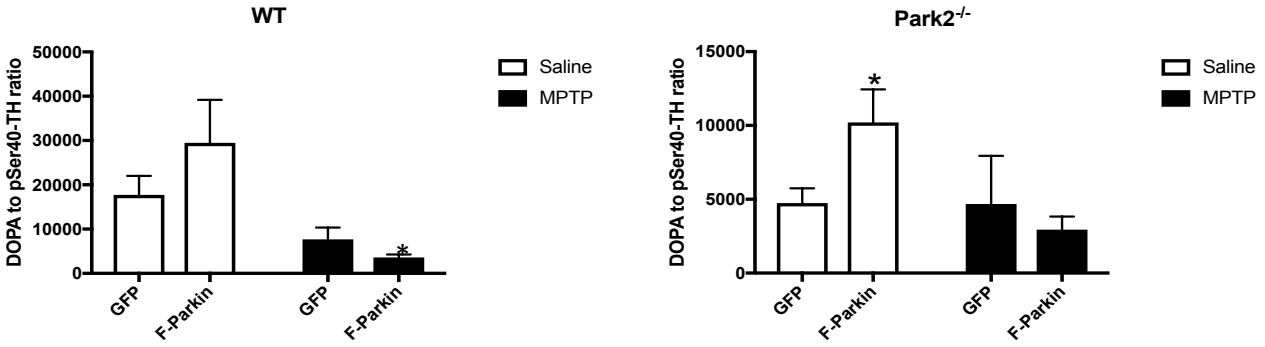

Figure 5-12 Effect of F-hParkin over-expression in the SN on TH activity in the ST of C57BL/6J and Park2$^{-/-}$ mice treated with MPTP. Male C57BL/6J and Park2$^{-/-}$ mice (n=15) received unilateral 500 nL stereotaxic injections of rAAV-F-Parkin (3.4 X 10^{12}) into the SN. The contralateral SN was injected with 3.4 X 10^{12} rAAV-GFP and was used as an internal control. Four weeks following injection mice were treated with either vehicle (saline) or 20 mg/kg MPTP. Two h following MPTP injection, the animals were injected with 100 mg/kg NSD-1015. Thirty min following NSD-1015 injection, the mice were decapitated and the ST was analyzed for DOPA. The columns represent mean concentration of DOPA +1 SEM. *DOPA concentrations were significantly different ($p < 0.05$) from the respective controls.

decreased following MPTP exposure regardless of F-hParkin over-expression. The ST of rAAV-GFP/rAAV-hParkin over-expressing WT and Park2$^{-/-}$ mice were also analyzed for neuro-

chemical content (**Figure 5-13**). In both WT and the Park2$^{-/-}$ mice, a single acute exposure to MPTP resulted in sustained depletion of DA, decreased DOPAC and increased DOPAC to DA ratios, which is indicative of increased DA turnover. In both the WT and Park2$^{-/-}$ mice, there was no significant difference between the GFP and F-Parkin expressing sides of the ST for either DOPAC or DA. However, F-hParkin over-expression in WT mice did result in a decrease in the DOPAC to DA ratio following MPTP exposure. **Figure 5-13** also demonstrates that TH activity following MPTP exposure was not rescued by F-hParkin over-expression.

Discussion

Most neurodegenerative diseases involve the loss of a specific subpopulation of neurons. In PD, NSDA neurons are lost while TIDA neurons are resistant to degeneration (Langston and Forno 1978). The DA neuron specific neurotoxicant MPTP recapitulates the specific neuronal population susceptibility that is seen in PD (Langston and Forno 1978; Benskey et al. 2013). In mice, both NSDA and TIDA neuronal populations initially respond to MPTP, however, TIDA neurons recover from neurotoxic insult, while NSDA neurons do not (Benskey et al. 2012; Benskey et al. 2013). The recovery of vesicular DA in the ME following MPTP exposure is dependent upon the expression of parkin in TIDA neurons (Benskey, Manfredsson, et al. 2015). Differential parkin expression following exposure to MPTP is of interest because mutations in the Park2 gene cause autosomal recessive juvenile PD (Hattori et al. 1998; Kitada et al. 1998; Sinha et al. 2005) and oxidative modifications causing parkin loss of function in the ST are found in patients with sporadic PD (Chung et al. 2004). Parkin is an E3 ubiquitin ligase that promotes the degradation of its specific protein substrates via the UPS (Shimura et al. 2000) as

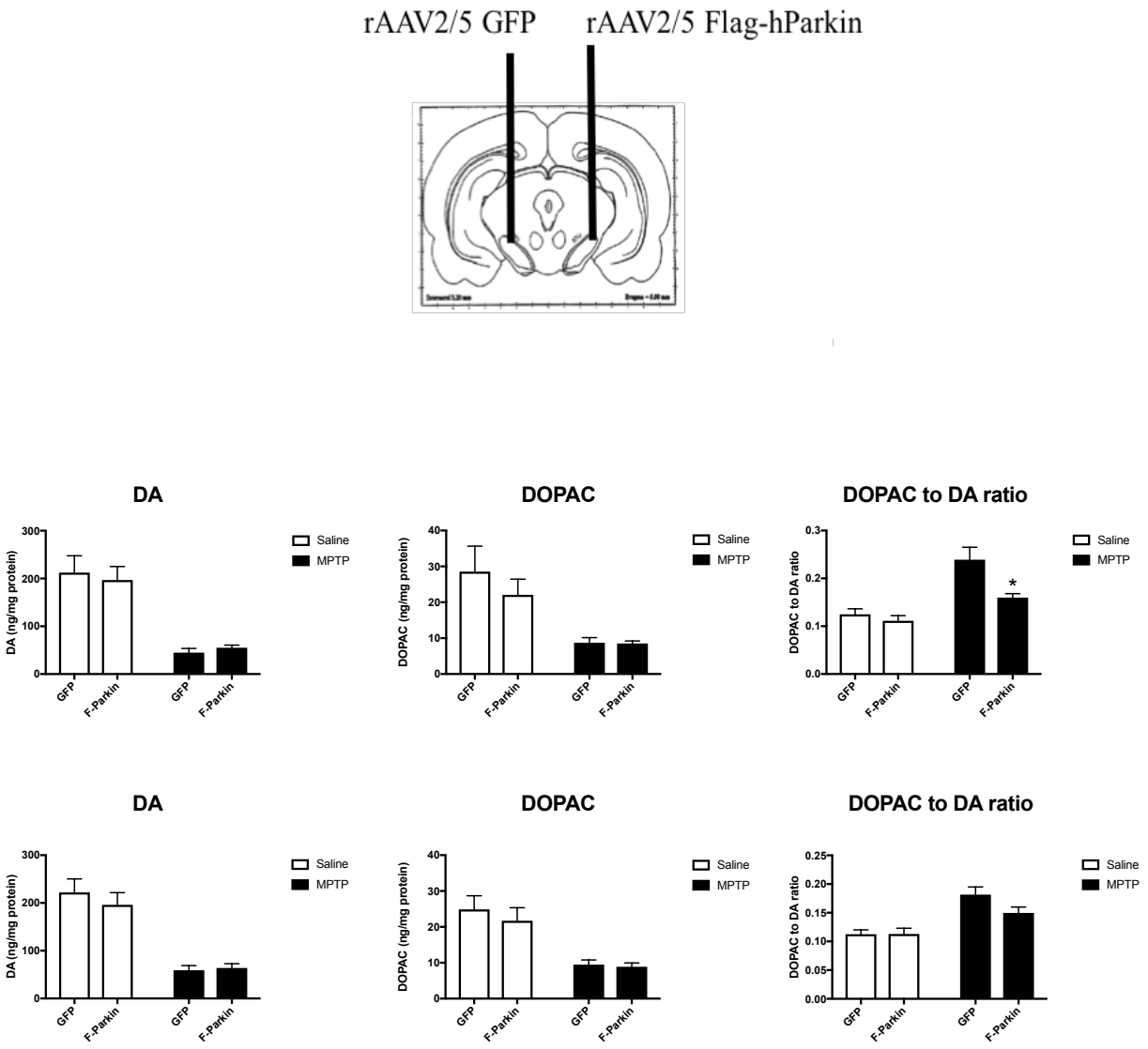

Figure 5-13 Effect of F-hParkin over-expression in the SN on DA turnover in the ST of mice treated with MPTP. C57BL/6J (**top panels**) and Park2$^{-/-}$ mice (**bottom panels**) received unilateral 500 nL stereotaxic injections of rAAV-F-hParkin (3.4 X 10^{12} vg/mL) into the SN. The contralateral SN was injected with 1 X 10^{12} vg/mL of rAAV-GFP and used as a control. Four weeks following injections, mice were treated with saline or 20 mg/kg MPTP. Twenty-four h later, the ST was analyzed for DA, DOPAC, and the DOPAC to DA ratio. Bars represent the mean neurochemical concentration. Error bars represent +1 SEM. * Indicates significant difference ($P < 0.05$, n=10) from the saline treated controls.

well as through autophagy (Olzmann and Chin 2008; Narendra et al. 2008). In addition to its E3 ligase function, the N-terminal UBL domain of parkin interacts with proteasome regulatory subunits Rpn10 (Sakata et al. 2003), RPN13 (Aguileta et al. 2015), Rpt6 (Tsai et al. 2003), and α4 (Dachsel et al. 2005) and directly enhances proteasome activity (Um et al. 2010; Hyun et al. 2002; Dachsel et al. 2005). In the previous two chapters, it was revealed that following MPTP exposure the increase in parkin expression is essential for the maintenance of proteasome activity in the ME and for the efficient turnover of the PD related UPS substrate, TH. The current chapter shows that increased parkin expression is sufficient to maintain proteasome activity in regions containing NSDA axon terminals under conditions of neurotoxicant induced oxidative stress.

It was hypothesized that increased expression of parkin would be sufficient to enhance proteasome activity. Parkin over-expression in HEK cells expressing ubiquitinated GFP have increased degradation of the GFP substrate and this effect is independent of the E3 ligase function of parkin (Um et al. 2010). To determine if parkin causes a direct effect on the proteasome, purified 26S proteasome was treated with purified 6His-Parkin and tested for CT-L activity. 6His-Parkin had a modest increase in CT-L activity while BSA had no effect. The lack of effect in the BSA treated controls indicates that the shift in activity was not due to the addition of protein but specifically due to 6His-Parkin. In addition, MPP^+ dose response curves in the presence of 6His-Parkin were shifted upward, and the IC50 of MPP^+ increased from 1 μM to 3 μM. This indicates that even though MPP+ directly impairs proteasome activity, 6His-Parkin was able to enhance unimpaired proteasome function.

There is a possibility that 26S proteasome preparation is co-purified with parkin or other UBL domain containing proteins. However, 26S proteasome was isolated from erythrocytes

which do not express parkin (Uhlen et al. 2010; Uhlen 2010). This does not rule out the presence of other UBL containing proteins. In fact, Rad23 is expressed in these cells (Kakhniashvili, Bulla, and Goodman 2004). Rad23 contains a UBL domain and could be isolated along with the 26S proteasome (Liang et al. 2014). Despite this possibility, addition of parkin to the 26S proteasome preparation enhanced proteasome activity. Further Western blot would need to be done in order to identify any endogenous UBL containing proteins that were co-purified with the 26S proteasome. Never-the-less, these data are consistent with the conclusion that 6His-Parkin can directly modulate proteasome activity *in vitro*.

NSDA neurons are susceptible to MPTP toxicity and do not up-regulate parkin expression following neurotoxicant exposure (Benskey et al. 2012). In addition, parkin expression is necessary to maintain proteasome activity following neurotoxic insult (**Figure 4-5**). Experiments described in this chapter were designed to determine if AAV mediated exogenous parkin expression in the SN could restore proteasome activity to the ST in MPTP-treated WT and Park2$^{-/-}$ mice. To achieve increased parkin expression in NSDA neuron axon terminals in the ST, rAAV2/5 F-hParkin was unilaterally injected into the SN and rAAV2/5 GFP was injected into the contralateral SN and used as an internal rAAV injected control. The best candidate for a rAAV injection control would have been rAAV Flag, however it was discovered that Flag antibodies were highly nonspecific in the mouse brain (Shevtsova et al. 2006). GFP afforded the ability to confirm the localization of the injection by both IHC and Western blot. Expression of GFP had no effect on proteasome activity compared to the ST of un-injected control mice. While rAAV2/5 mediated GFP expression within the contralateral SN of Park2$^{-/-}$ mice resulted in reduced proteasome activity compared to the GFP expressing ST of WT mice, expression of F-hParkin to the ipsilateral SN of Park2$^{-/-}$ mice restored proteasome activity to the ipsilateral ST

indicating that expression of exogenous F-hParkin was sufficient to restore proteasome activity in parkin deficient mice to the basal state similar to that observe in WT mice.

