BEHAVIORAL, NEUROCHEMICAL, AND HISTOLOGICAL

CHARACTERIZATION OF MICE DEFICIENT FOR

PARKIN, DJ-1, AND ANTIOXIDANT PROTEINS

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DEDICATION

To my mentor, Dr. Matthew Goldberg, for his guidance and support.

My gratitude is beyond words.

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BEHAVIORAL, NEUROCHEMICAL, AND HISTOLOGICAL CHARACTERIZATION OF MICE DEFICIENT FOR PARKIN, DJ-1, AND ANTIOXIDANT PROTEINS

by

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BEHAVIORAL, NEUROCHEMICAL, AND HISTOLOGICAL CHARACTERIZATION OF MICE DEFICIENT FOR PARKIN, DJ-1, AND ANTIOXIDANT PROTEINS

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Parkinson's disease is a progressive neurodegenerative disease characterized by a loss of dopaminergic neurons in the substantia nigra. The cause of Parkinson's disease remains uncertain, however, evidence implicates mitochondrial dysfunction and oxidative stress with selective vulnerability of dopaminergic neurons. Although most cases of Parkinson's disease are sporadic, 5-10% of cases are caused by mutations in a single gene. Loss-of-function mutations in *parkin* and *DJ-1* were the first to be linked to recessively inherited parkinsonism. Surprisingly, mice bearing similar loss-of-function mutations in *parkin* and *DJ-1* do not show age-dependent loss of nigral dopaminergic neurons or depletion of dopamine in the striatum. Although the normal cellular functions of Parkin and DJ-1 remain unclear, we hypothesized that Parkin and DJ-1 protect cells from oxidative stress and that loss-of-function mutations in these genes cause neurodegeneration in Parkinson's disease by rendering cells more sensitive to

mitochondrial dysfunction and oxidative stress. We crossed mice deficient for Parkin and DJ-1 with mice deficient for the major mitochondrial antioxidant protein Mn-superoxide dismutase or Cu/Zn-superoxide dismutase. Previous studies have shown that mice with reduced levels of Cu/Zn-superoxide dismutase or Mn-superoxide dismutase are more sensitive to dopaminergic neurotoxins whereas mice with increased levels of superoxide dismutase are more resistant to dopaminergic neurotoxins. We predicted that reducing levels of antioxidant proteins in *parkin*$^{-/-}$*DJ-1*$^{-/-}$ mice would result in age-dependent nigral cell loss, striatal dopamine depletion or behavioral abnormalities. Characterization of these mice for behavioral abnormalities, neurotransmitter defects and neuropathology, revealed significant behavioral abnormalities in the mutant mice even in the absence of significant changes to dopamine levels in the striatum, dopamine receptor density, or dopaminergic neuron numbers. Aged *parkin*$^{-/-}$*DJ-1*$^{-/-}$ and Mn-superoxide dismutase triple deficient mice have a surprising enhanced rotorod performance without the presence of an anxiety phenotype or hyperactivity. Cu/Zn-superoxide dismutase and Mn-superoxide dismutase triple deficient mice have elevated levels of dopamine in the striatum, however none of the mice present with nigral cell loss. Levels of D1-like and D2-like dopamine receptors in the striatum were unchanged. It is evident from our studies that on a *parkin/DJ-1* null background, additional loss of major antioxidant proteins does not lead to a progressive loss of dopaminergic neurons in mice.

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Lee DHS, Seamans KW. The Nogo66 receptor pathway and CNS axon regeneration: New hopes for treating CNS injuries and neurodegeneration. *Expert Op. Therapeutic Patents*. **16**(8):1041-1050 (2006).

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LIST OF ABBREVIATIONS

3-MT - 3-methoxytyramide

6-OHDA - 6-hydroxydopamine

8-OHdG - 8-hydroxyguanosine

AADC – L-aromatic amino acid decarboxylase

AAV – adeno-associated virus

AD – Alzheimer's disease

ANOVA - one-way analysis of variance

ASR - acoustic startle response

ATP – adenosine triphosphate

COMT - catechol-O-methyl-transferase

DA - dopamine

DAergic - dopaminergic

DAT - dopamine transporter

DBS - deep brain stimulation

DOPAC - 3,4-dihydroxyphenylacetic acid

Drp1- dynamin-related protein

EDL - extensor digitorum longus

ETC – electron transport chain

FST – forced swimming test

GDNF - glial cell-line derived neurotrophic factor

GFRα-1 - GDNF receptor α-1

Gpx1 – glutathione peroxidase 1

GstS1 - glutathione S-transferase S1

H & E - hematoxylin and eosin staining

HD – Huntington's disease

HVA - homovanillic acid

L-DOPA - L-3,4-dihydroxyphenylalanine

LRRK2 - leucine-rich repeat kinase 2

MAOA & MAOB - monoamine oxidase A and B

Mfn1 - mitofusion 1

Mfn2 - mitofusion 2

MPTP - 1-methyl 4-phenyl 1,2,3,6-tetrahydropyridine

NAc - nucleus acumbens

NE - norepinephrine

NET – norepinephrine transporter

NSAIDS - non-steroidal anti-inflammatory drugs

Opa1 - optical atrophy 1

PD - Parkinson's disease

PET – positron emission topography

PFC – prefrontal cortex

PINK1 – PTEN (phosphatase and tensin homolog)-induced putative kinase 1

PPAR - peroxisome, proliferator-activated receptor

ROS – reactive oxygen species

SOD1 – Cu/Zn-superoxide dismutase

SOD2 - Mn-superoxide dismutase

SOL - soleus muscle

SNpc - substantia nigra pars compacta

SN - substantia nigra

TH - tyrosine hydroxylase

TH-ir - tyrosine hydroxylase immunoreactive

VMAT2 - vesicular monoamine transporter

VTA - ventral tegmental area

UCHL1 - ubiquitin carboxy-terminal hydrolase 1

UPDRS - united Parkinson's disease rating scale

UPS - ubiquitin-proteasome system

CHAPTER ONE

INTRODUCTION TO PARKINSON'S DISEASE

Etiology, Pathology, and Clinical Symptoms of Parkinson's Disease

Parkinson's disease (PD) is a progressive neurodegenerative disease and the greatest risk factor is age. About 3-5% of the population over the age 65 years is afflicted with the disorder in the United States (Whitton 2007). In his 1817 essay "Essay on the Shaking Palsy", the English apothecary James Parkinson described the disorder in detail (Charcot 1872; Parkinson 2002). PD is characterized by motor dysfunctions including resting tremor, muscle rigidity, bradykinesia, and postural instability. Symptoms can also include anxiety, depression, cognitive impairment, autonomic dysfunction, and sleep abnormalities (Jankovic and Aguilar 2008). The motor symptoms of PD are attributed to the progressive degeneration of dopaminergic neurons primarily in the substantia nigra pars compacta (SNpc), which project to the dorsal striatum (Trétiakoff 1919; Ehringer and Hornykiewicz 1960; Riederer and Wuketich 1976). The mechanisms behind the manifestation of non-motor symptoms are not well understood as of yet. Another neuropathological hallmark of PD is the presence of intracytoplasmic α-synuclein-rich inclusions, Lewy bodies, in post-mortem brains of PD patients. The functional significance of these inclusions is yet to be determined.

PD pathology has been researched using post-mortem human brains and non-human animal models. Animal models are the best means of studying the neurodegenerative process in PD. Non-human models can provide significant results that will lead to better prevention and

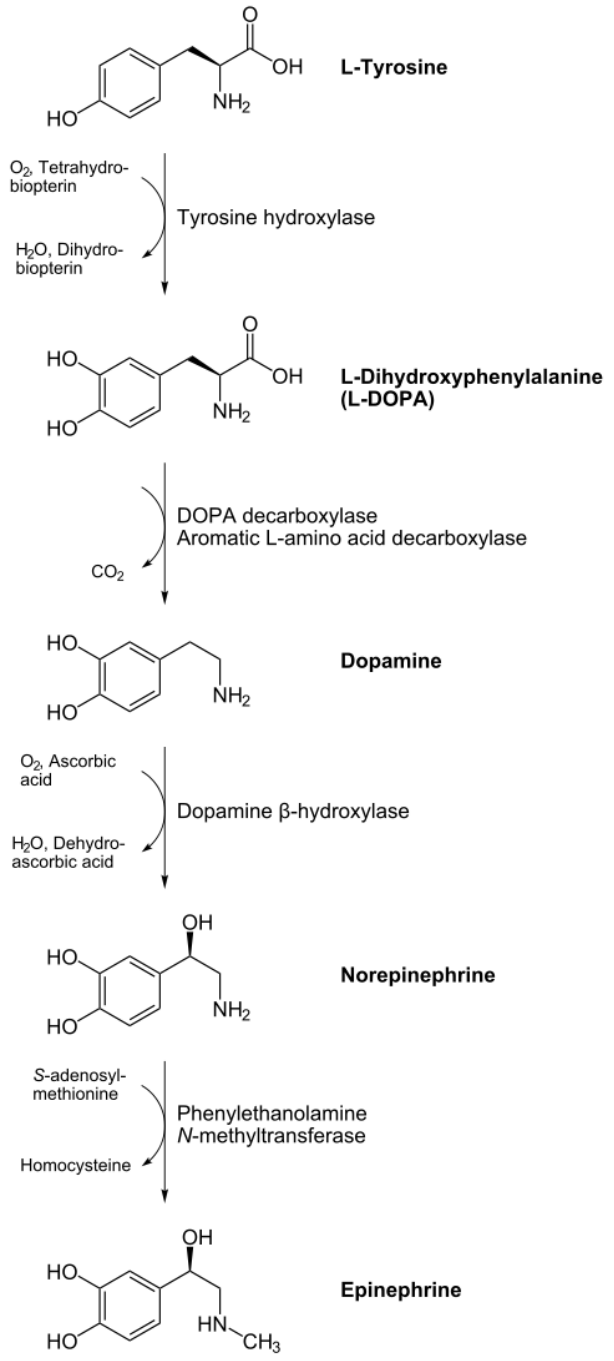


Figure 1.1: Synthesis of dopamine. Tyrosine hydroxylase and AADC synthesize DA from tyrosine. Norepinephrine and epinephrine are synthesized from DA.

treatment options for PD patients. Gaining a deeper knowledge of the pathology, biochemistry, genetics, histology, anatomy, and symptomology of PD is needed to develop a cure. To understand PD, it is important to first understand dopamine signaling and pathways in the brain, current treatment options, possible genetic and environmental causes, the effects of cellular mutations and oxidative stress on neurons, and current animal models. A thorough review of literature reveals vital information on these points and provides the basis for future studies.

Dopamine Pathways, Synthesis and Metabolism

Dopamine neurons in the nigrostriatal pathway project from the substantia nigra (SN) to the striatum. This pathway is essential for the initiation of movement. The meso-limbic dopamine pathway is

associated with social/emotional behavior, motivation, pleasure and reward. Dopamine (DA) neurons associated with this pathway project from the ventral tegmental area (VTA) to the nucleus accumbens (NAc), the amygdala, and the dorsolateral prefrontal cortex. This pathway regulates attention, initiative, motivation, planning, decision making, working memory, and other higher cognitive functions (Moore and Bloom 1978).

Dopamine Synthesis and Metabolism

Dopamine is synthesized from the amino acid tyrosine. Tyrosine hydroxylase (TH) is the rate limiting enzyme in the synthesis of DA (Figure 1.1). It adds a hydroxyl group to tyrosine to create L-DOPA (L-3,4-dihydroxyphenylalanine), which is then decarboxylated by AADC (aromatic amino acid decarboxylase) to form DA. DA is cleared from the synapse via metabolism or reuptake by the dopamine transporter (DAT) and broken down by monoamine oxidase (MAOA and MAOB) into 3,4-dihydroxyphenylacetic acid (DOPAC) (Figure 1.2). In the striatum, DAT is localized to the plasma membrane of axon terminals outside the synaptic active zone. Localization to the plasma membrane is facilitated by a direct interaction of DAT with the dopamine D2 autoreceptor (Lee, Pei et al. 2007). In the prefrontal cortex, the norepinephrine transporter (NET) is responsible for most of the DA clearance from the synapse (Moron, Brockington et al. 2002). The magnitude of DAT clearance of DA is about 10 times faster than NET clearance of DA. DOPAC and 3-MT are

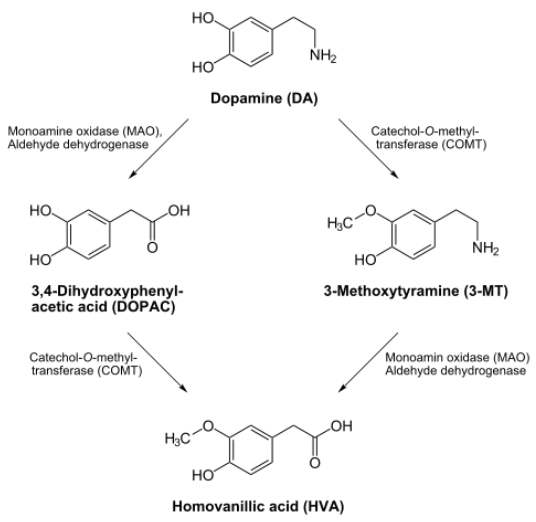


Figure 1.2: Dopamine is metabolized by MAO and COMT to form HVA.

catabolized to homovanillic acid (HVA) by the subsequent action of COMT or MAO, respectively. DA that is not metabolized by enzymatic activity is repackaged into synaptic vesicles by VMAT2 (vesicular monoamine transporter).