In order to further characterize the effect of F-hParkin expression on the proteasome, all three proteolytic activities of the proteasome were measured in the control and F-hParkin expressing ST. Exogenous parkin expression resulted in increased CT-L and T-L activity compared to the contralateral GFP expressing ST. Although it is likely that F-hParkin enhances T-L activity of the proteasome, it should be noted that, other serine proteases are abundant in the brain (Wang, Luo, and Reiser 2008) and although unlikely, F-hParkin expression might have contributed to increased non-proteasome specific T-L activity. Proteins that bind to non-catalytic sites on the proteasome and either enhance or inhibit its activity are considered allosteric modulators of the proteasome. It is common for allosteric modulators to increase the activity of one catalytic site while decreasing another catalytic site (Gaczynska and Osmulski 2014). For example, activation of the 20S proteasome occurs upon allosteric binding to PA28 and results in varied effects of the three catalytic activities (Realini et al. 1997).

Since parkin binds to the 19S regulatory complex and enhances proteasome activity, we examined if F-hParkin might participate in the assembly of the 26S proteasome. Native in gel CT-L assays and Western blots revealed that 26S activity was increased and that there was more 26S proteasome in the presence of F-hParkin than in the presence of GFP. These data demonstrate that parkin facilitates the assembly of the 19S and 20S complexes to form more 26S proteasome. This indicates that as parkin is upregulated in the TIDA neurons following neurotoxicant exposure, it is stimulating the assembly of more 26S proteasome.

While 26S activity is increased with exogenous F-hParkin expression, concurrent changes in 20S proteasome activity cannot be ruled out. While there is far more 20S proteasome

complex than 26S proteasome complex there was very little 20S proteasome activity detectible on the in-gel assay. The paucity of detectable 20S proteasome activity could reflect that most of the 20S proteasome complex is in a closed/inactive state in our activity buffer containing an excess of ATP-Mg^{2+} (Osmulski, Hochstrasser, and Gaczynska 2009). In the native in-gel assay, MPTP decreased CT-L activity in the GFP expressing ST as expected. 26S proteasome activity was diminished on the GFP over-expressing side of the ST, however, this was not due to decreased amounts of 26S complex. 26S CT-L activity is maintained in the side ipsilateral to F-hParkin over expression and this was associated with an increase in the amount of 26S. Taken together, these results suggest parkin facilitates the assembly of 26S proteasome and increases proteasome activity during neurotoxicant induced oxidative stress.

TH is the rate limiting enzyme in the biosynthesis of DA and is efficiently degraded by the UPS. Chapter 4 demonstrated that phosphorylated and ubiquitinated TH accumulates in the ST of Park2$^{-/-}$ mice, suggesting a role for parkin in this process. Exogenous expression of F-hParkin results in decreased pSer40-TH with increased activity of existing TH compared to the GFP expressing control ST. Normal DA and DOPAC levels in the F-hParkin expressing side of the ST suggests that the smaller amount of pSer40-TH is equally capable of producing the same amount of DOPA as the larger amount in the GFP expressing ST.

In mice, exposure to MPTP results in depletion of DA and loss of THir in the ST. The hypothesis was tested that increased F-hParkin expression might facilitate the efficient turnover of damaged TH following exposure to MPTP and allow the DA vesicles to refill with DA. Despite successful expression of F-hParkin in the ST, ST DA content remained depleted 24 h following MPTP and was not different from the control ST. The lack of effect of parkin on axon terminal vesicular stores is likely related to the mechanism of toxicant action. MPP^+ binds

to VMAT2 and is transported into the storage vesicle where it displaces DA into the cytoplasm (Lotharius and O'Malley 2000). It is thought that these vesicles release MPP^+ into the synapse and the toxicant is recycled back into axon terminals via DAT and repackaged into the DA vesicle.

One possible reason why DA is not replenished in the F-hParkin expressing ST is that parkin ubiquitinates cdcRel1/Sept5 targeting it for degradation by the UPS (Zhang et al. 2000). Sept5 competes with alpha-SNAP for binding to the SNARE complex and suppresses membrane fusion and exocytosis (Beites, Campbell, and Trimble 2005). It is possible that vesicles cannot release MPP^+ and consequently they cannot be refilled with DA. It is interesting to note that at least in the WT ST expressing F-hParkin, the DOPAC to DA ratio is normalized, which suggests that DA turnover is restored in the unlesioned neurons. This might be a result of DA feedback to TH resulting in decreased activity. Indeed, the DOPA to pSer40-TH ratio in the F-hParkin expressing ST of MPTP treated mice appears to be decreased compared to the control ST.

Conclusion

In PD, NSDA neurons are affected while TIDA neurons are not susceptible. MPTP recapitulates the regional susceptibility that is seen in PD. Following neurotoxicant exposure, recovery of TIDA neurons is dependent upon the increased expression of parkin (Benskey et al. 2012). Parkin is an E3 ligase that can also bind to the 26S proteasome and enhance proteasome activity. In Chapter 4, maintenance of proteasome activity in the ST and ME following MPTP exposure was found to be dependent upon the expression of parkin and the state of parkin deficiency resulted in accumulation of ubiquitinated pSer40-TH. Here it was demonstrated that exogenously expressed F-hParkin was sufficient to restore proteasome activity in $Park2^{-/-}$ mice

and this resulted in a decrease in pSer40-TH accumulation in the ST of Park2$^{-/-}$ mice. However, following MPTP exposure, DA vesicles were not replenished in the ST expressing F-hParkin. However, the DOPAC to DA ratio was diminished on the F-hParkin side of the ST suggesting that exogenous parkin expression might limit the pool of active TH and enhance feedback of cytosolic DA on TH. If so, this enhanced feedback on TH might be important in controlling the deleterious effects of toxic DA metabolites on the DA neuron.

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Chapter 6. General Discussion and Concluding Remarks

General Discussion

PD is a chronic and progressive neurodegenerative disease that is characterized by resting tremor, slowness of movement, rigidity, and impaired balance and coordination. PD is a common disease that affects nearly one million people in the United States (National Parkinson's Foundation). The disease is categorically an age related disease and as our aging population shifts to more advanced age, we can expect to see the prevalence of this debilitating disease rise (Pringsheim et al. 2014). There is currently no cure for PD and despite our increasing aging population there are limited therapies available. While these therapies temporarily address the primary motor symptoms of PD, none of these can stop the progressive nature of this disease.

Most neurodegenerative diseases involve the loss of a specific subpopulation of neurons. The primary symptoms of PD are motor in nature and are due to the chronic and progressive degeneration of NSDA neurons. NSDA axon terminals supply DA to the medium spiny neurons in the ST and modulate the basal ganglia circuitry allowing the selection of movement. While NSDA neurons are affected in PD, hypothalamic TIDA neurons are not susceptible (Langston and Forno 1978). An understanding of the molecular mechanisms that afford protection to TIDA neurons may facilitate the development of neuroprotective strategies that could slow, or halt the progressive neurodegeneration of NSDA neurons in patients with PD.

MPTP Paradigm

In this dissertation, early events that occur in DA neurons following a single acute exposure to MPTP are described. MPTP is a DA neuron specific toxicant and exposure results in

increased ROS production largely through inciting mitochondrial dysfunction and metabolism of DA displaced from storage vesicles within the DA neuronal axon terminals. Exposure to MPTP closely parallels the pattern of susceptibility that is observed in PD (Jackson-Lewis and Przedborski 2007; Langston et al. 1983). Despite the similarities between MPTP toxicity and PD, it is important to note that there are differences between the MPTP neurotoxicant model of PD and idiopathic PD including: pathological progression, acute onset, and lack of Lewy Body formation. However, MPTP recapitulates the selective neuronal population sensitivity that is seen in PD (Langston and Forno 1978; Benskey et al. 2013). In mice, both NSDA and TIDA neuronal populations initially respond to MPTP, however, TIDA neurons recover from the initial neurotoxic insult, while NSDA neuronal toxicity persists (Benskey et al. 2012; Benskey et al. 2013). The ability of MPTP toxicity to cause similar molecular pathology within NSDA neurons makes this neurotoxicant a useful tool for studying the early molecular events that may be involved in PD.

The recovery of vesicular DA in TIDA neurons following MPTP exposure is dependent upon the increased expression of parkin (Benskey, Manfredsson, et al. 2015). While parkin is increased in TIDA neurons it is decreased in NSDA neurons following MPTP exposure (Benskey et al. 2012). Differential parkin expression following exposure to MPTP is of interest because mutations in the PARK2 gene cause autosomal recessive juvenile PD (Hattori et al. 1998; Kitada et al. 1998; Sinha et al. 2005) and oxidative modifications causing parkin loss of function in the ST are found in patients with sporadic PD (Chung et al. 2004).

Parkin is an E3 ubiquitin ligase that promotes the degradation of its specific protein substrates via the UPS (Shimura et al. 2000) as well as autophagy (Olzmann and Chin 2008; Narendra et al. 2008). In addition to its E3 ligase function, the N-terminal UBL domain of parkin

interacts with proteasome regulatory subunits Rpn10, RPN13, Rpt6, and α4 (Sakata et al. 2003; Aguileta et al. 2015; Tsai et al. 2003; Dachsel et al. 2005) and directly enhances proteasome activity in cell culture (Um et al. 2010; Hyun et al. 2002; Dachsel et al. 2005). This dissertation describes regional differences in proteasome activity which are associated with differential parkin expression in regions containing TIDA and NSDA axon terminals under conditions of neurotoxicant induced oxidative stress *in vivo*.

In many experiments presented here, a single low dose of MPTP with a relatively short time course was used. The purpose was to identify early events that occur in TIDA axon terminals that are associated with increased parkin expression. Following the identification of these early molecular events that occur during the recovery of TIDA neurons, it could be determined if parkin protein expression was necessary and sufficient to reproduce these events within and protect NSDA neurons.

Regarding the use of Park2$^{-/-}$ Mice

To determine the necessity of parkin in maintaining proteasome activity and limiting oxidative damage, Park2$^{-/-}$ mice were used. These mice have the exon 3 deletion mutation that is most prevalent in early onset familial PD. Park2$^{-/-}$ mice have increased extracellular DA in the ST and reduced postsynaptic excitability compared to WT mice (Goldberg et al. 2003). As a result, Park2$^{-/-}$ mice demonstrate motor deficits related to dysfunction of the NSDA neuronal pathway (Goldberg et al. 2003). While PD patients with this mutation have significant loss of NSDA neurons, the number of TH-ir neurons in the SN in Park2$^{-/-}$ mice remain comparable to WT mice (Goldberg et al. 2003). This difference between mice and humans may be due to different lifetime environmental exposures. Alternatively, it is possible that Park2$^{-/-}$ mice have

compensatory mechanisms available that humans do not and these prevent the loss of NSDA neurons. Future studies might benefit from utilizing either rAAV Parkin-shRNA or an inducible parkin knockout which would not be subject to lifetime compensation for loss of parkin expression

Oxidative Stress, Parkin, and Proteasome Dynamics

There is a growing body of evidence that parkin is necessary for maintenance of central DA neurons (Benskey, Manfredsson, et al. 2015; Petrucelli et al. 2002; Narendra et al. 2008; Jiang et al. 2004) and that parkin can act to enhance proteasome activity (Tanaka et al. 2001; Sakata et al. 2003; Aguileta et al. 2015; Tsai et al. 2003; Dachsel et al. 2005; Um et al. 2010; Hyun et al. 2002). It was hypothesized that distinct parkin expression profiles in NSDA and TIDA neurons would be associated with differential proteasome activity within the ST and ME. To test this hypothesis mice were treated with MPTP to induce distinctive parkin profiles in the ST and ME. In fact, 24 h following MPTP exposure, proteasome activity is decreased in the ST where parkin is decreased, but not the ME where parkin is increased following MPTP treatment (**Figure 3-4; Figure 3-7**) Further, proteasome activity is decreased in the ST of Park2$^{-/-}$ mice while activity is not changed in the ME (**Figure 4-2**). An increase in oxidative damage in the ST but not the ME of Park2$^{-/-}$ mice was observed, which corresponds with decreased proteasome activity in the ST and maintained proteasome activity in the ME (**Figure 4-4**). This suggests that parkin is necessary in the ST to maintain proteasome activity under basal conditions while it is not necessary in the ME. Interestingly, when Park2$^{-/-}$ mice are exposed to MPTP, proteasome activity is no longer maintained in the ME (**Figure 4-5**). Therefore, parkin is necessary to

maintain proteasome activity in the ME following MPTP exposure associated with oxidative stress.

The proteasome is composed of the 20S catalytic core particle and the 19S regulatory complex. Although the 20S core particle can degrade small disordered proteins in an ATP independent manner, larger ordered proteins require a polyubiquitin tag that is recognized by the 19S regulatory complex before they are translocated into the 20S catalytic chamber to be degraded in an ATP dependent process. The 26S and 20S proteasome exist in a dynamic equilibrium within the cell. In order to limit damage caused by oxidative stress and loss of ATP, changes in proteasome composition and structure modulate the 26S and 20S dynamics and promote the degradation of oxidized proteins. **Figure 6-1** illustrates a proposed model of proteasome dynamics under conditions of oxidative stress and how these changes modulate proteasome activity in response to ROS (Aiken et al. 2011). Under non-oxidative conditions, the 26S proteasome is the major degradative complex. In the early phase of oxidative stress, 26S proteasome activity is increased. With persistent oxidative stress, the 26S proteasome disassembles to the 20S proteasome so that disordered proteins can be degraded independent of ATP and ubiquitin. Under prolonged oxidative stress, a decrease in 26S activity triggers the *de novo* synthesis of new proteasome components and the assembly of functional proteasome complexes (**Figure 6-1**).

In the studies presented here, WT mice were exposed to MPTP which caused a decrease in 26S proteasome activity in the ST and a compensatory increase in *de novo* synthesis of the β5 catalytic subunit of the 20S complex and the RPN10 subunit of the 19S regulatory complex and an increase in the amount of assembled 20S proteasome complex. The events described here indicate that 24 h following MPTP represents a snapshot of prolonged oxidative stress in the ST

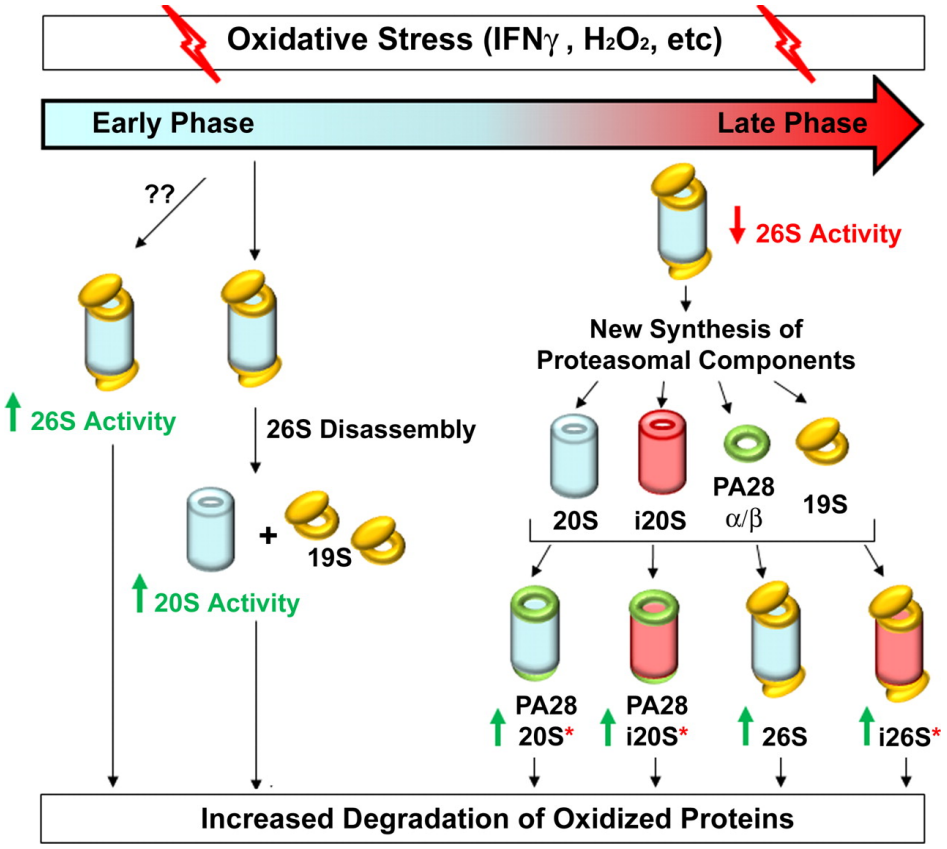

Figure 6-1 Model of oxidative stress and proteasome dynamics. Under non-oxidative conditions, the 26S proteasome is the major degradative complex. Early in an oxidative insult, the 26S proteasome is activated by an unknown mechanism. With persistent oxidative stress, the 26S proteasome disassembles into the 20S proteasome so that disordered proteins can be degraded independent of ATP and ubiquitin. Under prolonged oxidative stress, a decrease in 26S activity triggers the *de novo* synthesis of new proteasome components and the assembly of functional proteasome complexes (Aiken et al. 2011).

as previously proposed (Aiken et al., 2011). Additional time-course data (**Appendix 2)** indicate that proteasome assembly and activity in the ST of WT mice treated with MPTP follow the response described in **Figure 6-1**. In contrast, 26S proteasome assembly and activity also increase in parkin deficient mice, but to a lesser extent than WT mice. In addition, the increase in 26S assembly and activity is delayed in the ST of Park2$^{-/-}$ mice compared to WT mice. Therefore, the early increase in 26S assembly and activity in the ST of MPTP treated mice are dependent upon the presence of parkin

The Role of Decreased Proteasome Activity in the Degeneration of DA Neurons

Mutations in the PARK2 gene explain about half of all the familial cases of PD (Lucking et al. 2000) and oxidative modifications causing loss of parkin function are found in the ST of patients with sporadic PD (Chung et al. 2004). Mutations in parkin produce rare forms of early onset familial PD (Kitada et al. 1998) which indicates the importance of parkin for NSDA neuronal survival. While mutations in parkin only account for 5-10% of all cases of PD (de Lau and Breteler 2006) oxidative damage to parkin results in its loss of function. S-nitrosylation of cysteines within the RING domains of parkin are found in the SN of PD patients (Chung et al. 2004). In addition, DA can covalently modify parkin causing it to become insoluble and aggregate. Brains of patients with sporadic PD have DA-modified inactive parkin in the SN (LaVoie et al. 2005).

Parkin contains an N-terminal UBL domain that can bind to and enhance proteasome function. Data from this dissertation provide support that parkin is both necessary and sufficient to maintain proteasome activity and limit the accumulation of oxidatively modified proteins in the ST. Indeed, tissue from the ST of Park2$^{-/-}$ mice have decreased proteasome activity and

increased oxidatively modified proteins compared to WT mice while tissue from the ME was not affected (**Figure 4-2 and Figure 4-4**). This likely reflects the relatively high state of activity and higher energy needs of NSDA neurons, which comes with the consequence of increased intrinsic ROS production and reduced antioxidant capacity (Voets et al. 2012; Castro Mdel et al. 2012; Alexeyev 2009; Enochs et al. 1994).

The various pathogenic mechanisms that are associated with PD (i.e. mitochondrial dysfunction, abnormal DA metabolism, and neuroinflammation) all result in increased ROS production and oxidative stress. Oxidative modifications of proteins causes protein misfolding, and if damaged and modified proteins are not removed they will form toxic aggregates that lead to neuronal dysfunction and degeneration (Ross and Poirier 2004). There are two pathways that regulate the presence of toxic protein species, the lysosomal degradation pathway and the UPS. In neurons, late endosomes and lysosomes are localized in the soma and dendrites (Parton, Simons, and Dotti 1992). Proteolytic organelles that are contained in portions of the neuron where the surface area to volume is high, such as the axon and the highly arborized terminals of NSDA neurons, do not degrade materials efficiently (Tai and Schuman 2008). Accordingly, a large portion of endocytosed proteins must be pooled into multi-vesicular bodies and transported to the soma for breakdown. Because of this, these neurons are largely dependent upon the UPS to facilitate the rapid removal of damaged and unneeded proteins. This is important because protein aggregates that are the result of impaired degradation have been observed in the neuropathology of PD (Ross and Poirier 2004).

The data presented here are in line with the hypothesis that parkin serves a protective function in NSDA neurons by maintaining proteasome activity and allowing the efficient degradation of oxidized proteins. Following MPTP exposure, the amount of parkin mRNA is not

increased in NSDA neurons and parkin protein is decreased in the ST (Benskey et al. 2012), perhaps due to oxidative modifications that render the protein inactive and insoluble or due to its normal turnover. While over-expression of F-hParkin over-expression restored proteasome activity in the ST following MPTP exposure, it is important to know for the further development of AAV-hParkin studies if F-hParkin also appears in the insoluble fraction of the tissue lysate.

Neuronal Activity and Oxidative Damage

Results from studies presented here demonstrate that increased parkin expression is both necessary and sufficient to maintain proteasome activity in regions containing TIDA and NSDA axon terminals under conditions of neurotoxicant induced oxidative stress (**Figure 4-5; Figure 5-7; Figure 5-8**). However, under basal conditions, parkin is necessary in the ST, but not the ME to maintain proteasome activity (**Figure 4-2**). This phenomenon may be a result of the high energy requirements of NSDA neurons compared to TIDA neurons.

Due to the length of and the extensive branching of NSDA axons (less than 1% of the NSDA neuron cell volume is accounted for by the soma), these neurons have extremely high metabolic requirements that increases the risk of increased ROS production and susceptibility to oxidative stress (Sulzer 2007; Braak et al. 2004; Matsuda et al. 2009). In addition, NSDA neurons have higher basal neuronal activity than TIDA neurons, which is reflected by the DOPAC to DA ratio (**Figure 3-1C**).

The relatively high state of activity in NSDA neurons comes with the consequence of increased ROS production and reduced antioxidant capacity. To prevent toxic DA quinone formation, cytosolic DA must be metabolized to DOPAL by MAO, a reaction that results in the two-electron reduction of oxygen and the production of superoxide and hydroxyl radical

(Przedborski et al. 2000). Buffering of superoxide and hydroxyl radical results in the depletion of glutathione which can render these neurons susceptible to a modest increase in ROS. Despite the fact that NSDA neurons intrinsically produce more ROS than other DA neuronal populations (Voets et al. 2012; Castro Mdel et al. 2012; Alexeyev 2009; Enochs et al. 1994), there are no regional differences in oxidative protein modification (as measured by protein carbonylation; **Figure 3-3**), parkin expression (**Figure 3-4**), or proteasome activity (**Figure 3-7**) in untreated WT mice. These results suggest that under basal conditions the antioxidant system is sufficient to neutralize the ROS that is produced in both regions. However, the theory that NSDA neurons intrinsically produce more ROS than other DA neuronal populations and are more susceptible to oxidative stress (Voets et al. 2012; Castro Mdel et al. 2012; Alexeyev 2009; Enochs et al. 1994) is supported by the observation that parkin deficient mice have increased oxidative protein modifications (as measured by protein carbonylation; **Figure 4-4**) in the ST. The observation of elevated oxidatively damaged proteins in the ST of Park2$^{-/-}$ mice also indicates that parkin plays a role in limiting the amount of oxidatively damaged protein in brain regions containing axon terminals of NSDA neurons. The fact that there is a correlation between the increase in oxidatively modified proteins in the ST but not the ME and decreased proteasome activity in the ST and maintained proteasome activity in the ME of parkin deficient mice (**Figure 4-5**) supports the possibility that parkin is necessary in the ST to maintain proteasome activity and limit the presence of oxidatively damaged proteins under basal conditions. Further studies will be needed to measure protein aggregation in these neurons following neurotoxicant exposure and the effect of F-hParkin over-expression in the SN.

The production of ROS markedly increases in the ST and ventral midbrain following MPTP exposure (Jackson-Lewis and Przedborski 2007). A single acute exposure of MPTP

results in a compensatory increase in NSDA neuronal activity of the unlesioned NSDA axon terminals in the ST which is reflected by the DOPAC to DA ratio (**Figure 3-1**). The increase in activity of the NSDA neurons is likely mediated by loss of DA receptor mediated feedback regulation (either long-loop post-synaptic or autoregulatory presynaptic), which is absent in TIDA neurons that lack a robust presynaptic DA feedback mechanism (Lookingland and Moore 2005). TIDA neurons are instead, regulated by changes in circulating prolactin which tonically activates the TIDA neurons. MPP^+ triggers release of DA from the TIDA axon terminals to the anterior pituitary where it inhibits prolactin release from lactotrophs (Durham et al. 1997). It is feasible that the decrease in neuronal activity of TIDA neurons following MPTP is due to loss of prolactin feedback activation of TIDA neurons.

Despite the differing neuronal activity states of NSDA and TIDA neurons following MPTP exposure, protein carbonylation is elevated to the same extent in both the ST and the ME 24 h following MPTP exposure (**Figure 3-3**), indicating that the antioxidant capacity of both regions is overwhelmed following this single low dose MPTP exposure. Accordingly, it would be interesting to determine the antioxidant threshold for NSDA and TIDA terminals using lower doses of MPTP. Regardless, the observation that parkin is elevated in the ME and not the ST following MPTP exposure (**Figure 3-4**) suggests that changes in parkin expression are not triggered by the oxidative modification of proteins within the region. In this regard, differential expression of parkin could be due to differential expression of FosB and dFosB which are upregulated in regions containing TIDA soma, but not NSDA soma (Patterson et al. 2016).

Susceptibility to increased ROS production due to MPTP exposure is much harder to interpret in the $Park2^{-/-}$ mice. While there is a decrease in proteasome activity in both the ST and ME of MPTP treated $Park2^{-/-}$ mice, the amount of oxidatively damaged proteins did not

correspond to these changes. Surprisingly, parkin expression does not appear to be necessary to limit oxidized proteins in the ST and ME following neurotoxic insult (**Figure 4-6**), as the state of parkin deficiency results in reduced oxidative protein modifications in the ST and no change in the ME following MPTP exposure. A compensatory shift in degradation pathways for oxidatively damaged proteins may have occurred in the context of impaired proteasome activity. This is not an uncommon feature of proteasome impairment, inhibition of the UPS often leads to a compensatory increase in autophagic activity that might account for this decrease in oxidatively damaged proteins (Pandey et al. 2007; Ding et al. 2007; Rzymski et al. 2009). Future studies observing autophagic shifts in TIDA and NSDA neurons could be beneficial in understanding the roles of both the UPS and the ALP under conditions of oxidative stress.