Arvid Carlsson was awarded the Nobel Prize in 2000 for his pioneering work on dopamine. In the 1950's, the discovery that DA was not only a precursor of norepinephrine, but a putative neurotransmitter in its own right, was groundbreaking research (Carlsson, Lindqvist et al. 1957; Carlsson and Waldeck 1958). His work also determined that DA was necessary for signal transmission in the motor pathway. DA is released from the presynaptic terminals in the striatum, as well as from dendrites in the SN (Cheramy 1978; Glowinski, Chéramy et al. 1988; Caboni, Sherer et al. 2004). Carlsson showed that supplementation of the DA precursor L-DOPA to DA-depleted cells restored motor function in animals, which led to clinical use of L-DOPA as a therapy for Parkinson's disease.

Current Treatment and Therapies

L-DOPA

Dopamine replacement therapy with the DA precursor L-DOPA is the most widely used and effective treatment for PD. Clinical efficacy of short term L-DOPA treatment for PD was first reported in 1961 (Birkmayer and Hornykiewicz 1962), and following a long-term study in 1967, it came into more widespread clinical use (Cotzias, Van Woert et al. 1967). L-DOPA is now considered the gold standard for treating the motor symptoms of PD. L-DOPA is extremely effective for symptomatic relief in early stages of PD, however, it is a palliative intervention and

does not arrest the progression of the disease. L-DOPA is usually administered orally as levadopa along with the AADC inhibitor carbidopa which prevents conversion of L-DOPA to DA in the periphery prior to crossing the blood-brain barrier. Approximately 95% of a dose of levadopa is metabolized in the gut and liver by COMT, so selective COMT inhibitors such as Entacapone may be administered with levadopa. Limitations of chronic levadopa oral therapy include the development of levadopa-associated motor complications. Progression of nigral-striatal neurodegeneration results in an inability to convert levadopa to DA, package DA in pre-synaptic vesicles, and release it in regulated response to stimuli. This results in a narrowing of the therapeutic dose window and more pronounced "wearing off" effects as well as side effects manifesting as dyskinesias; (Pezzoli and Zini 2010) for review.

MAO-B and COMT Inhibitors

Monoamine oxidase-type B (MAO-B) is the major enzyme in the catabolism of DA into DOPAC and HVA. MAO-B inhibitors are used clinically to reduce metabolism of endogenous DA and increase DA receptor stimulation providing symptomatic relief. MAO-B inhibitors, such as selegiline, rasagilin, or lazabemide, are best used as combination therapy with L-DOPA. However, some studies haves shown that the degree of symptomatic relief conferred by MAO-B inhibitors alone is indistinguishable from placebo (Goetz, Leurgans et al. 2002; Parkinson Study Group 2002).

Anti-cholinergics

Prior to the discovery of levadopa, one of the primary treatments for PD was the administration of anti-cholinergic alkaloid extracts, such as hyoscyamine derived from the plant *Atropa belladonna* (Ordenstein 1868; Lehmann, Hartung et al. 2007). Anti-cholinergics are effective in treating early stage PD and act by countering the imbalance between DA and acetylcholine following DA neuron degeneration. Basal forebrain cholinergic neurons play a definitive role in the cognitive and memory deficits of PD patients (Whitehouse, Hedreen et al. 1983). Cholinergic neurons project directly into the SN and the striatum to regulate DA release and transmission (Zhou, Liang et al. 2001). Cholinergic interneurons in the dorsal striatum express D2 and D5 dopamine receptors which may be involved in mediating DA regulation of cholinergic transmission (Alcantara, Chen et al. 2003; Berlanga, Simpson et al. 2005). Clinical usefulness of this class of drugs is limited by secondary neuropsychiatric adverse effects.

A2A Receptor Antagonists

In PD animal models, degeneration of noradrenergic neurons is one of the primary pathophysiological events leading to motor deficits. Preclinical trials have shown that A2a receptor antagonists consistently reverse motor deficits and enhance neuronal transmissions when given in conjunction with dopaminergic agents in animal models and can bind to receptors in the striatum of human subjects (Gillespie, Bamford et al. 2008; Brooks, Papapetropoulos et al. 2010). Administration of A2a receptor antagonists including vipadenant, KF17837, KW-6002 and MSX-3 potentiate the contralateral turning behavior induced by levodopa and other dopaminergic agonists in a 6-hydroxydopamine- (6-OHDA) lesioned rat model of PD.

Stimulation of the motor system by an A2a receptor antagonist showed no tolerance after repeated treatment (Koga, Kurokawa et al. 2000; Calon, Dridi et al. 2004; Schwarzschild, Agnati et al. 2006). These findings provide the basis for ongoing pharmaceutical research to develop A2a receptor antagonists as a therapeutic treatment for PD.

Deep Brain Stimulation

High-frequency deep brain stimulation (DBS) of the subthalamic nucleus is a surgical treatment available for advanced-stage PD patients and has proven highly successful in mitigating PD symptoms in most patients (Benabid, Chabardes et al. 2009). It specifically improves dopaminergic drug-sensitive symptoms in PD and enables the physician to reduce drug dosage, preventing side effects such as dyskinesias. This procedure is not free from adverse effects, primarily neurocognitive effects due to over stimulation of structures surrounding the electrical stimulus. Quality of life, however, can improve substantially with DBS and this therapy is becoming more prevalent.

NSAIDs and Inflammation

Inflammatory processes have been implicated in the pathogenesis of PD. Non-steroidal anti-inflammatory drugs (NSAIDs) may confer some neuroprotection against the development of PD. A meta-analysis of seven human studies reported that the use of non-aspirin NSAIDs is associated with a 15% reduction in risk of PD, whereas aspirin and acetaminophen were not beneficial (Gagne and Power 2010). NSAIDs also have beneficial effects in animal models of

PD (Esposito, Di Matteo et al. 2007). Recently, ibuprofen has been shown to lower the risk of PD by about 30%, by activating the peroxisome, proliferator-activated receptor (PPAR) pathway and reducing oxidative stress (Gao, Chen et al. 2011).

DA Cell Transplant Clinical Trials

Rat fetal DA neurons from the ventral midbrain can be successfully transplanted into the striatum and restore impaired motor function in a 6-OHDA lesioned rat model (Björklund, Dunnett et al. 1980). This discovery led to experimental treatments using DA neuron grafts in PD patients. A randomized, double-blind, placebo-controlled clinical trial evaluated the usefulness of neurotransplantation in PD (Freed, Greene et al. 2001). Mesencephalic DA neurons from four human embryos were transplanted bilaterally into the putamen of PD patients without immunosuppressant drugs. After 1 year, analysis showed that the transplanted cells grew in 85% of recipients, and, in patients under 60 years old, improved their United Parkinson's Disease Rating Scale (UPDRS) motor "off" (off L-DOPA) scores by 34%. There was no symptomatic change in patients who received the sham surgery. The response to L-DOPA was a predictor of the magnitude of improvement following the transplant. Adverse effects of the surgery included severe dyskinesias in a subset of patients, probably due to the excessive and poorly regulated release of DA from the transplanted cells. Clinical outcome and ^{18}F-DOPA uptake as measured by PET was assessed in a subset of these original patients, two or four years after transplant. Researchers found that ^{18}F-DOPA uptake increased in the grafts over time and correlated with improved clinical outcome (Ma, Tang et al. 2010). An independent group also conducted a similar double-blind trial, but failed to see a significant improvement in the transplant patients

(Olanow and Tatton 1999). In an analysis 11-14 years post transplantation, the transplanted neurons had decreased DAT staining and developed Lewy body inclusions, suggesting that despite originating from a different genetic background, the transplant was eventually affected by the disease process (Kordower, Chu et al. 2008; Li, Englund et al. 2008).

GDNF Intraputamenal Infusion

Glial cell line-derived neurotrophic factor (GDNF) is a target-derived growth factor in the Transforming Growth Factor-beta superfamily that signals through a two-component receptor complex, GDNF receptor α-1 and c-Ret (Tomac, Lindqvist et al. 1995; Trupp, Arenas et al. 1996). There is a significant correlation between the expression of *RET* mRNA in the adult mammalian nervous system and the response to GDNF in spinal motorneurons, SN DA neurons, and peripheral ganglia (Trupp, Belluardo et al. 1997). GDNF promotes survival of midbrain dopaminergic neurons during normal development and following neurotoxic damage to the substantia nigra (Lin, Doherty et al. 1993; Åkerud, Canals et al. 2001; Borlongan, Zhou et al. 2001). GDNF is neuroprotective and reduces oxidative stress caused by lesions or toxins in animal models and *in vitro* (Beck, Valverde et al. 1995; Tomac, Lindqvist et al. 1995; Hou and Mytilineou 1996; Palfi, Leventhal et al. 2002; Smith and Cass 2007). Levels of GDNF are reduced in the substantia nigra of PD patients (Mogi, Togari et al. 2001). Studies on rats and non-human primates indicate that GDNF administration reduces the dopaminergic neuron damage associated with normal aging conditions and acute lesions (Choi-Lundberg and Bohn 1995; Granholm, Mott et al. 1997; Mandel, Spratt et al. 1997; Date, Aoi et al. 1998; Kordower, Emborg et al. 2000; Ericson, Georgievska et al. 2005).

The number of tyrosine hydroxylase immunoreactive (TH-ir) interneurons in the striatum is augmented in non-human primates on the ipsilateral and contralateral side following administration of the dopaminergic neurotoxin MPTP (Palfi, Leventhal et al. 2002). Glia may release GDNF as a compensatory response to DA depletion, which in turn stimulates the TH gene (Francis, Von Visger et al.). GDNF delivered via a lentiviral vector enhances TH-ir neuron proliferation in aged Parkinsonian nonhuman primates more than sevenfold. No behavior data was reported in this study. These data suggest that GDNF may be a strong mediator of plasticity following dopamine depletion.

Unilateral intraputamenal infusion of GDNF has been explored as a treatment for PD. At least two human studies showed beneficial results (Gill, Patel et al. 2003; Slevin, Gerhardt et al. 2005); however other studies have shown no significant improvements in the treatment group (Nutt, Burchiel et al. 2003; Lang, Gill et al. 2006). In addition to its effects on DA neurons, GDNF also increases choline acetyltransferase activity in cranial motor nuclei and spinal motoneurons (Tseng, Baetge et al. 1997). Releasing a continual low-dose of GDNF near the substantia nigra protects the nigral dopaminergic neurons from an axotomy-induced lesion (Tseng, Baetge et al. 1997). These studies provide a basis for further research toward the use of GDNF as a treatment for PD.

Gene Therapy Clinical Trials

Gene therapy has been attempted in PD patients using an adeno-associated virus. The first was the glutamic acid decarboxylase enzyme injected into the subthalamic nucleus, the target region

for DBS surgery. This study is now in Phase II clinical trials (as of 2010) without reports of any adverse effect (Kaplitt, Feigin et al. 2007). Clinical assessments of its efficacy are expected within the year.

Neurturin is an alternative treatment explored by Ceregene (San Diego, CA) for its therapeutic efficacy treating PD. Neurturin is a neurotrophin in the GDNF family. The mode of administration is an intrastriatal gene transfer technique which places AAV-neurturin into the putamen. Although clinical improvement was noted in some patients, neurturin failed to meet the expected primary clinical end point in Phase II trials (Marks, Bartus et al. 2010).