Another possibility is that Park2$^{-/-}$ mice have adapted mechanisms that are aimed at compensating for the presence of ROS and the accumulation of damaged proteins. Eighty-seven proteins are differentially expressed in the ST of WT and Park2$^{-/-}$ mice (Periquet et al. 2005). Several of these proteins are antioxidants that might be important in neutralizing ROS following exposure to MPTP. For example, one such protein is a carbonyl reductase which would have obvious implications for measuring carbonylated proteins as an index of oxidative stress. GSH S-transferase is also elevated in the ST of Park2$^{-/-}$ mice (Periquet et al. 2005). Under conditions of oxidative stress, TH can be modified by glutathionylation and this results in its reduced activity (Borges et al. 2002) and consequential reduction in cytosolic DA and the resultant DA oxidation products. In addition, glutathionylation can influence cell signaling pathways implicated in the antioxidant response. For example, Nrf2 is a transcription factor that regulates the expression of antioxidant proteins. Under non-oxidative conditions, Nrf2 is sequestered in the cytoplasm by Keap1 so that Cullin 3 can ubiquitinate Nrf2 and allow its degradation by the

proteasome (Zhang 2006). Glutathione S-transferase can potentiate the glutathionylation of Keap1 so that it is degraded by the proteasome and releases Nrf2 (Carvalho et al. 2016). Nrf2 is then free to translocate to the nucleus and bind to the antioxidant response element and increases the expression of other antioxidant genes.

Mitochondrial Dysfunction and Decreased Proteasome Activity

The maintenance of assembled 26S proteasome complex is dependent upon sufficient ATP within the cell. Both the state of parkin deficiency and exposure to MPP+ result in mitochondrial dysfunction and it could be argued that depletion of ATP is the cause of decreased proteasome activity. MPP^+ is a mitochondrial complex I inhibitor and while ATP is transiently decreased by about 10% shortly (1-3 h) after MPTP exposure, complex I inhibition is limited and by 24 h the amount of ATP is not noticeably different from control mice (Cosi and Marien 1998; Chan et al. 1991; Irwin, DeLanney, and Langston 1993). Consistent with this time course of MPTP effects, proteasome activity was impaired at 24 h – a time when there was no change in the amount of assembled 26S proteasome (**Figure 3-12**). Since maintenance of assembled 26S proteasome is ATP-dependent, the unaltered levels of assembled 26S proteasome in the ST following MPTP treatment are consistent with the conclusion that there is sufficient ATP to keep the 26S proteasome from disassembling following MPTP treatment.

Along the same lines, parkin has an important role in mitochondrial quality control and a reduction in mitochondrial respiration is observed in synaptosomes derived from the ST of $Park2^{-/-}$ mice (Exner et al. 2007; Narendra et al. 2008; Springer and Kahle 2011; Hawong 2014). Although the rate of ATP synthesis is decreased in ST synaptosomes from $Park2^{-/-}$ mice compared to WT mice, the decreased rate is compensated for and there is no difference in the

amount of ATP (Pickrell and Youle 2015). However, if ATP levels had been depleted, it is important to note that physiologic levels of ATP (i.e. mM range) have a negative effect on 26S proteasome activity. Under stress conditions, where ATP levels are lower, 26S proteasome activity actually increases from the basal level (Huang et al. 2010). Based on these reasons, it is unlikely that ATP depletion accounts for either Park2$^{-/-}$ related or MPTP induced decrease in 26S proteasome activity.

AAV Mediated F-hParkin Over-expression and Maintenance of Proteasome Activity in NSDA Neurons

NSDA neurons are susceptible to MPTP toxicity and do not up-regulate parkin expression while TIDA neurons are resistant to MPTP and do upregulate parkin. Data presented in this dissertation implicate increased parkin expression in TIDA neurons in the maintenance of proteasome activity and alleviation of oxidatively modified proteins from the axon terminal following neurotoxic insult (**Figures 4-5 and Figure 4-6**). Based on these findings, it was hypothesized that over-expression of F-hParkin in neurons in the SN would be sufficient to maintain proteasome activity in the axon terminals of NSDA neurons following neurotoxicant exposure.

Gene therapy is a treatment paradigm in which populations of cells are genetically modified in order to relieve the disease symptoms. To determine if exogenous parkin expression in the SN could restore proteasome activity to the ST, we employed viral vector mediated gene transfer. Adeno-Associated Viral (AAV) mediated gene transfer is the most common genetic therapy approach taken in the central nervous system and has been used in over 20 human clinical trials (Lim, Airavaara, and Harvey 2010). Viral vectors are engineered from WT viruses

so that the genes that are needed for their replication are removed from the genome. Once injected, these viral particles are unable to replicate themselves but can infect cells and transfer the genetic material to the nucleus leading to irreversible genetic modification within the host tissue. Adeno-Associated viruse (AAV)2/5 has specific tropism for neurons (Aschauer, Kreuz, and Rumpel 2013), has been used in human clinical trials, and was therefore used in these studies.

rAAV F-hParkin was unilaterally injected into the SN and rAAV GFP was injected into the contralateral SN and used as an internal AAV injected control. To determine if GFP expression had any effect on proteasome activity, CT-L proteasome activity of the GFP expressing ST was compared to that of the un-injected side. Expression of GFP has no effect on proteasome activity compared to the ST of un-injected control mice (**Figure 5-5A**). As is expected, proteasome activity within the contralateral SN of Park2$^{-/-}$ mice with AAV mediated GFP expression had reduced proteasome activity compared to the GFP expressing ST of WT mice (**Figure 5-5B**). However, expression of F-hParkin in the SN of Park2$^{-/-}$ mice restores proteasome activity compared to the contralateral GFP expressing side and the WT control.

The increased proteasome activity following rAAV-hParkin expression is associated with increased amounts of assembled 26S proteasome, although changes in 20S proteasome activity cannot be ruled out. Indeed, there is far more 20S proteasome complex than 26S proteasome complex (**Figure 5-8B**). There was, however, very little 20S proteasome activity detectible on the in-gel assay (**Figure 5-8A**). The paucity of detectable 20S proteasome activity likely reflects that most of the 20S proteasome complex is in a closed/inactive state in the activity buffer containing an excess of ATP-Mg^{2+} (Osmulski, Hochstrasser, and Gaczynska 2009). In fact, atomic force microscopy imaging of the 20S proteasome indicates that most of the

20S proteasome complex exists in the closed/inactive state with a small percentage in the open/active conformation at any given time (Gaczynska and Osmulski 2011).

F-hParkin over-expression in the SN results in increased proteasome activity in the ST of both the saline and MPTP treatment groups (**Figure 5-6 and Figure 5-7**), which is likely due to increased amounts of 26S proteasome (**Figure 5-8B**). MPTP exposure results in decreased CT-L activity associated with the 26S proteasome complex in the GFP expressing ST. However, the decrease in 26S activity is not due to decreased amounts of 26S complex (**Figure 3-12 and Figure 5-8**). 26S CT-L activity is maintained in the ST ipsilateral to F-hParkin over expression and this is associated with an increase in the amount of 26S complex. Taken together, these results suggest F-hParkin over-expression facilitates the assembly of 26S proteasome and increases 26S proteasome activity during neurotoxicant induced oxidative stress. F-hParkin over-expression reults in increases in assembled 26S proteasome which allows the maintenance of proteasome activity following neurotoxic insult. Specifically, these data demonstrate for the first time that increased F-hParkin expression is sufficient to allow the maintenance of proteasome activity following oxidative insult. Future work exploring functional point mutations within the UBL domain of parkin will shed light on whether parkin mediated modulation of the 26S proteasome occurs through a UBL domain dependent mechanism.

Parkin and Recovery of DA in the ST Following MPTP Exposure

While TIDA neurons up-regulate parkin expression and recover following MPTP exposure, NSDA neurons do not. We aimed to determine if over-expression of F-hParkin within the SN was sufficient to allow the maintenance of proteasome activity and TH levels following MPTP exposure.

TH is the rate limiting enzyme in DA synthesis (Levitt et al. 1965) and its activity is regulated by both short-term (allosteric regulation, tetrahydrobiopterin availability, catecholamine feedback inhibition and phosphorylation) and long-term transcriptional mechanisms (Fujisawa and Okuno 2005). In addition, TH activity is also regulated through its turnover by the UPS in the short-term and autophagy in the long-term (Congo Carbajosa et al. 2015; Nakashima et al. 2013). While TH is not a specific substrate of parkin E3 ligase function, Park2$^{-/-}$ mice had increased levels of TH phosphorylated at Ser19 and Ser40, post-translational modifications that are essential for TH activity (**Figure 4-7 and Figure 4-10)**. These data would seem to be in contrast to immunohistochemistry data that demonstrates that the number of THir neurons in the SN or their projections in the ST of Park2$^{-/-}$ mice are similar to that of WT mice (Goldberg et al. 2003; Perez and Palmiter 2005). However, their work quantitated the number of neurons that were THir and not the actual amount of TH or amount of TH per cell. A separate proteomic study did find that Park2$^{-/-}$ mice have increased TH compared to WT mice (Periquet et al. 2005).

Parkin expression is necessary to maintain proteasome activity and facilitate the normal degradation of TH in the ST (**Figure 4-7 and Figure 4-10**). While Park2$^{-/-}$ mice appear to have more phosphorylated TH in the ST which might indicate more activated TH, Park2$^{-/-}$ mice do not have different amounts of DA, or TH in situ activity (i.e. DOPA accumulation), compared to WT controls. However, when the level of DOPA is normalized to the amount of Ser40 phosphorylated TH, the amount of DOPA per TH was decreased in the ST of Park2$^{-/-}$ mice compared to the WT controls. It appears that there is more TH in Park2$^{-/-}$ mice but it is likely that the TH is ubiquitinated and perhaps inactive. In fact, TH immunoprecipitated from the ST of Park2$^{-/-}$ mice has increased K48 linked polyubiquitin as well as K63 linked polyubiquitin (**Figure**

4-10). These differences in the amount of TH likely reflect delayed turnover of ubiquitinated TH due to decreased activity of the proteasome in Park2$^{-/-}$ mice.

While parkin is necessary for the efficient turnover of TH, the results from these studies reveal that F-hParkin over-expression is sufficient for the recovery of proteasome activity, and normal levels of TH which were previously impaired in the ST of Park2$^{-/-}$ mice (**Figure 5-9**). It is interesting to note that in the ST expressing F-hParkin, the compensatory increase in the DOPAC to DA ratio in MPTP treated WT mice is normalized (**Figure 5-13**), which suggests that DA turnover is dampened in the remaining unlesioned neurons. This might be a result of restored cytosolic DA feedback onto TH resulting in decreased TH activity. Indeed, the DOPA to pSer40-TH ratio in the F-hParkin expressing ST of MPTP treated mice appears to be decreased compared to the control ST.

Cytosolic DA is prone to auto-oxidation and forms severely toxic DA quinones (Lotharius and O'Malley 2000). The ability to mitigate increases in cytosolic DA is important for the recovery of DA neurons. F-hParkin over-expression increases proteasome activity in NSDA neurons and may prevent cytosolic DA related oxidative damage to the neuron caused by a compensatory increase in neuronal activity in the unlesioned neurons, a major component of MPTP toxicity as well as PD. Although it was found that loss of parkin expression in the ST results in increased protein oxidation, it remains to be seen if F-hParkin over expression reduces protein oxidation in the ST following MPTP. The initial protein carbonylation assays in the ST of Park2$^{-/-}$ mice exposed to MPTP were muddied by possible compensatory antioxidant mechanisms in these mice. Future work in measuring protein carbonylation in MPTP exposed WT mice expressing F-hParkin will be useful in determining the ability to prevent oxidative protein damage. The effectiveness of F-hParkin over-expression in reducing cytosolic DA

turnover may give validity to the idea that proteasome activating drugs may be an effective strategy in both reducing oxidatively damaged due to oxidation of cytosolic DA and increased DA turnover.

Despite the successful over-expression of F-hParkin within the SN, concentrations of DA were not replenished within 24 h following MPTP exposure (**Figure 5-13**). The inability of F-hParkin over-expressing NSDA neurons to recover DA following MPTP exposure is in line with reports from other investigators (Benskey, Kuhn, et al. 2015; Paterna et al. 2007). It is likely that the lack of effect of parkin on axon terminal vesicular stores of DA is related to the mechanism of MPP^+. Once in the DA terminal, MPP^+ binds to VMAT2 and is transported into the storage vesicle where it displaces DA into the cytoplasm (Lotharius and O'Malley 2000). It is believed that these vesicles release MPP^+ into the synapse and the toxicant is recycled back into axon terminals via DAT and repackaged into the DA vesicle. Parkin over-expression in SH-SY5Y and DAT expressing HEK293 cells causes increases cell surface expression of DAT (Jiang, Jiang, and Feng 2004). This might allow increased recycling of MPP^+ as it is released from the vesicle into the synapse. Further studies are needed to demonstrate the effect of F-hParkin over-expression and increased DAT surface expression on both DA and MPP^+ recycling.

Another possible reason why DA is not replenished in the F-hParkin expressing ST is that exogenous F-hParkin differs from endogenous parkin. The human parkin that was expressed in these experiments contained an N-terminal FLAG tag which consists mostly of a string of charged aspartic acid residues. The FLAG tag was originally intended to be used for enhanced immunodetection and immunopurification of the human protein as opposed to the endogenously expressed murine parkin; although, antibodies directed toward the FLAG tag proved to be highly nonspecific in the mouse brain (Shevtsova et al. 2006). It is possible that the N-terminal FLAG

tag could have disrupted proper folding of the parkin protein. In endogenously expressed parkin, the UBL domain folds back on the parkin protein and blocks the ubiquitin binding site, Cys431 (Dove and Klevit 2013). The N-terminal tag may present a more open tertiary structure which may allow ubiquitin binding and E3 ligase function including auto-ubiquitination of parkin, a process that regulates the activity and amount of parkin within the cell (Caulfield, Fiesel, and Springer 2015; Burchell, Chaugule, and Walden 2012). However, the F-hParkin construct has previously been functionally validated in mechanistic studies (Imai et al. 2001; Imai, Soda, and Takahashi 2000; Moore 2006; Geisler et al. 2010; Benskey, Manfredsson, et al. 2015). In addition, the studies presented here demonstrate that F-hParkin is detected in the ipsilateral ST by Western blot (**Figure 5-4**) and is able to rescue proteasome activity (**Figure 5-5, Figure 5-7 and Figure 5-8)** and allow efficient TH turnover (**Figure 5-9**). This suggests that the UBL domain was functional at least in binding to the 26S proteasome. Regardless, future studies may benefit from expression of a hParkin construct that does not utilize an N-terminal tag.

Model of ROS Induced Parkin Expression and Maintenance of Proteasome Activity

In this dissertation, it was hypothesized that distinct parkin expression profiles in NSDA and TIDA neurons would be associated with differential proteasome activity within the ST and ME following neurotoxic insult with MPTP. Indeed, parkin expression was found to be both necessary and sufficient to maintain proteasome activity in the ME following MPTP exposure. While parkin expression is associated with corresponding changes in proteasome activity in these two regions, it does not appear to limit the amount of carbonylated proteins in the ME. **Figure 6-2** illustrates the role that parkin might play in limiting protein aggregation in the ME following

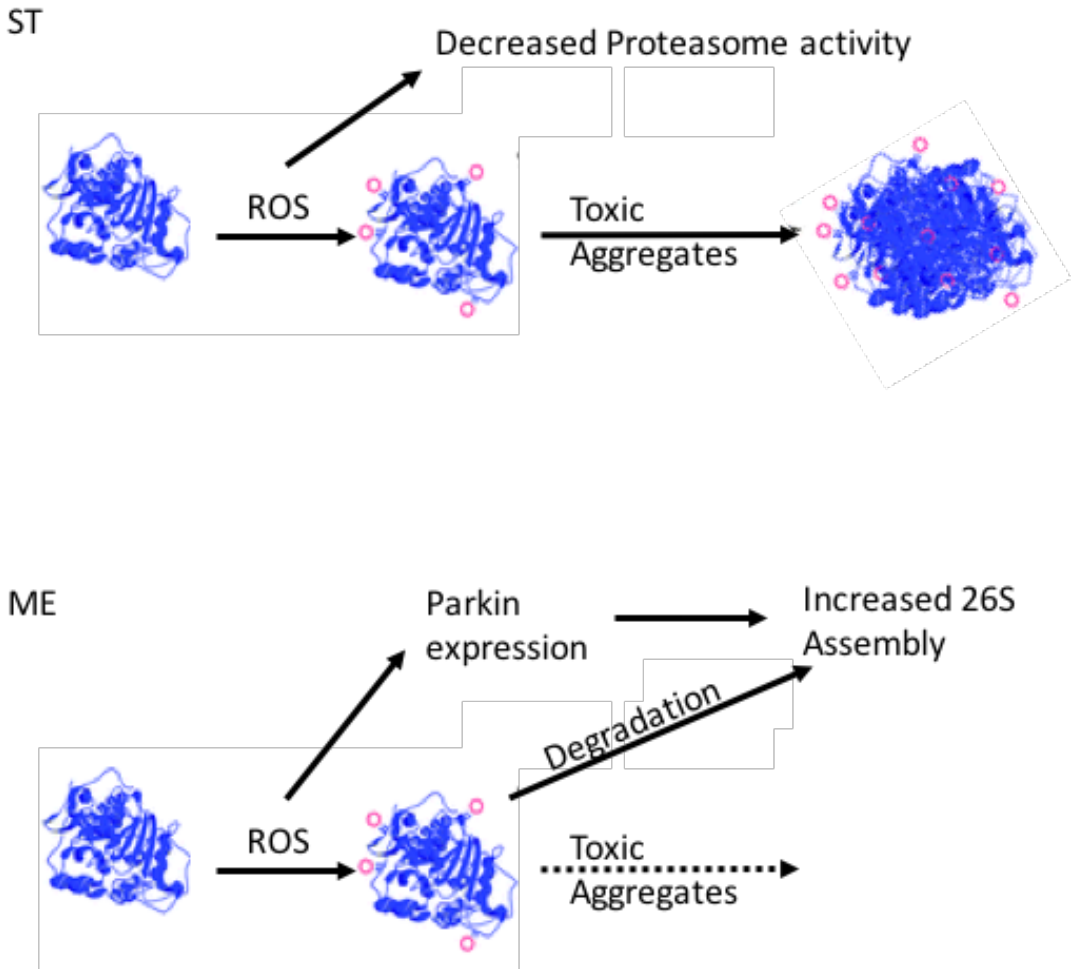

Figure 6-2 Model of ROS induced parkin expression, maintenance of proteasome activity, and prevention of aggregate formation. In the ST (top panel), reactive oxygen species result in oxidative modifications to proteins. Oxidized proteins that are not degraded will form toxic and insoluble protein aggregates. In the ME (bottom panel) In, reactive oxygen species also result in oxidative modifications to proteins. Parkin expression is increased allowing increased assembly of 26S proteasome. Increased 26S proteasome allows the degradation of oxidized proteins.

an oxidative insult. A single acute exposure to MPTP results in a similar increase in the amount of protein carbonyls in the ST and ME. Oxidatively modified and misfolded proteins must be degraded or they will form toxic and insoluble protein aggregates within the neuron. In this way, the amount and activity of the 26S proteasome plays a key role in limiting the formation of these aggregates. Twenty-four h following MPTP exposure, parkin protein expression was decreased in the ST and proteasome activity also decreased. Since there was no change in the amount of oxidized protein compared to the ME, this might indicate that more of the oxidized proteins has formed insoluble protein aggregates. On the other hand, parkin expression and 26S proteasome activity is increased in the ME. Maintained proteasome activity in the ME may allow increased degradation of oxidized proteins and prevent the formation of protein aggregates. To test this hypothesis, further experiments could be done to demonstrate the presence, or lack of, protein aggregates within the insoluble fraction of the ST and ME tissue lysates.

Concluding Remarks

The motor symptoms of PD are due to the chronic and progressive degeneration of NSDA neurons. While NSDA neurons are affected in PD, hypothalamic TIDA neurons are not susceptible (Langston and Forno 1978). An understanding of the molecular mechanisms that afford protection to TIDA neurons may facilitate the development of neuroprotective strategies that could slow, or halt the progressive neurodegeneration of NSDA neurons in patients with PD. Exposure to MPTP closely parallels the pattern of molecular dysfunction that is observed in PD (Jackson-Lewis and Przedborski 2007; Langston et al. 1983) and recapitulates the specific neuronal population sensitivity that is seen in PD (Langston and Forno 1978; Benskey et al. 2013). In mice, both NSDA and TIDA neuronal populations initially respond to MPTP, however,

TIDA neurons recover from the initial neurotoxic insult, while NSDA neuronal toxicity persists (Benskey et al. 2012; Benskey et al. 2013).

The recovery of vesicular DA in TIDA neurons following MPTP exposure is dependent upon the increased expression of parkin (Benskey, Manfredsson, et al. 2015). While parkin is increased in TIDA neurons it is decreased in NSDA neurons following MPTP exposure (Benskey et al. 2012). Differential parkin expression following exposure to MPTP is of interest because mutations in the PARK2 gene cause autosomal recessive juvenile PD (Hattori et al. 1998; Kitada et al. 1998; Sinha et al. 2005) and oxidative modifications causing parkin loss of function in the ST are found in patients with sporadic PD (Chung et al. 2004).

Parkin is an E3 ubiquitin ligase that promotes the degradation of its specific protein substrates via the UPS (Shimura et al. 2000) as well as autophagy (Olzmann and Chin 2008; Narendra et al. 2008). In addition to its E3 ligase function, the N-terminal UBL domain of parkin interacts with proteasome regulatory subunits Rpn10, Rpn13, Rpt6, and α4 (Sakata et al. 2003; Aguileta et al. 2015; Tsai et al. 2003; Dachsel et al. 2005) and directly enhances proteasome activity in cell culture (Um et al. 2010; Hyun et al. 2002; Dachsel et al. 2005). Experiments presented in this dissertation demonstrate that regionally specific parkin expression results in regionally specific increases in 26S proteasome activity following neurotoxic insult. Specifically, these data demonstrate for the first time that maintenance of proteasome activity in the ME following oxidative insult is dependent upon parkin expression. Parkin deficient mice have decreased proteasome activity in the ST and the decrease in proteasome activity in the absence of parkin is associated with increased oxidative damage. Based on this protective ability, F-hParkin over-expression in NSDA neurons proved successful in restoring proteasome activity in the ST of parkin deficient mice and maintaining proteasome activity following neurotoxicant exposure.

The work presented in this dissertation suggest the utility of future research into whether Park2$^{-/-}$ mice are more susceptible to α-synuclein preformed fibril aggregation and if parkin over-expression in the SN can protect NSDA neurons from degeneration in this model of PD. Additionally, work presented here suggest that parkin over-expression results in increased proteasome activity and that proteasome activation may be an idea target for small molecule therapeutics aimed at preventing further degeneration of neurons.

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APPENDICES

APPENDIX A DIFFERENCES IN GAIT BETWEEN C57BL/6J AND PARK2$^{-/-}$ MICE AND DEPENDENCE ON PARKIN

Introduction

Mutations in the Parkin gene, Park2, cause autosomal recessive juvenile onset parkinsonism. In 2004 Perez and co-workers (Perez & Palmiter, 2005) examined if mutations in the mouse parkin gene also caused a parkinsonian phenotype in mice. An extensive behavioral screen was used to evaluate neurological function, motor ability, emotionality, learning and memory in aged Parkin deficient mice. While these mice exhibit nigrostriatal deficits including increased extracellular DA, and a reduction in synaptic excitability, there is no loss of dopaminergic nigral neurons (M. S. Goldberg et al., 2003). While Goldberg et al described behavioral impairments in measuring slips per step, rotorod behavior, and somatosensory adhesive removal, Perez et al (2004) indicated that the behavioral profile of the parkin deficient mice was similar to that of control mice and they argue that parkin deficient mice are not a robust model of parkinsonism.

In PD patients, observations of gait dynamics can be useful in characterizing motor changes. These patients tend to have increased variability in stride compared to healthy control subjects (Blin, Ferrandez, & Serratrice, 1990; Hausdorff, Cudkowicz, Firtion, Wei, & Goldberger, 1998; Hausdorff et al., 2003; Schaafsma et al., 2003; Wu & Krishnan, 2010). While gait dynamics have been reported in mice exposed to acute doses of MPTP (Amende et al., 2005; N. R. Goldberg, Hampton, McCue, Kale, & Meshul, 2011; Rommelfanger et al., 2007), observations in temporal and spatial gait indices have not been reported for parkin deficient mice. The goal of the current study is to investigate the effects of parkin deficiency and the dependence on parkin on gait dynamics using the DigiGait treadmill and analysis software.

Methods

Experiment 1: Do Park2-/- mice have increased variability in gait compared to C57BL/6J mice

Adult (20 weeks; n = 10 per group) male C57BL/6J mice (Jackson Labs, Bar Harbor, ME) and $Park2^{-/-}$ mice ($Park2^{tm1Shn}$) had continuous access to standard lab chow and water and were maintained on a 12:12-hr light : dark cycle. Gait dynamics were assessed using the DigiGait treadmill and software (Mouse Specifics). Mice were acclimated to the treadmill for 10 min prior to gait analysis. Ventral plane videography captured the gait of each mouse through a transparent treadmill belt over a time period of about 6 s (Amende et al., 2005; Kale, Amende, Meyer, Crabbe, & Hampton, 2004). Digital images of the paws of each mouse were taken at 150 frames/sec as mice ran at a speed of 25 cm/sec with no incline or decline. Each mouse was tested individually on the treadmill, in an enclosed acrylic compartment (5 cm in width, 25 cm in length). Temporal and spatial measurements were indicated by the area of the paw relative to the treadmill belt at each frame. The following gait parameters were measured: swing time, % swing stride, brake time , % brake stride, propel time , % propel stride, stance, % stance stride, stride time, % brake stance, % propel stance, stance/swing, stride length, stride frequency, paw angle, absolute paw angle, paw angle variability, stance width, step angle, stride length variability, step angle variability, stride length CV, stance width CV, step angle CV, swing duration CV, paw area at peak stance, paw area variability at peak stance, hind limb shared stance time, % shared stance, stance factor, gait symmetry, max dA/dT, min dA/dT, Tau propulsion, overlap distance, paw placement positioning, ataxia coefficient, midline distance, axis distance, and paw drag.