An additional experimental treatment is the injection of aromatic L-amino acid decarboxylase (AADC) into the putamen to increase conversion of levadopa into dopamine (Christine, Starr et al. 2009; Muramatsu, Fujimoto et al. 2010). Phase I of the trial has reported clinical improvement. Gene therapy of AADC alone in non-human primates is associated with a 35% increase in normalized response to levadopa up to 8 years post-injection (Bankiewicz, Forsayeth et al. 2006; Hadaczek, Eberling et al. 2010).

A further approach in gene therapy is the administration of lentiviral vector containing three genes required for DA synthesis: TH, AADC and GCH1 (Guanosine 5’-triphosphate cyclohydrolase 1) (Jarraya, Boulet et al. 2009). Injected into macaques treated with MPTP, the gene therapy resulted in stable reduction of tremor and rigidity up to one year following

treatment, and up to four years in one follow-up animal. The safety profile of lentiviral delivery in humans is unknown as yet. Phase I-II trials are underway for TH-AADC-GCH1.

In summary, despite the treatment options available, there remains no cure for PD. It is important to have an animal model of the PD neurodegenerative process to understand the underlying causes and develop neuroprotective or neuroregenerative treatments to cure PD.

Pathogenesis of Parkinson's Disease

Age is the greatest risk factor for developing PD, which implicates accumulation of oxidative damage as a contributing factor. Exposure to environmental toxins that inhibit mitochondrial complex I, such as paraquat and rotenone, cause parkinsonism. Current research provides evidence that oxidative damage and mitochondrial dysfunction play a significant role in the etiology of PD. A small percentage of PD cases are caused by dominant or recessive heritable mutations. There are 5 main genes that have been reported to cause PD, each with multiple documented mutations: *α-synuclein, LRRK2, parkin, DJ-1,* and *PINK1*. Rare cases of patients carrying multiple mutations have been reported. Digenic mutations in *parkin* and *LRRK2* do not alter progression, onset, or severity of PD (Dächsel, Mata et al. 2006). Patients with digenic *parkin* and *PINK1* mutations had a lower age of onset than those carrying the same single *parkin* mutation (Funayama, Li et al. 2008). In another clinical report, individuals with digenic *DJ-1* and *PINK1* mutations developed early-onset PD. Mechanistically, *in vitro* wild type DJ-1 and PINK-1 interact and stabilize each other, whereas cells with PD-associated *DJ-1A39S* and

PINK1L399P mutations lose their ability to protect cells against MPP^+-induced oxidative stress and have reduced PINK1 levels (Tang, Xiong et al. 2006). Digenic mutations in some recessive PD genes appeared to modify the clinical course and hasten the disease process, suggesting a synergistic effect among these genes. Research has linked Parkin, DJ-1 and PINK1 to mitochondrial function, strengthening the hypothesis that mitochondrial dysfunction plays a prominent role in the genesis of PD.

Genetic Causes of Parkinson's Disease

Parkinson's disease was long-characterized as idiopathic, but recent studies have identified distinct genetic mutations that cause familial forms of PD. Genes with mutations causally linked to inherited forms of parkinsonism encode the proteins α-synuclein, LRRK2 (Leucine-rich repeat kinase 2), Parkin, DJ-1, PINK1 (PTEN-induced putative kinase 1), and ATP13a2. We will focus our studies on two of these proteins whose loss-of-function mutations cause PD: Parkin and DJ-1. We hope that studying the effects of PD-linked mutations in laboratory animals will reveal the pathogenic mechanisms of PD.

α-synuclein

Mutations in the *α-synuclein* gene lead to autosomal dominant PD. Disease causing point mutations in the promoter include A53T, A30P, and E46K (Polymeropoulos 1997; Kruger 1998; Zarranz, Alegre et al. 2004). Duplication or triplication of the gene also causes PD (Ross, Braithwaite et al. 2008). It appears that patients with higher levels of α-synuclein are at a greater

risk of developing PD. Although a rare cause of PD, its identification was extremely beneficial as it also led to the discovery that α-synuclein is present in Lewy bodies and neurites in most PD cases (Goedert 2001). α-synuclein may play a role in mitochondrial dysfunction. *In vitro*, α-synuclein binds to mitochondria and leads to mitochondrial fragmentation and reduced mitochondrial fusion that can be rescued by coexpression of *PINK1*, *parkin* or *DJ-1* but not the PD-associated mutations *PINK1G309D, parkinΔ1–79* or *DJ-1C106A* (Kamp, Exner et al. 2010).

LRRK2

LRRK2 mutations, the most frequent cause of genetic PD, have been linked to autosomal dominant PD with incomplete penetrance and account for a small percentage of idiopathic PD cases (Paisán-RuIz, Jain et al. 2004; Zimprich, Biskup et al. 2004; Albrecht 2005). The most common *LRRK2* mutation is the *G2019S* mutation in the kinase domain observed in 1% of sporadic PD cases and 4% of all familial cases (Healy, Falchi et al. 2008; Biskup and West 2009). Patients with one or two *G2019S* mutations have a similar age of onset and asymmetric onset of symptoms (Ishihara, Warren et al. 2006). The similarities between patients with homozygous and heterozygous mutations do not support a gene dosage effect. LRRK2 function is undetermined, but PD causing mutations in *LRRK2* have shown an increase in kinase activity *in vitro* (Guo, Gandhi et al. 2007). LRRK2 also plays a role in synaptic vesicle storage and mobilization, which may be secondary to effects on microtubules and axonal maintenance, implicating a role for LRRK2 in plasticity (Sämann, Hegermann et al. 2009; Piccoli, Condliffe et al. 2011).

Parkin

Loss-of-function mutations in *parkin* have been linked to autosomal recessive early-onset familial PD (Kitada, Asakawa et al. 1998). *Parkin* mutations account for 50% of all early onset familial PD cases, and about one fifth of all early onset sporadic cases (Lücking, Dürr et al. 2000). Over 100 loss-of-function mutations in *parkin* have been reported following its initial identification in four Japanese families as a genetic cause of juvenile Parkinsonism (Kitada, Asakawa et al. 1998). All types of mutations including deletions, insertions, truncations and point mutations in *parkin* have been linked to recessive parkinsonism. *Parkin* is a large (1.3Mb) gene encoding 465 amino acids; (Dawson and Dawson 2010) for review. Not all PD patients with *parkin* mutations develop Lewy bodies (Kitada, Asakawa et al. 1998; Shimura, Hattori et al. 1999; Farrer, Chan et al. 2001). Patients bearing *parkin* mutations develop earlier onset PD than patients without mutations, however progression of the disease is slower, and these patients are more sensitive to levadopa therapy. Patients with at least one missense mutation had a higher UPDRS motor score than those carrying two truncating mutations (Lohmann, Periquet et al. 2003). Parkin has E3 ubiquitin ligase activity, allowing modification of proteins through mono- or poly-ubiquitination (Shimura, Hattori et al. 2000). In humans, *parkin* mutations are related to impaired protein degradation, increased free radical accumulation and abnormal neurotransmitter release (Solano, Casarejos et al. 2008; Casarejos, Solano et al. 2009). Human fibroblasts from PD patients carrying *parkin* mutations have abnormal mitochondrial morphology and impaired function (Mortiboys, Thomas et al. 2008).

Parkin may be able to selectively target dysfunctional mitochondria for degradation and initiate mitochondrial autophagy (mitophagy). Disease-causing mutations in *parkin* impair

mitochondrial ubiquitination and mitophagy (Lee, Nagano et al. 2010). Parkin is recruited from the cytosol to the mitochondria following mitochondrial membrane potential uncoupling (Narendra, Tanaka et al. 2008). This recruitment requires PINK1 activity (Narendra, Jin et al. 2010). Interestingly, α-synuclein and Parkin expression are predominantly neuronal, however, *parkin* can be upregulated in glia in response to unfolded protein stress (Ledesma, Galvan et al. 2002). Parkin enhances mitochondrial biogenesis through direct interaction with the mitochondrial transcription factor TFAM, which may be neuroprotective as over expression of Parkin enhanced mitochondrial transcription and replication and overexpression of TFAM ameliorates delayed neuronal death following transient forebrain ischemia (Kuroda, Mitsui et al. 2006; Hokari, Kuroda et al. 2010). *Parkin* deficient *Drosophila* have severe mitochondrial defects in flight muscles and mild degeneration of a subset of dopaminergic neurons (Greene, Whitworth et al. 2003; Whitworth, Theodore et al. 2005). *Parkin* deficient mice do not exhibit severe mitochondrial defects; however they do have reduced mitochondrial respiratory activity (Palacino, Sagi et al. 2004). Although Parkin may be able to selectively target dysfunctional mitochondria for degradation, how Parkin influences mitochondria function and integrity is still unclear.

In vitro, *parkin*$^{-/-}$ midbrain neurons are resistant to induction of apoptosis by epoxomicin, a ubiquitin proteasomal system (UPS) irreversible inhibitor (Casarejos, Solano et al. 2009). This resistance may be due to increased glutathione peroxidase 1 (Gpx1) and DJ-1 protein levels in *parkin*$^{-/-}$ mice. The study suggests that mild UPS inhibition is compensated for by other mechanisms in *parkin*$^{-/-}$ midbrain neurons. *Parkin*$^{-/-}$ mice were crossed with mice expressing

mutant human α-synuclein. These mice showed significant age-related mitochondrial degeneration in dopaminergic neurons (Stichel, Zhu et al. 2007).

PINK1

Mutations in *PINK1* have been identified as the second most frequent cause of autosomal recessive inherited PD. *PINK1* encodes a serine/threonine kinase with a mitochondrial targeting sequence. In mitochondria, the kinase domain of PINK1 is cytoplasmic, whereas the N-terminal tail is inside the mitochondria (Zhou, Huang et al. 2008). PINK1 is present throughout the brain and co-localizes with mitochondria preventing its dysfunction, perhaps by phosphorylating Parkin or the mitochondrial chaperone protein TRAP1 (Pridgeon, Olzmann et al. 2007; Kim, Park et al. 2008). Most disease-causing mutations in *PINK1* are localized to the kinase domain. Experiments in *Drosophila* have shown that loss of Parkin or PINK1 results in flight muscle degeneration and mitochondrial dysfunction. This phenotype in *PINK1* null flies can be rescued by *parkin* overexpression, but the converse is not true (Clark, Dodson et al. 2006; Park, Lee et al. 2006; Yang, Gehrke et al. 2006). Parkin and PINK1 thus appear to be in the same pathway, and Parkin may regulate the stability of PINK1 through direct interaction of the two proteins (Shiba, Arai et al. 2009). In p*arkin* null or *PINK1* null *Drosophila*, mitochondrial phenotypes can also be rescued by overexpression of *Drp1* or decreased expression of *Opa1* or *Mfn2,* regulators of mitochondrial fission and fusion, suggesting that the Parkin/PINK1 pathway regulates mitochondrial fission. (Deng, Dodson et al. 2008; Poole, Thomas et al. 2008; Yang, Ouyang et al. 2008). Heterozygous loss-of-function *Drp1* mutations cause lethality in combination with *parkin* or *PINK1* deficiency in flies.

DJ-1

Loss-of-function mutations in *DJ-1* are linked to autosomal recessive early-onset familial Parkinson's disease (Bonifati, Rizzu et al. 2003). Originally identified for its interactions with the proto-oncogene *c-myc*, and as an RNA binding protein, DJ-1 may be a weak protease which can act as an oxidative stress-activated chaperone for synuclein, preventing its aggregation (Shendelman, Jonason et al. 2004; Zhou, Zhu et al. 2006). DJ-1 is predominantly expressed in the cytoplasm and nucleus of glial cells in the human brain, however *in vitro* it has been found to be concentrated in hippocampal neurons and astrocytes (Bandopadhyay, Kingsbury et al. 2004). Northern blots of *DJ-1* show ubiquitous expression across tissue types and it is highly conserved evolutionarily, indicating that DJ-1 has an important cellular function, however the specific details of this function are unknown (Bonifati, Rizzu et al. 2003; Bandyopadhyay and Cookson 2004). DJ-1 expression is upregulated in Alzheimer's disease (AD) brains and under conditions of oxidative stress in zebrafish (Baulac, Lu et al. 2009). DJ-1 may transcriptionally or translationally regulate gene expression and stabilize the antioxidant transcriptional regulator *Nrf2* (Clements, McNally et al. 2006; van der Brug, Blackinton et al. 2008). DJ-1 has been reported to function as an atypical peroxiredoxin-like peroxidase that scavenges H_2O_2 and can inhibit apoptosis via its interaction with Daxx (Junn, Taniguchi et al. 2005; Andres-Mateos, Perier et al. 2007).