Experiment 2: Is parkin over-expression sufficient to restore gait dynamics in Park2-/- mice?

Surgery

Twenty-five male Park2$^{-/-}$ mice, age 10-20 weeks were used in this experiment (**Table 1**). Fifteen Park2$^{-/-}$ mice (Group 1, Park2^{tm1Shn} from our in house breeding colony) received a right SN injection of rAAV2/5-F-Parkin (3 X 10^{12} vg/mL) and a left SN injection of of 3.4 X 10^{12} vg/mL rAAV2/5 GFP. Analgesic (ketoprofen, 5 mg/kg, s.c.) was administered prior to surgery. The mice were anesthetized using 120 mg/kg ketamine and 18 mg/kg xylazine and the surgical site was shaved. The animals were placed in a stereotaxic frame and the surgical site was scrubbed three times with alcohol and then betadine prior to making the incision. A single incision was made along the rostrocaudal axis of the skull and the tissue overlying the skull was retracted to expose the skull surface. A Hamilton syringe with a 30 gauge blunt tip needle was fitted with parafilm, and then a siliconized pulled glass micropipette with an opening of 60-80 μm was placed over the parafilm to form a seal. Unilateral SN injections of 500 nL of rAAV2/5–F-parkin or rAAV2/5 GFP was performed using the following coordinates from Bregma: R/C -3.0, L/M -1.6 and D/V -4.6 from the skull. The volume was injected at a rate of 125 nL/min using an automated micropump. The needle was left in place for an additional 5 min to prevent reflux and the needle was slowly withdrawn.

The hole in the skull was filled with sterile bone wax and the skin was replaced and closed using surgical staples. For identification, ears will be marked using an ear punch. Mice will be kept on a heating pad until recovery from anesthesia and then returned to their home cages. Mice were given ketoprofen, for pain, 24 h following surgery and checked daily for signs of infection or distress. Staples were removed 10-14 d following surgery. Animals were housed for 4 weeks prior to the experiment.

Gait Analysis

Adult (20 weeks; n = 10 per group) male C57BL/6J mice (Group 2, Jackson Labs, Bar Harbor, ME) and Park2$^{-/-}$ mice (Group 3, Park2^{tm1Shn} from our in house breeding colony) had continuous access to standard lab chow and water and were maintained on a 12:12-hr light : dark cycle. Four weeks after surgery, gait dynamics of the mice were assessed using the DigiGait treadmill and software (Mouse Specifics) as described above.

Statistics

Each parameter for variability in gait was analyzed by ANOVA, using one-way comparisons between C57BL/6J and Park2$^{-/-}$ groups for gait dynamics. For gait dynamics, left fore, right fore, left hind and right hind paws were analyzed individually unless otherwise specified. Mice that did not run were excluded from the study.

Group	N	Strain	Description
1	15	Park2$^{-/-}$	3.4 X 10^{12} vg/mL rAAV2/5-F-Parkin, ipsilateral 3.4 X 10^{12} vg/mL rAAV2/5-GFP, contralateral
2	10	C57BL/6J	Naive
3	10	Park2$^{-/-}$	Naive

Table A1 Treatment groups for F-hParkin over-expression and gait analysis.

Results and Discussion

Characterization of temporal and spatial gait indices have not been reported for Park2$^{-/-}$ mice. The goal of the current study was to investigate the effects of parkin deficiency on gait dynamics. The Digigate treadmill and software allows the analysis of each digital paw print and provides a dynamic gait signal; giving the history of paw placement through the sequence of strides that are recorded. The signals are comprised of a stride interval, which includes the stance duration (when the paw is in contact with the walking surface), plus the swing duration (when the paw is not in contact with the surface) (**Figure AA-1**).

Analysis of gaits and comparison of the Parkin deficient mice with the WT control mice indicate that there is no difference in primary gait measures including the durations of the stride, swing, stance, propulsion, brake or paw angle. In addition, there is no difference in the mean stride length, or stride frequency. While these parameters were not changed in the parkin deficient mice, the step-to-step placement of the paws was irregular. Increased variability in paw angle, stride length, paw area at peak stance, and swing duration were all significantly increased in the hind limbs of Park2$^{-/-}$ mice compared to the C57BL/6J mice (**Figure AA- 2**). One measurement of variability, the ataxia coefficient, is a cumulative measure of step-to-step variability that is increased in human PD patients. This coefficient was significantly increased in the hind paws of Park2$^{-/-}$ mice compared to WT mice. These differences are of interest because PD patients tend to have increased variability in their step placement compared to healthy control subjects (Blin et al., 1990; Hausdorff et al., 1998; Hausdorff et al., 2003; Schaafsma et al., 2003; Wu & Krishnan, 2010). In mice, measures of step to step variability are also increased in a toxin-induced mouse models of PD (Amende et al., 2005).

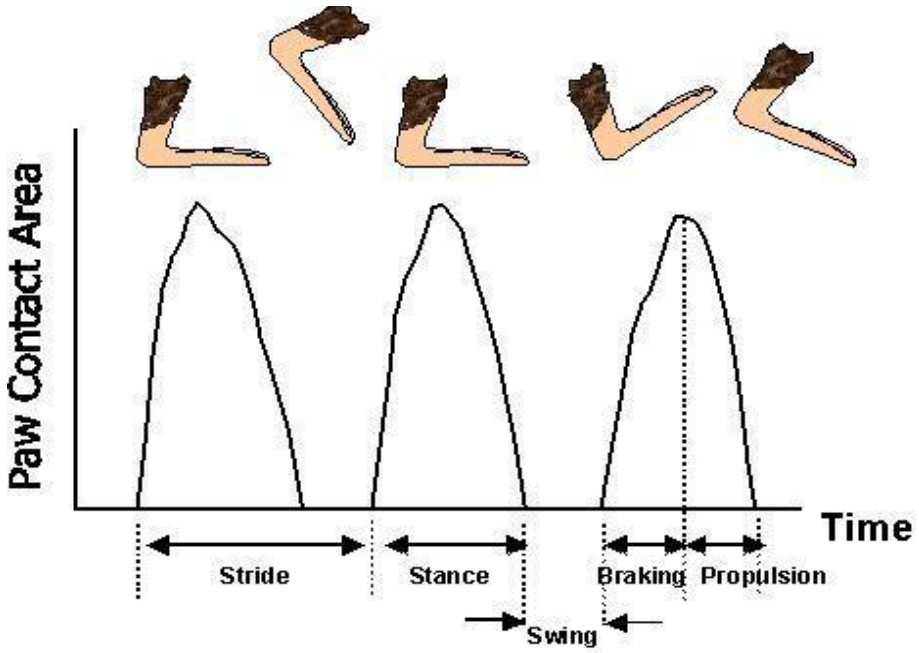


Figure A1 The digigait signals. The signals are comprised of the stride interval which includes the stance duration (when the paw is in contact with the surface), plus the swing duration (when the paw is not in contact with the surface). The stance phase is comprised of braking and propulsion phases. (Image taken from Mouse Specifics).

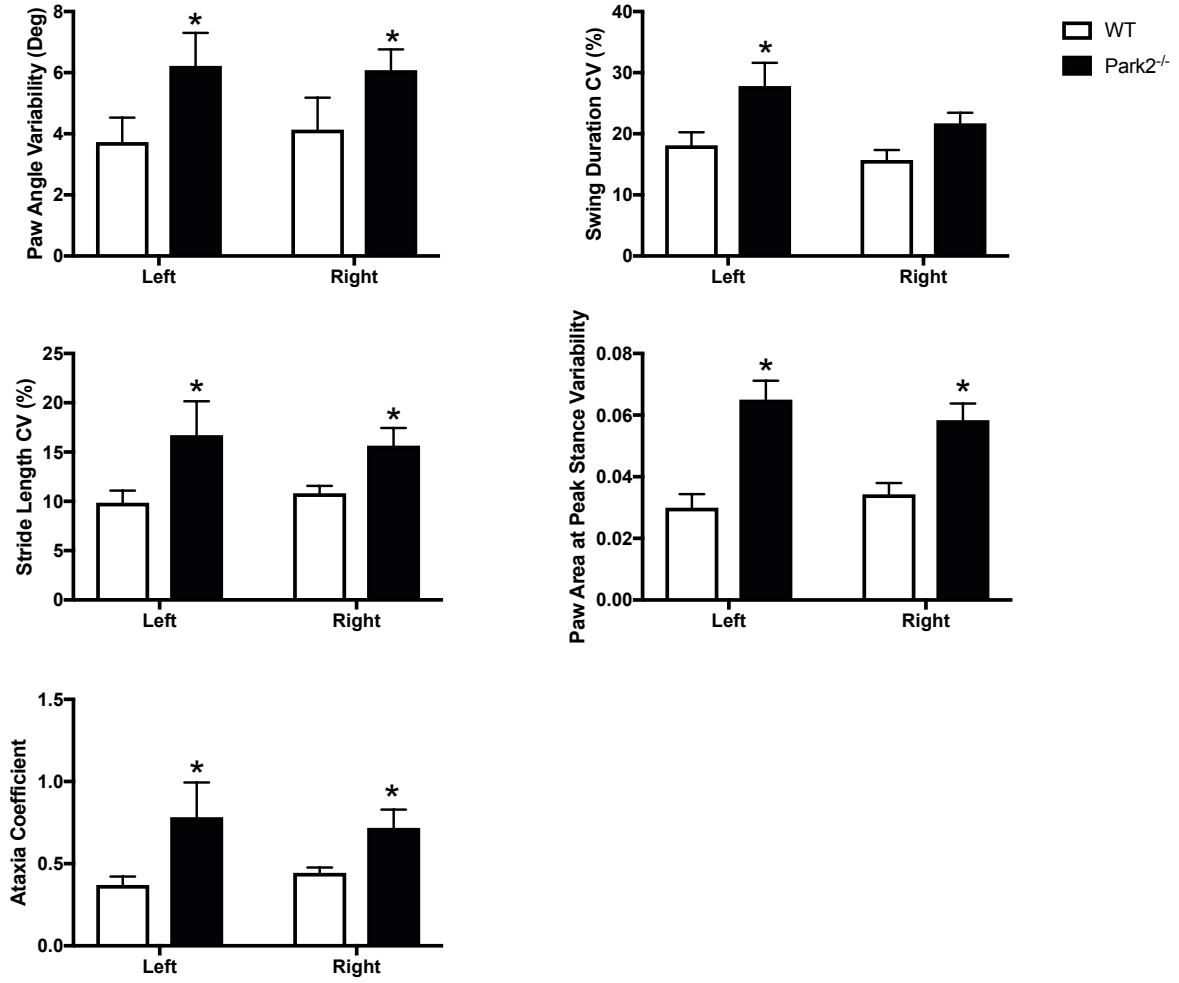


Figure A2 Effect of parkin deficiency on gait variability. Ventral plane videography captured the gait of WT C57BL/6J and Park2$^{-/-}$ mice (N=10 per group) while walking on a treadmill at 25 cm/s. Bars represent the mean. Error bars represent +1 SEM. * indicates significant difference from the C57BL/6J control mice ($p < 0.05$, n=10).

Under normal conditions, mice will place their hindpaw so that they overlap the previous forepaw positions. Paw overlap distance is the distance in which the hindpaw is sustained within the area of forepaw across successive steps. Overlap distance was significantly increased in the right fore and hindlimb of the Park2$^{-/-}$ mice (**Figure AA-3**). Weaker hindlimbs can result in altered dynamics of paw rise and placement during the entire stance. The fact that this change was unilateral suggests that there have been asymmetric changes in the brain. Paw placement positioning (PPP) is a similar measurement that indicates changes in balance. This metric measures the extent of ipsilateral paw overlap while the mouse is at peak stance. PPP was increased on the left side of the Park2$^{-/-}$ mice. A larger contact area of hindpaw with the area of forepaw suggests a potential shift in the center of gravity. Taken together the data might suggest that weakness in the right hindlimb is compensated for by shifting the center of gravity to the left side.

As mice that are deficient in parkin have increased step-to-step variability, the hypothesis that changes in gait dynamics are parkin dependent was tested. Parkin deficient mice were unilaterally injected with rAAV2/5-F-Parkin in the SN and four weeks later were subjected to gait analysis and compared to naïve C57BL/6J and Park2$^{-/-}$ mice. **Figure AA-4** demonstrates that unilateral over-expression of F-hParkin in the SN reduces the variability on the opposing side of the body.

Conclusion

In these studies, gait dynamics of C57BL/6J and Park2$^{-/-}$ mice were compared. It was found that Park2$^{-/-}$ mice have increased step to step variability and unilateral weekness that may result in a shift in the center of gravity. Changes in gait may not be directly related to deficiency of parkin

protein. In the next study, mice were unilaterally injected with rAAV2/5-F-Parkin and gait was analyzed to determine if over-expression of parkin in the SN can rescue Park2$^{-/-}$ mice from this phenotype. Unilateral F-hParkin over-expression in the SN is able to rescue the normal gait phenotype on the opposing side of the injection. Further studies should be completed with bilateral injections of rAAV2/5 F-hParkin compared to the vector control. In addition, it should be determined if parkin maintains normal gait through a DA dependent mechanism.

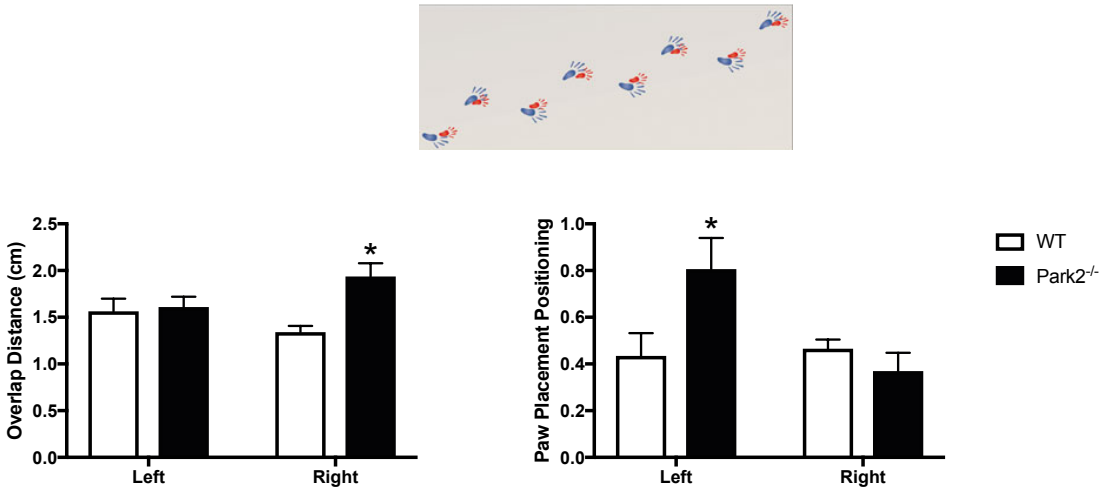


Figure A3 Unilateral changes in Paw overlap distance and paw placement positioning. A. Under normal conditions, mice tend to place their hindpaw so that they overlap the previous forepaw positions at peak stance. Picture from *Nature Reviews Neuroscience* 10, 519-529 (July 2009). B. Park2$^{-/-}$ mice have a right sided increase in overlap distance. **B**. Park2$^{-/-}$ mice have a Left sided increase in PPP.

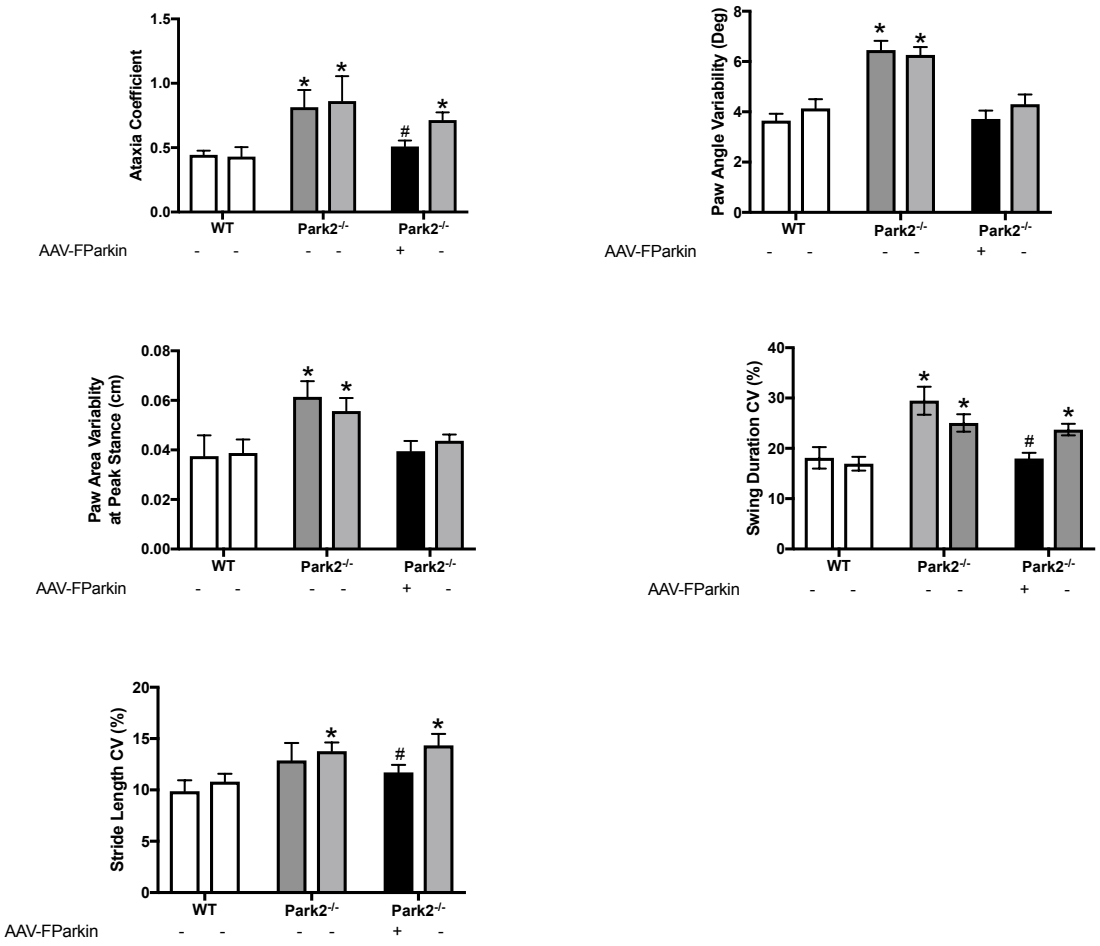


Figure A4 Effect of F-hParkin over-expression on gait variability. Park2$^{-/-}$ mice (n=10) received unilateral 500 nL stereotaxic injections of rAAV-F-Parkin (3.4 X 10^{12}) into the right, SN (+). The right, contralateral SN was injected with 3.4 X 10^{12} rAAV-GFP and was used as an internal control. Four weeks following injection mice were subjected to Ventral plane videography on a treadmill running at 25 cm/s to capture the gait of each mouse. Gait was compared to naïve WT C57BL/6J and Park2$^{-/-}$ mice (n=8). The columns represent mean gait index measurement+1 SEM. *indicates that gait index is significantly different ($p < 0.05$) from the WT controls. # indicates that the gait index is significantly different ($p < 0.05$) from the GFP injected side.

APPENDIX B TEMPORAL CHANGES IN 26S AND 20S PROTEASOME ACTIVITY IN STRIATAL SYNAPTOSOMES FOLLOWING AN ACUTE DOSE OF MPTP

Introduction

The neurotoxicant MPTP produces oxidative stress in DA neurons and can replicate the pattern of DA neuronal survival that is seen in PD (Behrouz, Drolet, Sayed, Lookingland, & Goudreau, 2007; M. Benskey et al., 2012). TIDA neurons are dependent upon increased parkin expression for their recovery (M. J. Benskey, Manfredsson, Lookingland, & Goudreau, 2015). To determine the timing in which changes in proteasome activity occurred, studies were undertaken to characterize the temporal effects of MPTP in C57BL/6J and Park2$^{-/-}$. For these studies, groups of C57BL/6J and Park2$^{-/-}$ mice were treated with MPTP for various amounts of time and changes in neurochemistry, TH, and proteasome activity were measured. In some cases, measurements were made in more than one study and the times measured were slightly different. Due to the large degree of freedom in these studies the groups were underpowered and the data was difficult to validate statistically. Because of this and the incomplete nature of these studies, the results have been placed in this appendix for future reference.

Results and Discussion

Temporal Changes in Neurochemistry

MPTP exposure results in a loss of NSDA axon terminal DA stores, a decrease in DA metabolism and an increase in DA turnover which is due to increased activity of the remaining NSDA neurons (Benskey et al., 2012). To determine the response to MPTP over time, samples collected from the ST of saline and MPTP treated animals were used to compare neurochemical

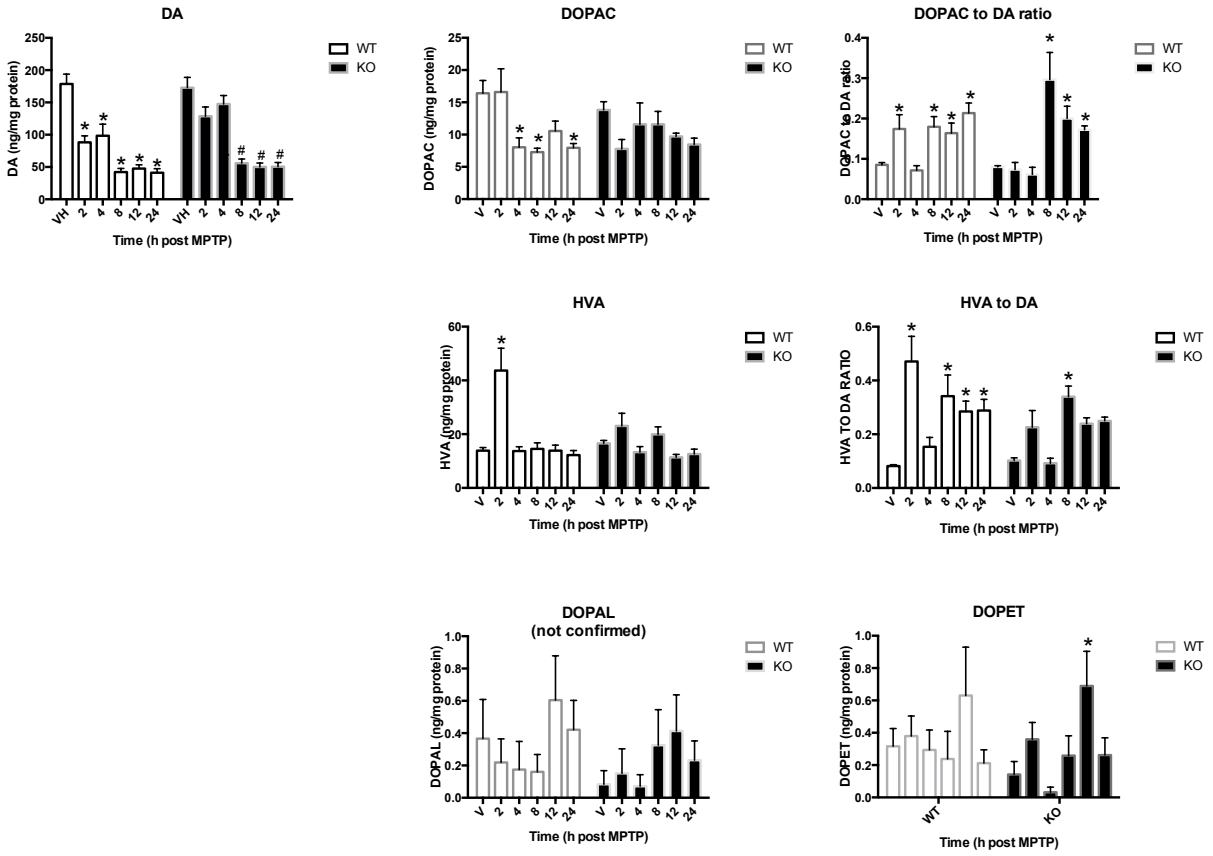


Figure B1 Temporal effect of MPTP on ST neurochemistry.. C57BL/6J and Park2$^{-/-}$ mice were treated with MPTP (20 mg/kg; sc) for the indicated time. The mice were decapitated and the striatum was analyzed for DA, DOPAC, DOPAL, DOPET and HVA content (n=10). Bars represent the mean +SEM. * Indicates statistically significant ($p < .05$) from the respective vehicle control.

profiles of C57BL/6J and Park2$^{-/-}$ mice (**Figure B1**). Upon treatment with MPTP, C57BL/6J and Park2$^{-/-}$ mice behave differently neurochemically. First, depletion in DA occurs differently between the two strains. DA is depleted early in the C57BL/6J mice (about a 50% reduction compared to the saline control). Park2$^{-/-}$ mice maintain the basal level of DA until 8 h following MPTP exposure. This may be because Park2$^{-/-}$ mice have increased and more active TH (**Figure B2**, **Figure 4-7**). By 8 h (in both the C57BL/6J and Park2$^{-/-}$ animals) DA was decreased to about 25% of the respective controls.