Mitochondrial hypothesis of PD

Mitochondria generate the majority of the cell's supply of chemical energy, adenosine triphosphate (ATP), via cellular respiration with reactive oxygen species (ROS) as a byproduct of ATP synthesis. Mitochondria have their own genome, however most mitochondrial proteins

are encoded in the nucleus and must be transported into the mitochondria. There are elevated levels of acquired mitochondrial DNA deletions in SN neurons from PD patients (Bender, Krishnan et al. 2006). The inner mitochondrial membrane contains the four membrane-bound complexes of the electron transport chain (ETC) necessary for cellular respiration: Complex I, II, III, and IV. In addition to ATP synthesis, mitochondria are also involved in signaling, cell death, cell cycle and cell growth (McBride, Neuspiel et al. 2006). Mitochondria can undergo fission requiring the GTPase dynamin-related protein (Drp1) and fusion requiring mitofusion 1 (Mfn1), mitofusion 2 (Mfn2), and optical atrophy 1 (Opa1). In an animal model of stroke, nitrosative stress causes mitochondrial fission in neurons preceding neuron loss, and treatment with antioxidants has been shown to reduce mitochondrial fission (Barsoum, Yuan et al. 2006). It is possible that profound mitochondrial fission is a presymptomatic marker in PD, preceding the loss of DA neurons.

Inhibition of complex I by the environmental toxins rotenone, paraquat, and MPTP causes a loss of DA neurons and increased ROS production. PD patients have reduced complex I activity and oxidatively damaged subunits in platelets, multiple brain areas including the SN, and other tissues (Schapira, Cooper et al. 1990; Mann, Cooper et al. 1994; Haas, Nasirian et al. 1995; Keeney, Xie et al. 2006). Mitochondrial swelling, rarefaction of cristae, discontinuous outer membranes, and intramitochondrial inclusions have been reported in human PD cybrids, cytoplasmic hybrid cells containing PD mitochondria (Trimmer, Swerdlow et al. 2000). Mitochondrial swelling and cristae disruption is usually an early change in acute animal or *in vitro* neurotoxin models (Song, Shults et al. 2004).

Normal mitochondrial function and morphology is critically dependent on some PD related genes (Figure 1.3). Accumulation of wild-type and A53T α-synuclein in the mitochondria of human dopaminergic neurons causes reduced mitochondrial complex I activity and increased production of ROS (Devi, Raghavendran et al. 2008). *In vitro*, α-synuclein overexpression can cause mitochondrial fragmentation that is rescued by wild type Parkin, PINK1, or DJ-1, but not the disease-causing mutant variants (Kamp, Exner et al. 2010). *PINK1* knockdown or mutation can cause accumulation of calcium in the mitochondria, resulting in calcium overload, increased ROS production, and enhanced mitochondrial fission (Gandhi, Wood-Kaczmar et al. 2009; Marongiu, Spencer et al. 2009; Sandebring, Thomas et al. 2009). *PINK1* deficiency causes mitochondrial swelling, reduced cristae, and fragmentation that are dependent upon the mitochondrial fission protein Drp1 (Poole, Thomas et al. 2008). The double *PINK1-W437X* and *A53T α-synuclein* mutation and the *parkin/α-synuclein* double knockdown lead to even more severe mitochondrial alterations than single mutations (Stichel, Zhu et al. 2007; Marongiu, Spencer et al. 2009).

Parkin is recruited to the mitochondria in a PINK1-dependent manner following mitochondrial depolarization and initiates mitophagy of dysfunctional mitochondria by ubiquitinating mitofusin and targeting mitochondria for proteasome-dependent degradation (Matsuda, Sato et al. 2010; Ziviani, Tao et al. 2010; Yoshii, Kishi et al. 2011). Parkin enhances mitochondrial biogenesis by direct interaction with TFAM (Kuroda, Mitsui et al. 2006).

DJ-1 deficiency leads to fragmented mitochondria and aberrant mitochondrial morphology that can be rescued by over-expression of wild-type *parkin* and *DJ-1* (Irrcher,

Aleyasin et al. 2010). Mitochondrial function and morphology has been shown to decrease with age in *DJ-1* deficient fly and mouse models (Krebiehl, Ruckerbauer et al. 2010). In the fly model, up-regulation of *DJ-1* can rescue *PINK1*$^{-/-}$, but not *parkin*$^{-/-}$ phenotypes, suggesting DJ-1 is imperative for mitochondrial function and acts in parallel or downstream of PINK1 (Hao, Giasson et al. 2010). Parkin, DJ-1 and PINK1 have been demonstrated to play a role in mitochondrial function and autophagy, which further highlights the importance of mitochondria function in the etiology of PD.

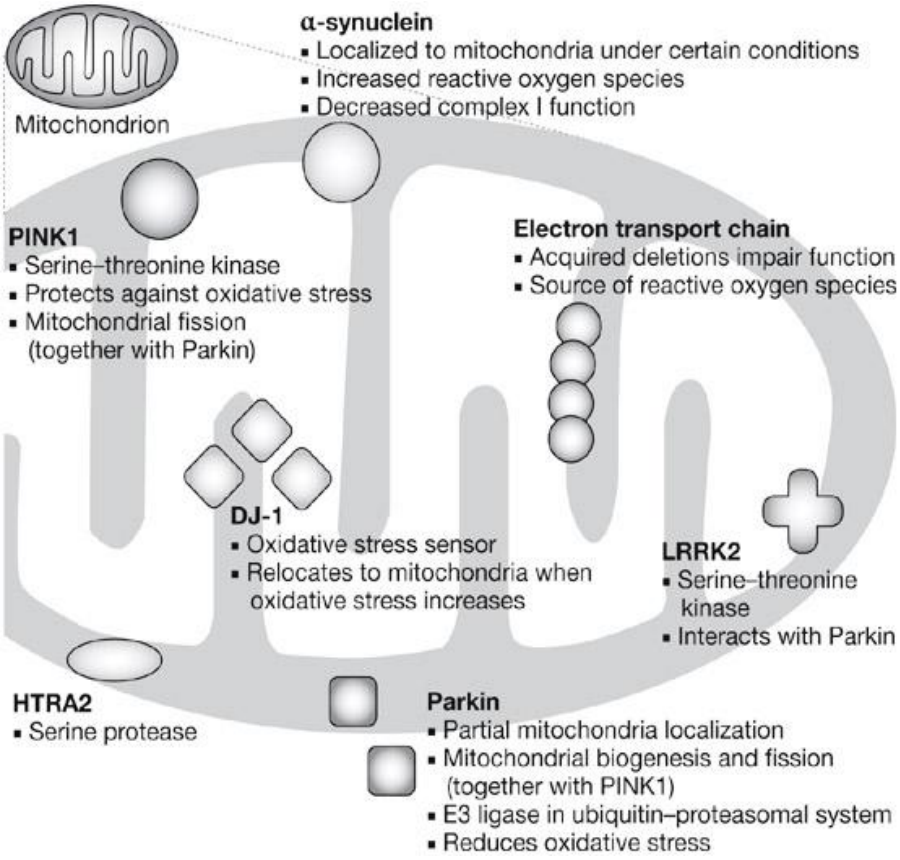


Figure 1.3: **Products of PD-associated genes affect mitochondrial function and oxidative stress.** Genes linked to familial PD such as parkin, DJ-1, PINK1, α-synuclein, LRRK2, and HTRA2 are localized to the mitochondrial following membrane depolarization and decrease complex I activity, increase ROS, enhance mitochondrial fission, enhance mitochondrial biogenesis, or target mitochondria for degradation.

Failure of the Ubiquitin Proteasome System in PD

Altered ubiquitination and degradation of proteins may play a pivotal role in DA neurodegeneration in PD. In patients, elevated levels of proteins damaged by oxidative stress increase protein aggregation and impair proteolysis (Alam, Jenner et al. 1997; McNaught, Olanow et al. 2001; Jenner 2003). The ubiquitin-proteasome system (UPS) is a natural intracellular protein degradation pathway that mediates targeted degradation of normal proteins and facilitates the removal of abnormal soluble proteins. Autophagy is another pathway responsible for degrading defective organelles, such as mitochondria. UPS proteolytic malfunction results in proteinopathies, which may lead to activation of autophagy and cell death.

Proteinopathies in most neurogenerative diseases occur due to toxic aggregation of metabolic products in the cell (Zheng, Li et al. 2009). Aggregates can also accumulate as a result of protein misfolding causing formation of inclusions. Ubiquitin present in these aggregates acts as a signal for proteolysis by the 26S proteasome, and serves to recruit proteins that are involved in the refolding of misfolded proteins. Mutations in genes coding for several ubiquitin-proteasome pathway proteins in PD suggests a link between the ubiquitin-proteasome system and neurodegeneration (Ross and Pickart 2004). Additionally, Parkin contains an E3-ligase domain and can function as an ubiquitin ligase associated with proteasomal degradation (Shimura, Hattori et al. 2000; Zhang, Gao et al. 2000; Imai, Gehrke et al. 2008). Alterations in protein homeostasis that promote the toxic accumulation of proteins has been implicated in the pathophysiology of PD (Lim and Tan 2007).

Oxidative Damage in PD

Oxidative stress has been implicated in neurodegeneration in PD (Jenner 2003). Oxidative damage can be caused by excessive ROS, a byproduct of cellular respiration. It has been hypothesized that excessive ROS in PD may be generated during DA metabolism or oxidative phosphorylation (Lotharius and O'Malley 2000; Hasegawa, Treis et al. 2008). Antioxidant proteins such as Gpx1 and superoxide dismutase (SOD) protect the cell from ROS.

In addition to damaging proteins and DNA directly, products of oxidative damage, such as 4-hydroxynonenal, can damage the 26S proteasome. Impairment of proteasomal function leads to more free radical generation, potentiating oxidative stress. PD patient postmortem brains exhibit oxidative damage to DNA, lipids, and protein (Dexter, Wells et al. 1989; Alam, Jenner et al. 1997; Floor and Wetzel 1998). 8-hydroxyguanosine (8-OHdG) is an oxidative derivative of guanosine and is used as a marker of oxidative stress and DNA damage. Levels of 8-OHdG are selectively increased in midbrain of PD patients (Zhang, Perry et al. 1999). Decreased levels of reduced glutathione in the PD brain indicate a failure to combat oxidative damage (Pearce, Owen et al. 1997).

Superoxide Dismutase

Reactive oxygen species (ROS), such as superoxide, are generated during aerobic metabolism and in response to external noxious stimuli which can damage cellular macromolecules. Antioxidant systems are present to mitigate the damage caused by ROS. One such system is comprised of the superoxide dismutase enzymes, which convert superoxide anion into molecular

oxygen and hydrogen peroxide. SOD1 (CuZn-superoxide dismutase) and SOD2 (Mn-superoxide dismutase) are the major cytoplasmic and mitochondrial antioxidant enzymes that protect neurons from oxidative damage.

SOD1 is the most abundant and ubiquitous isoform. The central nervous system (CNS) is particularly sensitive to injury by ROS. Transcription of the *SOD1* gene is regulated in response to stimuli such as stress, proinflammatory cytokines, and growth factors (Rojo, Salinas et al. 2004). The level of *SOD1* mRNA is significantly reduced in the substantia nigra of PD patients and marmosets treated with MPTP (Kunikowska and Jenner 2003). In mice, overexpression of SOD1 and SOD2 protect against MPTP and 6-OHDA toxicity (Andrews, Ladenheim et al. 1996; Asanuma, Hirata et al. 1998; Klivenyi, St. Clair et al. 1998; Callio, Oury et al. 2005). Surprisingly, *SOD1* deficiency is associated with enhanced recovery following brain trauma in mice, and locally silencing mutant *SOD1* protects against neurodegeneration in an Amyotrophic lateral sclerosis (ALS) mouse model (Beni, Tsenter et al. 2005; Ralph, Radcliffe et al. 2005).