In C57BL/6J mice, as DA is depleted, the concentration of DOPAC is also reduced. Following MPTP exposure, DOPAC levels were decreased by about 50% in the C57BL/6J animals by 8 h and remained decreased for the remainder of the experiment (**Figure B1**). However, there was no statistically significant change in the amount of DOPAC in the Park2$^{-/-}$ mice. Park2$^{-/-}$ mice have increased extracellular DA compared to C57BL/6J mice (M. S. Goldberg et al., 2003). Interestingly, the DOPAC to DA ratio, a measure of cytosolic DA turnover was increased early in the C57BL/6J mice and in both the C57BL/6J and Park2$^{-/-}$ animals, the DOPAC to DA ratio was increased at 8, 12, and 24 h following MPTP.

Cytosolic DA is converted by monoamine oxidase (MAO) to its toxic metabolite, DOPAL, which is further oxidized to DOPAC via aldehyde dehydrogenase (ALDH) or to DOPET via aldehyde reductase. DOPAL is significantly more toxic to DA neurons than DA. As a result of oxidative stress, major lipid peroxidation products (4-hydroxynonenal, malondialdehyde) can competivitly inhibit ALDH activity leading to toxic levels of DOPAL (Yinsmaa et al, 2009). Therefore, the decrease in the amount of DOPAC may reflect changes in the activity of ALDH due to competitive inhibition with other aldhehydes produced during oxidative stress.

DOPAL peaks were very small and thus the measurements were highly variable, with many samples having undetectable amounts of DOPAL. Because of this high degree of variability, no statistically significant changes in DOPAL were detected in this experiment. Although the changes in DOPAL appear to be at the limit of detection for our HPLC. DOPET was also highly variable. However, the amount of DOPET is increased by 12 h in the Park2$^{-/-}$ mice. This suggests that at least some of the DOPAL was shunted to the alternative AR pathway late after MPTP exposure.

Temporal Changes in TH

Based on the changes in DA seen early following MPTP exposure, the amount of TH in the ST of C56BL/6J and Park2$^{-/-}$ was measured (**Figure B2**). The amount of TH was higher in the ST of Park2$^{-/-}$ mice compared to C57BL/6J, and while the C57BL/6J mice sustain total TH for 8 h, the Park2$^{-/-}$ demonstrate decreases in TH after only 2 h. By 24 h, TH in the ST of both the C57BL/6J and Park2$^{-/-}$ mice was decreased and did not appear to be different from each other.

Temporal Changes in Proteasome Activity

Proteasome activity was measured in the ST of C57BL/6J and Park2$^{-/-}$ mice that were treated with either vehicle or 20 mg/kg MPTP . In the C57BL/6J mice, proteasome activity initially rose at 2 h following MPTP exposure and then declined over time. On the other hand, proteasome activity did not rise until 4 h in the Park2$^{-/-}$ mice (**Figure B3-A**). In a second experiment, proteasome activity in C57BL/6J mice increased by 4 h and then declined while in the Park2$^{-/-}$ mice, proteasome activity increased by 8 h (**Figure B3-B**). Despite the differences in

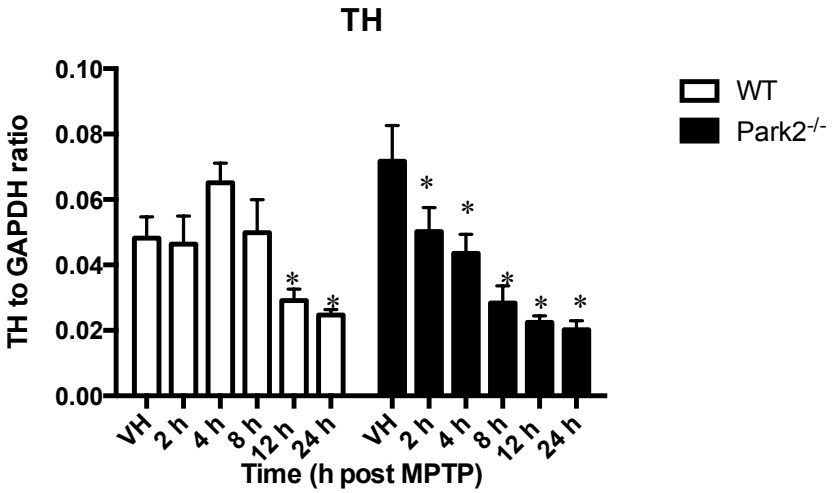
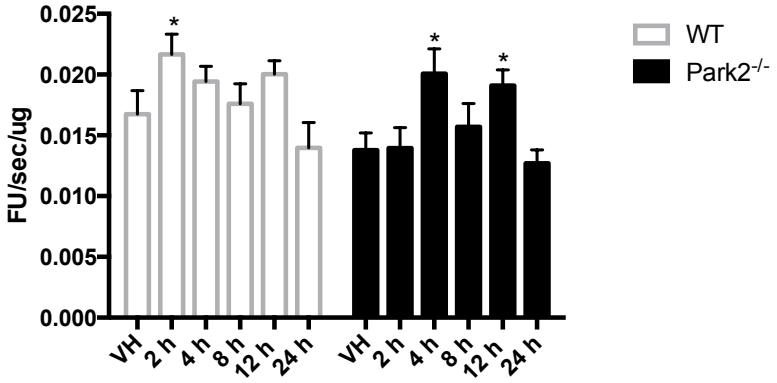


Figure B2 Effect of parkin deficiency on the amount of TH. C57BL/6J and Park2$^{-/-}$ mice were treated with MPTP (20 mg/kg; sc) for the indicated time. The mice were decapitated and striatal synaptosomes were prepared and subjected to denaturing electrophoresis and Western blot with an antibody directed toward TH and Pser40-TH. Bars represent the mean +SEM. * Indicates statistically significant ($p < .05$) from the respective vehicle control.

A



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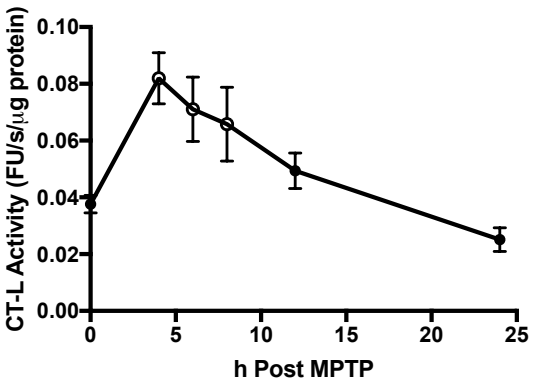

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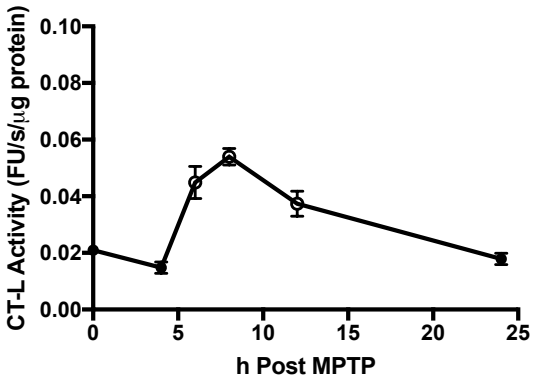

Figure B3 Temporal effect of MPTP on CT-L proteasome activity in the ST. C57BL/6J and Park2$^{-/-}$ mice were treated with MPTP (20 mg/kg; sc) for the indicated time. The mice were the ST was collected and proteasome activity was measured using the CT-L substrate Suc-LLVY-AMC and FU/sec/ug represents the linear portion of the degradation curve. **A**, Experiment 1 with C57BL/6J and Park2$^{-/-}$ mice **B**, Experiment 2 with C57BL/6J mice **C**, Experiment 3 with Park2$^{-/-}$ mice. Bars represent the mean +SEM. * Indicates statistically significant from the respective vehicle control ($p < .05$, n=10 per group).

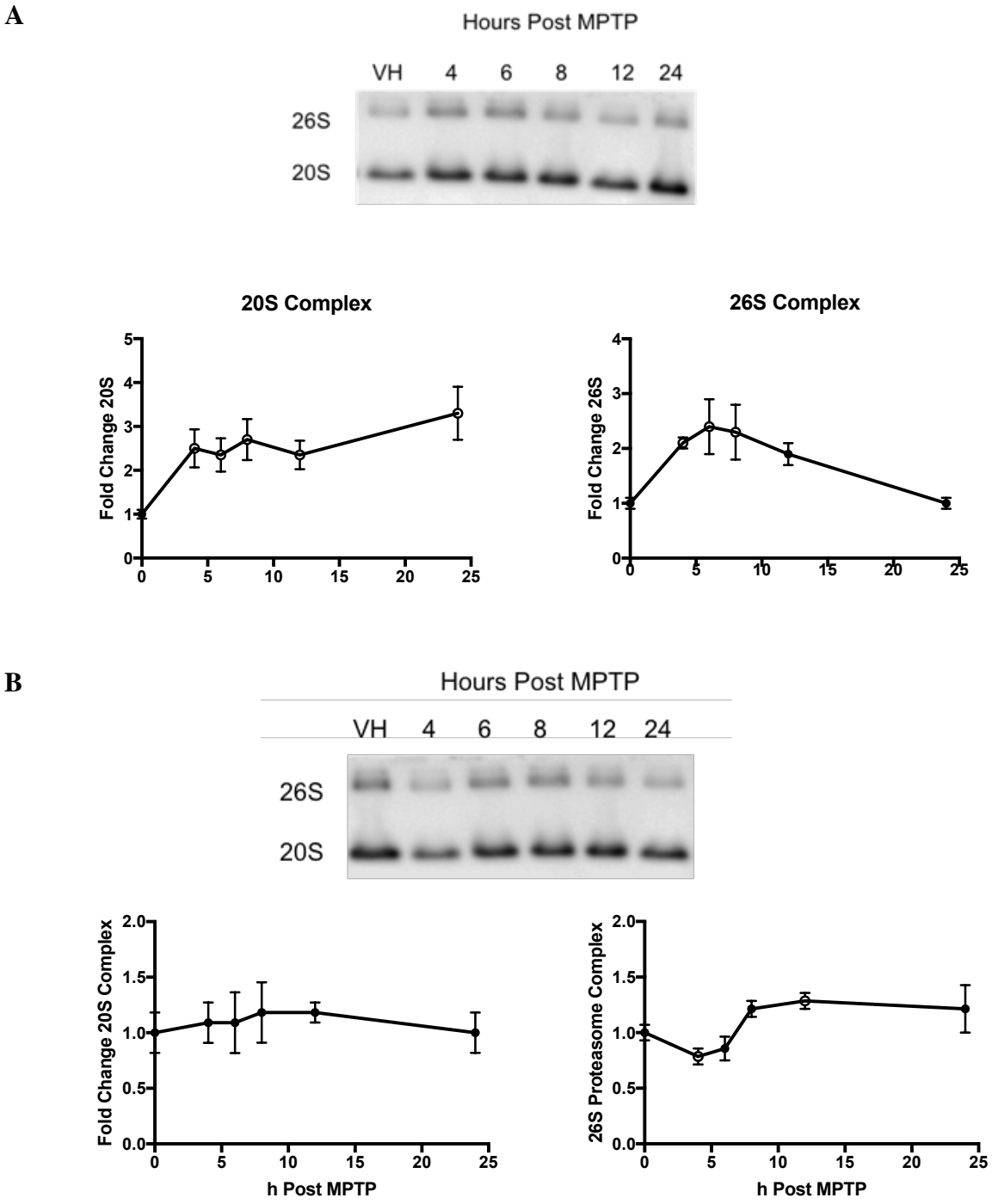

Figure B4 Temporal effects of MPTP on the amount of 20S and 26S proteasome in the ST. **A,** C57BL/6J and **B**, Park2$^{-/-}$ mice were treated with MPTP and 4, 6, 8, 12, and 24 h later the ST was collected and analyzed for 26S (right graph, presence of RPN10) and 20S (left graph, presence of b5 with no RPN10). By native gel electrophoresis followed by Western blot. The bars represent the mean fold change of 20S and 26S proteasome complexes. Error bars represent +1 SEM. Open symbols indicate time points that were significantly different from the vehicle control. ($p < 0.05$, n=10).

timing, both experiments indicate that there is a delay in the increase in 26S activity in the ST of Park2$^{-/-}$ mice following MPTP exposure.

Native gel electrophoresis of ST lysates allowed the determination of the relative amount of assembled 26S and 20S proteasome complex. **Figure B4** demonstrates that the amount of 26S proteasome complex in the ST of C57BL/6J mice increases early after MPTP exposure and then decreases over time while the 20S complex steadily increases over time. In the ST of Park2$^{-/-}$ mice, 26S complex is decreased early after MPTP exposure and then increases by 12 h. 20S complex in Park2$^{-/-}$ mice does not significantly change over the time of the experiment. This suggests that parkin is necessary for the initial rise in 26S proteasome complex. Data in chapter 5 (**Figure 5-8**) demonstrate that increased F-hParkin expression increases the assembly of the 26S proteasome and is likely responsible for the early increase in proteasome activity following MPTP exposure.

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