The SOD2 (Mn-superoxide dismutase) isoform is localized to the mitochondria and serves as the primary defense against mitochondrial superoxide. There are conflicting reports on the role of SOD2 activity in the risk of developing PD (Shimoda-Matsubayashi, S. et al., 1997, Bandmann O. et al., 1995, Farin, F.M. et al., 2001). Mice with homozygous *SOD2* deficiency are not viable and die within the first week of life from cardiomyopathy, however, $SOD2^{+/-}$ mice are viable and used in our study (Li, Huang et al. 1995). A different *SOD2* deficient mouse lives until about 3 weeks of ages and displays neuronal cell loss and motor dysfunction (Lebovitz,

Zhang et al. 1996). Fibroblasts from *SOD2*$^{+/-}$ mice do not show the increased sensitivity to paraquat that is seen in *SOD1*$^{+/-}$ or *SOD1*$^{-/-}$ mice (Huang, Yasunami et al. 1997).

Rodent Models of Parkinson's Disease

The exact pathophysiology of Parkinson's disease is unknown. The progressive loss of dopaminergic neurons in the SNpc is one of the primary causes of clinical symptoms. However, the therapeutic benefit of symptomatic treatments, such as DA replacement therapy, diminishes as the disease progresses. Although neurointoxication models cause an acute and selective loss of DA neurons, no neuroprotective or neurodegenerative agents have been identified using these models that are efficacious in human patients. The pathway leading to the progressive degeneration of nigral neurons is likely a complex one which may be better addressed by genetic models based on mutations of genes that are linked to PD.

None of the current genetic animal models of PD completely recapitulate the neuropathology and clinical symptoms seen in patients. An ideal genetic animal model should exhibit progressive and age-dependent neuronal cell loss in the substantia nigra, motor deficits including slowness of movement, rigidity, tremor, postural instability and an improvement of motor behavior deficits with L-DOPA therapy. Lewy bodies and α-synuclein or ubiquitin inclusions are hallmarks of PD found in some patients with *parkin* and *LRRK2* mutations, although they are not present in all cases of PD (West, Moore et al. 2007). No postmortem neuropathology studies have been reported for PD cases with *DJ-1*, *PINK1*, or *ATP13a2*

mutations. Currently, none of the numerous PD animal models available adequately reproduce all the symptoms, progression and pathology of PD.

Beal suggests that an ideal PD model must satisfy the following criteria:

1. The animal model should posses a normal complement of dopaminergic neurons at birth, more than 50% of which should be selectively, gradually and measurably lost in adulthood, as well as exhibit the presence of Lewy bodies.
2. The model must exhibit motor deficits akin to those observed in PD patients, i.e., bradykinesia, rigidity and resting tremor.
4. A genetic model should be based on a single mutation to allow robust propagation and facilitate crossing with enhancer or suppressor strains.
5. The course of the disease in the animal model should be of short duration in order to facilitate rapid experimentation (Beal 2010).

PD related neurodegeneration is not limited to the substantia nigra. Studies show that PD also affects the olfactory bulb and other brain regions (Braak, Bohl et al. 2006). This is supported by the clinical finding that many patients report a loss of the sense of smell prior to the onset of motor symptoms. In addition to motor symptoms, patients may experience cognitive symptoms such as depression and dementia which could be assessed in an animal model. Many groups have created transgenic animals utilizing genes linked to familial PD in the hopes of developing a model that recapitulates the disease and can predict the efficacy of treatments in patients. These models, along with chemical models of PD, are discussed below.

Genetic Models: a -synuclein

A *Caenorhabditis. elegans* model over expressing *α-synuclein* has a decrease in the DA neuron population, however this degeneration is not progressive (Lakso, Vartiainen et al. 2003; Kuwahara, Koyama et al. 2006). *Drosophila* and yeast models with *α-synuclein* mutations have shown that α-synuclein, an abundant presynaptic phosphoprotein, may be involved in intracellular cargo trafficking, specifically within the ER/Golgi network (Cooper, Gitler et al. 2006; Gitler, Bevis et al. 2008). α-synuclein normally exists in an unstructured state, however there are a number of variables which cause the protein to misfold, including mutations in the gene, oxidative stress, and mitochondrial dysfunction. Lewy bodies are formed as a result of α-synuclein aggregating into oligomers, protofibrils, fibrils, and filaments. Overexpression of wild type human *α-synuclein* in *Drosophila* has been studied in concert with A53T and A30P mutations. This model mirrors many of the anticipated phenotypes of PD: age-dependent selective DA neuron loss, Lewy body inclusions containing α-synuclein, and DA-responsive locomotor deficits (Feany and Bender 2000). The benefit of using the *Drosophila* model overexpressing *α-synuclein* is the presence of progressive nigral cell loss, however, this species is less complex than vertebrates, they do not express endogenous α-synuclein, and they do not exhibit other symptoms of the disease as outlined above. It may be valuable for identifying possible pharmacological or genetic modifiers of *α-synuclein* expression, such as Hsp70 and HDAC6, but these modifications would have to be evaluated in higher organisms that are more genetically, anatomically, and physiologically similar to humans (Auluck, Chan et al. 2002; Du, Liu et al. 2010).

There have been a number of *α-synuclein* mouse models developed, including a knock-out animal and various over-expression models (Abeliovich, Schmitz et al. 2000). The *α-synuclein* deficient mice did not exhibit any difference in DA neuron number, indicating that α-synuclein does not play a critical role in the development, pruning, or maintenance of DA neurons. Additionally, these mice are resistant to MPTP toxicity, indicating that selective DA neurodegeneration induced by mitochondrial complex I inhibition is closely linked to α-synuclein function (Dauer and Przedborski 2003; Klivenyi, Siwek et al. 2006). Mice overexpressing *α-synuclein* are more susceptible than wild type to the mitochondrial toxin paraquat. Mice overexpressing the A53T mutation, but not wild type *α-synuclein,* under the mouse prion promoter displayed age-dependent intracytoplasmic neuronal α-synuclein inclusions and degeneration of motor function over time (Giasson, Duda et al. 2002). None of the overexpressing transgenic mouse lines model the pathology or symptoms of PD. Most notably, there is no nigral cell loss, (Chesselet, Fleming et al. 2008) for review.

Genetic Models: LRRK2

Overexpression of *LRRK2* in *Drosophila* causes an age-dependent reduction of locomotor activity that is responsive to DA replacement therapy. *Drosophila* and *C. elegans* overexpression models both present with age-dependent loss of DA neurons (Ng, Mok et al. 2009; Saha, Guillily et al. 2009; Venderova, Kabbach et al. 2009). Since these lower organisms do not endogenously express α-synuclein, they cannot exhibit α-synuclein pathology. A number of groups have published knockouts of the single *LRRK2* homolog in *Drosophila* (*dLRRK*) or *C. elegans* (*LRK-1*) with varied results. One group showed that *dLRRK*$^{-/-}$ flies did not develop a reduction in DA neurons, and they are less sensitive to paraquat and H_2O_2 than wild type, while flies with

Y1383C or I1915T mutations were more sensitive to these toxins (Imai, Gehrke et al. 2008). A conflicting study reported a loss of DA neurons in *dLRRK*$^{-/-}$ flies, but no change in DA neuron number in flies expressing wild type or pathogenic variants of *dLRRK* (Lee, Kim et al. 2007). In *C. elegans*, over expression of human *LRRK2*, G2019S, and R1441C causes a loss of DA neurons that is not present with the loss of endogenous *LRK1* or over expression of the dead kinase K1347A (Yao, El Khoury et al. 2010). In a zebrafish model, the deletion of the WD40 domain of *LRRK2* causes neuron loss and a locomotor defect (Sheng, Qu et al. 2010).

Unlike fly models, *LRRK2* transgenic mice do not present with any DAergic neurodegeneration. *LRRK2*$^{-/-}$ mice do not exhibit any neuropathological changes, although aged *LRRK2*$^{-/-}$ mice exhibit an impairment of protein degradation pathways and an accumulation of α-synuclein in the kidneys (Tong, Yamaguchi et al. 2010). DA transmission and motor deficits were reported in a BAC transgenic overexpression model of wild type *LRRK2* or G2019S. Overexpression of wild type *LRRK2* leads to increased DA release and improved motor function, whereas G2019S mice have increased kinase activity and an age-dependent decrease in striatal DA with no corresponding change in motor function, TH activity, VMAT2, DAT, or D2R protein levels (Li, Patel et al. 2010). Mouse models expressing R1441*G/C LRRK2* mutations also have impaired DA transmission and D2 receptor function (Li, Liu et al. 2009; Tong, Pisani et al. 2009). Despite changes in striatal DA levels and impairment in DA transmission and D2 function, *LRRK2* mouse models are not a good model for PD because they lack age-dependent progressive and selective nigral cell loss.

Genetic Models: Parkin

Parkin deficient *Drosophila* have defects in flight and climbing abilities that can be ameliorated by DA replacement (Greene, Whitworth et al. 2003; Pesah, Pham et al. 2004). In these flies there is an age-dependant decrease of DA neurons in the protocerebral posterior lateral region, and flight muscle degeneration (Whitworth, Theodore et al. 2005). Mitochondria in *parkin*$^{-/-}$ *Drosophila* exhibit a loss of cristae structure and mitochondrial defects in muscle and sperm. A defect in the formation of spermatids renders the male *parkin*$^{-/-}$ flies sterile (Greene, Whitworth et al. 2003; Pesah, Pham et al. 2004). *Parkin*$^{-/-}$ flies also have a reduced lifespan. The defects in the *parkin*$^{-/-}$ flies can be rescued by overexpression of *Glutathione S-transferase S1* (*GstS1*), which may lead to a promising treatment with compounds that enhance *GstS1* expression (Whitworth, Theodore et al. 2005; Fett, Pilsl et al. 2010). In a zebrafish model, Parkin has been shown to be protective against proteotoxic stress, specifically heat shock (Fett, Pilsl et al. 2010).

Parkin deficient mice generated by Goldberg *et al.* with a targeted germline disruption of *parkin* do not exhibit nigral cell loss, the pathological hallmark of PD. The phenotype of these mice is subtle, including elevated extracellular DA levels in the striatum, reduced excitability of medium-spiny striatal neurons, and deficits in the beam walk motor task (Goldberg, Fleming et al. 2003). *Parkin*$^{-/-}$ mice have reduced levels of complex I and IV, leading to diminished respiratory capacity. Additionally, there is an increase in lipid peroxidation and a decrease in serum antioxidant capacity (Palacino, Sagi et al. 2004).

Genetic Models: DJ-1

DJ-1$^{-/-}$ mice have been independently generated and characterized by a number of different laboratories (Chen, Cagniard et al. 2005; Goldberg, Pisani et al. 2005; Kim, Smith et al. 2005). We used the mouse generated by Goldberg *et al.* which contains a deletion of exon 2, thereby eliminating the ATG start codon and rendering the gene product null. These mice have decreased evoked DA overflow in the striatum, increased DA uptake, impaired long term depression, and lower locomotor activity compared to wild-type mice. There is no loss of DA neurons, and D2 receptor binding is also normal (Goldberg, Pisani et al. 2005). Another *DJ-1*$^{-/-}$ mouse missing the first 5 exons and part of the promoter showed a progressive decline in motor tasks, increased striatal DA, but no change in DA neuron number in the SN (Chen, Cagniard et al. 2005). A third group also deleted exon 2 and found no change in striatal DA levels or neuron number. *DJ-1* deficient mice are more susceptible to MPTP and *DJ-1* overexpression can protect mice from MPTP-induced neurotoxicity (Kim, Smith et al. 2005). *DJ-1*$^{-/-}$ mice aged 24 months have normal numbers of DA neurons in the substantia nigra, and normal levels of DA in the striatum (Yamaguchi and Shen 2007).

Genetic Models: PINK1

PINK1$^{-/-}$ mice do not have DAergic neurodegeneration, abnormal levels of striatal DA, or changes in DA receptor expression (Kitada, Pisani et al. 2007). These mice exhibit mitochondrial dysfunction in the striatum which is exacerbated by oxidative stress (Gautier, Kitada et al. 2008). Studies of a different *PINK1*$^{-/-}$ mouse reported a similar phenotype as well as weight loss and mild movement deficiency (Gispert, Ricciardi et al. 2009). As in *DJ-1*$^{-/-}$ mice, evoked DA overflow in the striatum is reduced in *PINK1*$^{-/-}$ mice. Mutations in *DJ-1* and *PINK1* genes have

been identified concurrently in some patients with early onset PD. It has been suggested that DJ-1 works by stabilizing PINK1, thereby protecting cells against oxidative stress. Interactions of PINK1, Parkin and DJ-1 may be the focus of future study, however triple knockout mice generated in the Shen lab lacking *parkin*, *DJ-1*, and *PINK1* exhibited no signs of nigral degeneration (Kitada, Tong et al. 2009), making it an unsuitable model of progressive nigral cell loss for PD research.

Chemical Models of PD

Most of the animal research on PD has been conducted on chemical models in which toxins are used to selectively destroy the DAergic neurons in the SN. The most commonly used neurotoxins are 6-OHDA, rotenone and MPTP, which are each described below.

Chemical Models: 6-OHDA

6-hydroxydopamine (6-OHDA) is a noradrenergic analog sharing structural similarity with DA, with the ability to enter neurons and inflict damage to the catecholaminergic pathways in the peripheral as well as the central nervous systems. It destroys catecholaminergic pathways by the combined action of reactive oxygen species and quinines and has been used in rodents as well as non-human primates to induce SN neurodegeneration. The standard approach uses surgical techniques for the administration of the compound in the medial forebrain bundle or striatum of mice or rats in order to create a unilateral lesion of the nigrostriatal circuit. The compound is directly injected into the brain as it cannot be administered peripherally due to poor penetration of the blood brain barrier. After the lesion forms, peripheral injection of amphetamine induces rotation behavior in the mouse or rat with a tendency to rotate contralaterally to the lesion due to

the asymmetry of dopaminergic innervation. The rotational behavior can be alleviated by numerous interventions including implantation of DAergic stem cells which are able to restore balanced DA receptor activation within the nigrostriatal circuit. Quantification of the rotation behavior in rats and mice with 6-OHDA unilateral lesions has been used extensively to assess the anti-parkinsonian potency of new drugs, gene therapies and to study motor fluctuations in levodopa chronic treatment. A stepping test, where forelimb akinesia is the observable end point, has been used to simulate the slowness of movement observed in PD patients. Data suggest that this technique provokes molecular alterations comparable to those seen in PD, enhancing the model's use as a means to explore the mechanisms of neurodegeneration in PD (Olsson, Nikkhah et al. 1995). However, because the loss of DA neurons in this model is rapidly induced and neurodegeneration in PD patients is progressive, this animal model does not accurately model the disease process, making it difficult to test drugs that may halt the progression of early-stage PD.

Rotenone

Among the various environmental factors that may play a role in the pathogenesis of PD, exposure to agrochemicals such as rotenone has been investigated (Gao, Hong et al. 2003). Rotenone and deguelin are active ingredients and principal components obtained from *Lonchocarpus utilis* which is employed as an agricultural insecticide (Caboni, Sherer et al. 2004). Rotenone is a specific inhibitor of complex I and has been shown to induce parkinsonism in rodent models. Four weeks following initial infusion, rats show more than 70% reduction in spontaneous motor activity that can be reversed by L-DOPA administration. Mechanistically, the degeneration of nigral DA neurons induced by rotenone is likely dependent on the participation of microglia and other immune response cells in the brain (Gao, Hong et al. 2002). Animal

studies have shown that chronic systemic rotenone exposure reproduced the nigrostriatal dopaminergic lesions and caused Lewy body formation in SN neurons. The PD-like syndrome induced in rats by rotenone and deguelin is due to the parent compounds rather than metabolites, and deguelin is about half as active as rotenone in inducing the PD-like syndrome (Betarbet, Sherer et al. 2000). However, DAergic damage measured quantitatively across different doses of rotenone did not correlate with motor behavior in individual rats. It is not yet firmly established that the behavioral abnormalities are a result of loss of nigrostriatal DAergic neurons. A recently developed rotenone model of PD shows a progressive loss of DAergic neurons, enteric nervous system dysfunction, microgliosis and α-synuclein positive Lewy body pathology (Cannon et al. 2009).

MPTP

The MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) model is one of the best characterized toxin-based model for PD available (Beal 2010). It was discovered accidently, when opiate addicts in California developed PD-like symptoms after inadvertently injecting the compound along with a synthetic opiate. MPTP administration in mice and non-human primates acutely reproduces the selective loss of SN DAergic neurons characteristic of PD. It exerts its action by inhibiting complex I of the electron-transport chain in mitochondria, resulting in reduction in ATP generation and increased free-radical production. Toxicity models using MPTP are used to study the nigrostriatal circuitry involved in PD pathophysiology. MPTP causes loss of SN and locus coeruleus DA neurons, while neurons in other regions such as dorsal motor nucleus of the vagus nerve and nucleus basalis of Meynert are spared. Chronic administration of MPTP has been found to produce α-synuclein inclusions.

Experimental Design Rationale

We recognized the importance and the paucity of reliable animal models that recapitulate the main hallmarks of PD: progressive nigral cell loss and motor dysfunction. With a proper animal model of PD we will be able to study the disease process, develop early interventions to prevent the progression of neurodegeneration, and develop new therapeutics to restore higher function in patients. It is possible that mice bearing disease causing mutations in *DJ-1* or *parkin* do not develop nigral cell loss due to compensatory mechanisms, or limited accumulated of oxidative damage due to a short 2-year life span. We postulated that if a loss of *parkin* or *DJ-1* is sufficient in humans to cause PD, then a combined deficiency of *parkin* and *DJ-1* would lead to nigral cell loss by interrupting two potentially different neuroprotective pathways. Additionally, a *SOD* deficiency should increase oxidative damage and lead to dopaminergic neurodegeneration on a *parkin/DJ-1* deficient background. In the next chapter we assess the behavioral, neurochemical, and pathological phenotype of *parkin*$^{-/-}$, *DJ-1*$^{-/-}$, *SOD1*$^{-/-}$, *SOD2*$^{+/-}$, *parkin*$^{-/-}$*DJ-1*$^{-/-}$, *parkin*$^{-/-}$*DJ-1*$^{-/-}$ *SOD1*$^{-/-}$ and *parkin*$^{-/-}$*DJ-1*$^{-/-}$*SOD2*$^{+/-}$ mice.

CHAPTER 2

BEHAVIORAL, NEUROCHEMICAL, AND HISTOLOGICAL CHARACTERIZATION OF MICE DEFICIENT FOR PARKIN, DJ-1, AND ANTIOXIDANT PROTEINS

MATERIALS AND METHODS

Animals

Parkin and *DJ-1* knockout mice on a C57BL/6 background were generated by Dr. M.S. Goldberg (Goldberg, Fleming et al. 2003; Goldberg, Pisani et al. 2005) and transferred to The University of Texas Southwestern Medical Center under a Material Transfer Agreement. *SOD1* and *SOD2* knockout mice on a C57BL/6 background were obtained from Dr. Holly Van Remmen at The University of Texas Health Science Center at San Antonio, with permission from Dr. Ting-Ting Huang, who generated these mutant mice. Double and triple mutant strains were obtained via animal husbandry. All lines were backcrossed to strain C57BL/6 at least 10 generations. When possible, paired littermates were used as controls. Experimental procedures involving the use of animals or animal tissue were performed in accordance with the NIH Guidelines for Animal Care and Use and approved by the Institutional Animal Care and Use Committee at The University of Texas Southwestern Medical Center in Dallas. Animals were housed in a climate controlled facility with a twelve hour light/dark cycle.

Methods for Characterization of Mouse Behavior

We conducted a battery of behavioral tests using the Behavioral Core Facility at UT Southwestern. We tested wild type, single, double, and triple mutant mice. All of these techniques have been previously used and published and are widely accepted in the field.

Rotorod Learning

Mice are placed on a stationary rotorod (IITC Life Science Inc.) and must maintain their balance for approximately ten seconds before the rotation is initiated. The rod is then accelerated from 5 to 45 rpm over five minutes. The time that each mouse falls from the rod is recorded. If a mouse holds onto the rod and rotates completely around two times, it is treated as if it has fallen from the rod at that time. Each mouse is tested four times a day for two consecutive days with a 15-30 minute intertrial interval.

Locomotor Activity

Mice were placed individually into a new, plastic mouse cage (18 cm x 28 cm) which was located inside a dark Plexiglas box. Movement was monitored by five photobeams in one dimension (Photobeam Activity System, San Diego Instruments, San Diego, CA) for two hours, with the number of beam breaks recorded during each five minute interval.

Open Field Activity

Mice were placed in the periphery of a novel open field environment (44 cm x 44 cm, walls 30 cm high) and allowed to explore for five minutes. The animals were monitored from above by a video camera connected to a computer running video tracking software (Ethovision 3.0, Noldus, Leesburg, Virginia) to determine the time, distance moved and number of entries into two areas: the periphery (5 cm from the walls) and the center (14 cm x 14 cm). The open field arenas were wiped and allowed to dry between mice.

Elevated Plus Maze

Mice were placed in the center of a black Plexiglas elevated plus maze (each arm 30 cm long and 5 cm wide with two opposite arms closed by 25 cm high walls) elevated 31 cm and allowed to explore for five minutes. The animals were monitored from above by a video camera connected to a computer running video tracking software (Ethovision 3.0, Noldus, Leesburg, Virginia) to determine time spent in the open and closed arms, time spent in the middle, and the number of entries into the open and closed arm. The apparatus was wiped and allowed to dry between mice.

Dark-Light Activity

Mice were placed into a black Plexiglas chamber (25 cm x 26 cm) and allowed to explore for two minutes. After the habituation period, a small door was opened allowing them to access the light side of the apparatus (25 cm x 26 cm lit to approximately 1700 lux) for ten minutes. The

animals were monitored by seven photobeams in the dark compartment and eight photobeams on the light side connected to a computer which recorded the time spent in each compartment, latency to enter the light side and the number of entrances to each compartment (Med-PC IV, Med Associates, St. Albans , VT). The dark-light apparatus was wiped and allowed to dry between mice. Results are presented as m±SEM and analyzed using a one-way analysis of variance (ANOVA).

Acoustic Startle Response

Acoustic startle response behavior was measured using a San Diego Instruments SR-Lab Startle Response System (San Diego, CA). Mice were placed into the Plexiglas holders and allowed to acclimate to the chamber and background white noise (70 dB) for five minutes. After the acclimation period, startle stimuli (80, 90, 100, 100 and 120 dB, 40 ms, white noise) were presented with an average interstimulus interval of 20 seconds (range 13 - 27 seconds). The Plexiglas holders were wiped and allowed to dry between mice.

Forced Swim Test

Mice were placed in a beaker (16.5 cm diameter) of water (21-25° C) to a depth of seven inches. The mice remained in the water for 6 minutes and were then removed and allowed to dry in a clean dry cage before returning to their home cage. The water was changed between each subject. The mice were monitored from the side by a video camera and data was stored on a videotape for later analysis. Only the last four minutes of the test were scored for latency to the

first immobility and total time spent immobile. The experimenter scoring the behavior was blind to the genotypes. Immobility was defined as no body or limb movement other than a minimal forelimb movement required for keeping the head above water.

Grip strength

The mice were suspended by the tail until it clasps the grip bar with both forelimbs and gentle constant resistance is applied until the moue releases the bar. This test measures the maximum force at which the forelimb grip yields when undergoing increasing gentle traction applied to the tail. Force is measured in grams by a force transducer. The best three of five trials per mouse were averaged.

Wheel running

We used a voluntary wheel running paradigm to analyze the voluntary locomotion of wild type and *parkin*$^{-/-}$*DJ-1*$^{-/-}$ mice over a long period of time. Additionally, we used a light/dark paradigm to investigate any changes in circadian rhythm in the mutant mice. Mice were placed in individual cages with running wheels attached to a sensor that recorded revolutions over time. Mice were on a normal 12 hour light/dark cycle for 2 weeks and then shifted to a 24 hour dark cycle for four weeks to measure circadian rhythms in the absence of external cues.

Brain Tissue Preparation

Mice were sacrificed following IACUC protocol using asphyxiation by CO_2. The brain was removed and inserted into a Brain Block on ice. Brains were cut in the coronal plane at the 5th or 6th slot (5-6mm) from the rostral end of the brain, immediately caudal to the optic chiasm. For HPLC experiments, the striatum was removed from the rostral half of the brain on ice. The left and right half of the striatum were weighed in separate 1.7 ml tubes, frozen on dry ice, and stored at -80°C until processed. For stereological estimation of neuron numbers, the caudal half of the brain was drop-fixed in 10% formalin for 24 hours at 4°C, rinsed in PBS, and processed in the paraffin processor. Briefly, all steps in the process were for one hour, except paraffin, which was for 4 hours. The stages were as follows: 50% EtOH, 70% EtOH, twice in 95% EtOH, twice in 100% EtOH, twice in ClearRite®, and twice in paraffin. Eight brains were embedded in each paraffin block with wild-type and mutant brains embedded in the same block in random order by Dr. Goldberg so I was blind to genotype. For radioligand binding experiments, the rostral half of the brain was flash frozen on dry ice, wrapped in aluminum foil to prevent freezer burn, and stored at -80°C until sectioned. Brains were embedded in OCT freezing medium and 20 μm coronal sections were cut on a cryostat at -20°C. Slides were stored at -80°C.

Neurochemical analysis by HPLC

Striatum was removed from mouse, weighed, and frozen on dry ice. The samples were stored at -80°C until use. Tissue samples were sonicated in 49 volumes of 0.1M perchloric acid containing 0.2mM sodium metabisulfate, then centrifuged at 15,000 rpm for 20 minutes at 4°C to clear debris. 5 μl of cleared supernatant was injected onto a C18 HPLC column and separated by

isocratic elution at a flow rate of 0.6 ml/min with MD-TM mobile phase (ESA Inc., Chelmsford, MA). Neurotransmitter monoamines and metabolites were detected using and ESA CoulArray electrochemical and model 5014B cell set to a potential of +220 mV. Peak areas were compared to a standard curve of external standards to calculate quantities of dopamine and its metabolites per milligram of tissue.

Stereological estimation of dopaminergic neuron number in the SNpc

Dopaminergic neuron numbers in the SNpc, marked by tyrosine hydroxylase immunohistochemical staining, were estimated with rigorous and unbiased stereology using StereoInvestigator analysis software (MicroBrightField Inc., Williston, VT) and the optical fractionator method as previously published (West et al., 1991; McCoy et al., 2008). Boundaries of the SNpc were defined using a 4X objective in accordance with previously published anatomical studies in the rat (German and Manaye, 1991). Counting frames were designated by 50x50 micron frames arrayed on a 100x100 grid which resulted in 25% of each region being randomly sampled. The average mounted thickness was 16 μm, the optical dissector was set to 14 μm with a 1 μm upper and lower guard zones. Cells were counted using a 40X oil-immersion objective on a Nikon 80i microscope.

Tritiated Dopamine Ligand Binding to D_1 and D_2-like Receptors in the Striatum

The rostral half of the mouse brain was sectioned on a cryostat at a thickness of 20 μm, mounted on microscope slides (SuperFrost Plus, Fisher Scientific, Hampton, NH), and stored at -80°C. On

the day of the assay, slides were thawed at room temperature and then preincubated in assay buffer (50 mM Tris, 120 mM sodium chloride, 5 mM potassium chloride, 1 mM magnesium chloride, 40 nM ketanserin) for 20 min at room temperature. Slides were then incubated in buffer containing either 2 nM tritiated SCH 23390 (PerkinElmer, Boston, MA) or 5 nM tritiated spiperone (PerkinElmer, Boston, MA) to examine binding of the D1 or D2 dopamine receptor, respectively. Ketanserin blocks binding of the ligands to 5-HT_2 receptors. To assess non-specific signal, I used cold competition of tritiated siperone with 10μm siperone, or of tritiated SCH 23390 with 1μm SCH 23390. Following one hour incubation at room temperature, slides were washed twice for five minutes in ice-cold buffer and then rinsed in cold water. Slides incubated overnight at 4C in a desiccator with paraformaldehyde powder in the bottom to fix the tissue without washing away the ligand. The following day, slides were dried for 2 hours in a desiccator with Dri-Rite. Slides were exposed to Kodak BioMax MS film with the Kodak BioMax TranScreen-LE Intensifying Screen for seven days (D1) or 5-7 weeks (D2). Films were analyzed for both density and area of binding using Adobe Photoshop software. For analysis of binding density, a region of interest was drawn around the striatum, density determined by the software, and an identical region of interest used to evaluate subsequent atlas-matched sections. A background reading was also taken from each slide and subtracted from the given density. For analysis of binding area, a minimum binding density was determined for a given section, a region of interest drawn around the desired area, and then the total area of binding within that region determined. The measurements from twenty sections per animal were averaged prior to statistical analysis. Four mice per genotype were included in the analysis.

Tyrosine hydroxylase Immunohistochemistry

Paraffin embedded brains were sectioned on a microtome at a thickness of 20 μm sections. The coronal sections were placed in a water bath at 40°C until they were flat, then mounted on SuperFrost Plus slides. Serial sections were kept on numbered slides. Slides were dried overnight at room temperature, and then baked for 2 hours at 60°C. Slides were cooled, deparaffinized, and rehydrated as follows: slides were incubated for 5 minutes in each of the following solutions: CitriSolve, CitriSolve, 50% CitriSolve / 50% ethanol, 100% ethanol, 100% ethanol, 95% ethanol, 70% ethanol, 50% ethanol, and water. For staining dopamine neurons slides were incubated with 0.1% H_2O_2 in methanol to quench endogenous peroxidase. After rinsing in PBS, slides were incubated in blocking solution consisting of 5% normal goat serum in PBS, then incubated with primary antibody consisting of a polyclonal Rabbit anti-Tyrosine Hydroxylase (AB152, Chemicon) 1:1000 dilution in the above blocking over night at 4°C. Slides were washed 15 minutes in PBS, followed by a horseradish peroxidase conjugated goat anti-rabbit secondary antibody for 1 hour at room temperature. Slides were washed in PBS and followed by 1 hour incubation with fresh avidin-biotin complex solution (ABC Elite kit, Vector Labs). After washing in PBS a DAB substrate was applied until color developed. The reaction was stopped with water and the deparaffin sequence was followed in reverse. Slides were coverslipped and sealed with a non-aqueous mounting solution.

Hematoxylin and eosin staining (H&E)

Application of hemalum is oxidized to hematoxylin and stains nuclei blue. The counter stain eosin Y stains eosinophillic tissues shades of red or pink. Briefly, slides were incubated 4

minutes in hematoxylin stain followed by a rinse in water, then a 0.3% hydrochloric acid in ethanol rinse. Slides were incubated in eosin for 2 minutes, dehydrated and mounted for imaging with a light microscope, as above.

Muscle fiber typing

Slow twitch (Type I) and fast twitch (Type II) muscles can be distinguished in muscle sections using a metachromatic ATPase stain. This stain utilizes the difference in mitochondrial density and pH to differentiate between fiber types. Fibers with low ATPase activity and subsequently low phosphate content are stained metachromatically, and those with high phosphate content were stained orthochromatically. In other words, Type I fibers stain dark blue, whereas Type II fibers stain light blue. Extensor digitorum longus (EDL) and soleus (SOL) muscles were dissected from wild type and *parkin*$^{-/-}$*DJ-1*$^{-/-}$ mice and flash frozen. Muscle were cut into thin sections and maintained at -20°C. Sections were stained with Metachromatic ATPase as previously published (Ogilvie 1990). Briefly, sections were pre-incubated with ATPase 8 minutes (pH 4.5), rinsed twice for 3 minutes in Tris buffer (pH 7.8), then incubated with ATP (pH9.4) for 25 minutes at room temperature. Slides were rinsed 3 times with calcium chloride and counterstained with Toluene blue 0.1% for 1 minute, cleared in ethanol, then xylene, and mounted for imaging with a light microscope, as above.

RESULTS

Given that loss of *parkin* or *DJ-1* in humans results in PD, we postulated that simultaneous deletion of these two genes linked to PD would cause a motor behavioral phenotype, as seen in PD patients. We further speculated that an additional stressor, such as the loss of the major antioxidant protein SOD1 or SOD2, would exacerbate these symptoms, perhaps by accelerating degeneration of DA neurons, and model the pathology of Parkinson's disease. Homozygous *SOD1* deficient mice are viable, although females are infertile. By contrast, homozygous *SOD2* deficient mice are not viable due to perinatal cardiomyopathy. Therefore, we characterized the behavioral, neurochemical, and pathological phenotype of *parkin*$^{-/-}$*DJ-1*$^{-/-}$ mice on a *SOD1*$^{-/-}$ genetic and a *SOD2*$^{+/-}$ genetic background.

We assessed the locomotor abilities and related behaviors of wild-type and mutant mice using a battery of well-established behavioral tests. Because age is the greatest risk factor for PD and because symptoms typically worsen with age, we tested separate cohorts of mice at ages ranging from young to old to determine if the triple mutant mice develop age-dependent behavioral deficits. The double mutant *parkin*$^{-/-}$*DJ-1*$^{-/-}$ mice were littermates of the triple mutant mice tested. All lines were backcrossed at least 10 generations to the strain C57BL/6J.

Parkin/DJ-1 Deficient Mice Outperform Wild-Type Mice on the Rotorod Test

The rotorod test is an accepted method to measure the ability of a mouse to sustain complex coordinated movement over time. It has been used as a functional measure of neurodegeneration in mouse models of ALS, Huntington's disease (HD) and other neurodegenerative diseases (Ramaswamy, McBride et al. 2009). Rotorod is also a test of basal ganglia function. Because PD is a disease where patients have difficulty with ambulatory movement, we hypothesized that mice carrying multiple mutations in PD-linked genes would have ambulatory deficits and would not be able to maintain themselves on the rotorod as long or perform as well as wild type mice. No abnormal rotorod phenotypes have yet been reported for *parkin*$^{-/-}$, *DJ-1*$^{-/-}$, *SOD1*$^{-/-}$, or *SOD2*$^{+/-}$ mice (Goldberg, Fleming et al. 2003; Von Coelln, Thomas et al. 2004; Chen, Cagniard et al. 2005; Goldberg, Pisani et al. 2005; Sato, Chiba et al. 2006; Chandran, Lin et al. 2008; Perucho, Casarejos et al. 2010), however one report found that *parkin*$^{-/-}$ mice had a learning deficit that was not observed on an accelerating rotorod (Shiotsuki, Yoshimi et al. 2010). We assessed the rotorod performance of wild type, *parkin*$^{-/-}$, *DJ-1*$^{-/-}$, *parkin*$^{-/-}$*DJ-1*$^{-/-}$, *SOD1*$^{-/-}$, *SOD2*$^{+/-}$, *parkin*$^{-/-}$*DJ-1*$^{-/-}$*SOD1*$^{-/-}$, *parkin*$^{+/-}$*DJ-1*$^{-/-}$*SOD2*$^{+/-}$, and *parkin*$^{-/-}$*DJ-1*$^{-/-}$*SOD2*$^{+/-}$ at various ages.

Mice were placed on a rotorod (IITC Life Science Inc) at a starting speed of 5 rpm and the rotation speed was accelerated over 5 minutes to a maximum speed of 45 rpm. The time at which each animal fell off the 1.25 inch diameter rotating rod was automatically recorded by magnetic switches that stopped individual timers when each mouse dropped. Mice were given 4 trials per day over 2 days with at least 30 minutes rest between trials. Data were analyzed by

repeated measures two-way ANOVA. Typically, mice learn to stay on top of the rotating rod and the latency to fall off increases with each trial until their maximum ability is reached in about 6-8 trials. As expected, we found a main effect of trial for each experiment, indicating that there is a learning process associated with the task and all groups improve over the course of the trials. We also found a main effect of genotype in each experiment, but were surprised to find that the *parkin*$^{-/-}$*DJ-1*$^{-/-}$ mice have enhanced performance over wild type mice instead of impaired performance. The effect is apparent in all age groups, but becomes more significant and pronounced with age. The following is the report of our rotorod experiments with detailed statistical analysis for each experimental group.

Our youngest cohort of mice tested was an average of six months old with mice ranging in age from 4 to 8 months old. In this cohort consisting of wild type, *parkin*$^{-/-}$*DJ-1*$^{-/-}$, and *parkin*$^{+/-}$*DJ-1*$^{-/-}$*SOD2*$^{+/-}$ (Figure 2.1A), there was a main effect of genotype and an expected main effect of trial [2 way RM ANOVA; main effect of genotype: $F(2, 62) = 5.853$, $p = 0.005$; main effect of trial: $F(7, 432) = 65.682$, $p < 0.001$; genotype x trial interaction: $F(14, 432) = 2.740$, $p < 0.001$]. Further analysis (Tukey Test) revealed a significant and surprising difference between *parkin*$^{-/-}$*DJ-1*$^{-/-}$ mice and wild type ($P = 0.004$). The *parkin*$^{-/-}$*DJ-1*$^{-/-}$ mice performed better on the rotorod than their wild type counterparts. There was no difference between *parkin*$^{-/-}$*DJ-1*$^{-/-}$and *parkin*$^{+/-}$*DJ-1*$^{-/-}$*SOD2*$^{+/-}$ ($P = 0.227$), or *parkin*$^{+/-}$*DJ-1*$^{-/-}$*SOD2*$^{+/-}$ and wild type ($P = 0.201$). These intermediate data from the *parkin*$^{+/-}$*DJ-1*$^{-/-}$*SOD2*$^{+/-}$ are challenging to interpret; the enhanced rotorod performance phenotype may be different from the *parkin*$^{-/-}$*DJ-1*$^{-/-}$ because of one normal *parkin* allele or because of the loss of a single *SOD2* allele. Further testing would be needed to parse out the causative element in the intermediate phenotype. When the two age groups, 4

months and 8 months, were analyzed separately, none of the genotypes were statistically different from each other, possibly due to the small sample size.

In another 6 month old cohort of mice, we tested wild type mice with *parkin*$^{-/-}$*DJ-1*$^{-/-}$, and the two triple mutant lines: *parkin*$^{-/-}$*DJ-1*$^{-/-}$*SOD1*$^{-/-}$and *parkin*$^{-/-}$*DJ-1*$^{-/-}$*SOD2*$^{+/-}$ (Figure 2.1B). We found that there was no significant difference between the genotypes at this age, but a trend toward better performance is apparent despite the small sample size. There was a main effect of trial indicating that there is expected learning of the task, however no main effect of genotype indicating no difference between the mice in the performance of the task [2 way RM ANOVA; main effect of genotype: $F(3, 27) = 1.976$, $p = 0.141$; main effect of trial: $F(7, 184) = 35.537$, $p < 0.001$]. Any effect the mutations have on rotorod performance is not significant at this age.

In the nine month old cohort of mice (Figure 2.1C, ranging 7-14 months of age), we tested wild type, *parkin*$^{-/-}$*DJ-1*$^{-/-}$, and *SOD2*$^{+/-}$. We found a main effect of genotype and trial [2 way RM ANOVA; main effect of genotype: $F(2, 25) = 10.927$, $p < 0.001$; main effect of trial: $F(7, 174) = 19.359$, $p < 0.001$; genotype x trial interaction: $F(14, 174) = 1.452$, $p = 0.134$]. Further analysis found that the *parkin*$^{-/-}$*DJ-1*$^{-/-}$ mice again stayed on the rotorod longer than wild type (Tukey Test, $p < 0.001$), the opposite of the expected phenotype.

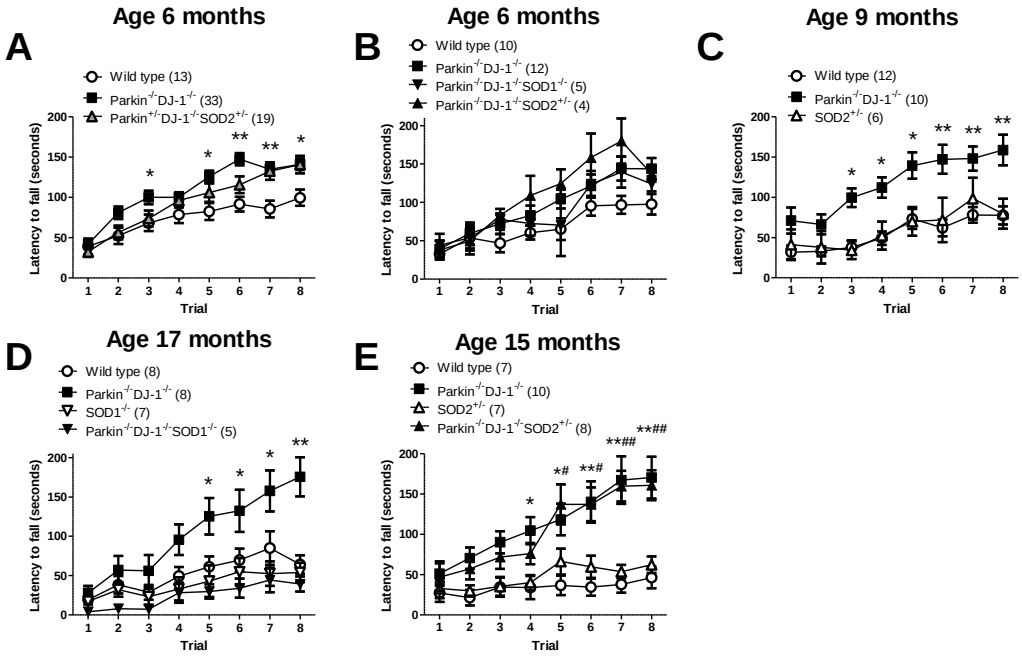


Figure 2.1: Unexpected enhanced rotorod performance in *parkin*$^{-/-}$*DJ-1*$^{-/-}$ mice. The *parkin*$^{-/-}$*DJ-1*$^{-/-}$ mice out-performed the wild type mice: *(A)* wild type, *parkin*$^{-/-}$*DJ-1*$^{-/-}$, and *parkin*$^{+/-}$*DJ-1*$^{-/-}$*SOD2*$^{+/-}$ age six months; *(B)* wild type, *parkin*$^{-/-}$*DJ-1*$^{-/-}$, *parkin*$^{-/-}$*DJ-1*$^{-/-}$*SOD1*$^{-/-}$, and *parkin*$^{-/-}$*DJ-1*$^{-/-}$*SOD2*$^{+/-}$ age six months; *(C)* wild type, *parkin*$^{-/-}$*DJ-1*$^{-/-}$ and *SOD2*$^{+/-}$ age 9 months; *(D)* wild type, *parkin*$^{-/-}$*DJ-1*$^{-/-}$, *SOD1*$^{-/-}$, and *parkin*$^{-/-}$*DJ-1*$^{-/-}$ *SOD1*$^{-/-}$ age 17 months; and *(E)* wild type *parkin*$^{-/-}$*DJ-1*$^{-/-}$, *SOD2*$^{+/-}$, and *parkin*$^{-/-}$*DJ-1*$^{-/-}$ *SOD2*$^{+/-}$ age 15 months. Data are presented as mean ± SEM. Statistically significant difference between wild type and *parkin*$^{-/-}$*DJ-1*$^{-/-}$ is denoted by * $p < 0.05$, ** $p < 0.001$; between wild type and *parkin*$^{-/-}$*DJ-1*$^{-/-}$*SOD2*$^{+/-}$ is denoted by # $p < 0.05$, ## $p < 0.001$.

To address the issue of the which alleles contribute to the enhancement in rotorod performance in *parkin*$^{-/-}$*DJ-1*$^{-/-}$ mice, and to determine the effect of a loss of SOD on the wild type and *parkin*$^{-/-}$*DJ-1*$^{-/-}$ phenotypes, we analyzed two aged cohorts consisting of wild type, *parkin*$^{-/-}$*DJ-1*$^{-/-}$, *SOD1*$^{-/-}$ or *SOD2*$^{+/-}$, and SOD1 or SOD2 triple mutants: *parkin*$^{-/-}$*DJ-1*$^{-/-}$*SOD1*$^{-/-}$ and *parkin*$^{-/-}$*DJ-1*$^{-/-}$*SOD2*$^{+/-}$ (Figure 2.1D, 2.1E). Consistent with previous cohorts, we found a main effect of genotype and trial in the 17-month old cohort [2 way RM ANOVA; main effect of

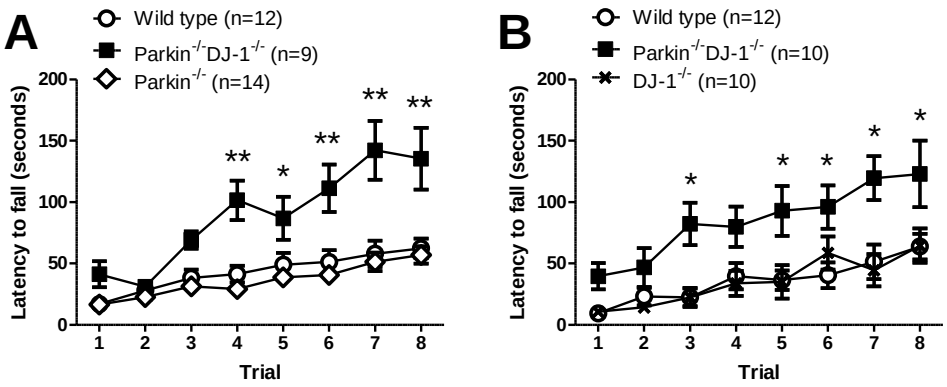


Figure 2.2: Rotorod enhanced performance of dKO mice over wild type is due to a synergistic effect of the loss of *parkin* and *DJ-1*. Single mutations in *parkin (A)* or *DJ-1 (B)*, are not sufficient for the enhanced performance phenotype. Data is shown as mean ± SEM. *parkin*$^{-/-}$*DJ-1*$^{-/-}$ is different from wild type as denoted by *p<0.05, **p<0.001.

genotype: F(3, 25) = 6.816, p = 0.002; main effect of trial: F(7, 174) = 24.337, p < 0.001; genotype x trial interaction: F(21, 174) = 3.629, p < 0.001]. Pair-wise comparison shows a significant increase in time spent on the rod by *parkin*$^{-/-}$*DJ-1*$^{-/-}$ over wild type [Tukey test, p = 0.034], SOD1$^{-/-}$ (n = 8) [Tukey test, p = 0.006], and *parkin*$^{-/-}$*DJ-1*$^{-/-}$*SOD1*$^{-/-}$ [Tukey test, p = 0.003]. The *SOD1*$^{-/-}$ mice deteriorate with age, so by 17 months their mobility has become limited. The *parkin*$^{-/-}$*DJ-1*$^{-/-}$*SOD1*$^{-/-}$ mice are similarly lethargic, which may account for poor coordination or stamina to stay on the rotorod and the different phenotype from the *parkin*$^{-/-}$*DJ-1*$^{-/-}$. Alternatively, the absence of SOD1 may reverse or override the phenotype normally generated by the combined loss of Parkin and DJ-1, but we do not believe this to be the case. Another older cohort, age 15 months, analyzed the SOD2 line with the *parkin*$^{-/-}$*DJ-1*$^{-/-}$, (Figure 2.1E). Again, we found a main effect of genotype and trial in the 15 month old cohort [2 way RM ANOVA; main effect of genotype: F(3, 28) = 7.954, p < 0.001; main effect of trial: F(7, 196) = 25.654, p < 0.001; genotype x trial interaction: F(21, 196) = 4.073, p < 0.001]. Pair-wise comparison shows a

difference between *parkin*$^{-/-}$*DJ-1*$^{-/-}$ and wild type [Tukey test, p = 0.002], and *parkin*$^{-/-}$*DJ-1*$^{-/-}$ and *SOD2*$^{+/-}$ [Tukey test, p = 0.012]. There is also a difference between *parkin*$^{-/-}$*DJ-1*$^{-/-}$*SOD2*$^{+/-}$ and wild type [Tukey test, p = 0.010] and *SOD2*$^{+/-}$ [Tukey test, p = 0.044]. Performance on the rotorod test by *parkin*$^{-/-}$*DJ-1*$^{-/-}$*SOD2*$^{+/-}$ is not different from *parkin*$^{-/-}$*DJ-1*$^{-/-}$, indicating that the loss of one allele of *SOD2* in addition to the combinatorial loss of *parkin* and *DJ-1* does not change the enhanced rotorod phenotype observed in *the parkin*$^{-/-}$*DJ-1*$^{-/-}$ mice.

We tested single *parkin*$^{-/-}$ and *parkin*$^{-/-}$*DJ-1*$^{-/-}$ dKO with wild type mice, age 13 months, to determine if a loss of Parkin is sufficient to elicit an enhanced rotorod phenotype. Previous studies of *parkin*$^{-/-}$ mice have not reported a rotorod phenotype different from wild type, just as we failed to see one in our trials. In this experiment, we found a main effect of genotype [2 way RM ANOVA; main effect of genotype: F(2, 32) = 14.417, p < 0.001; main effect of trial: F(7, 222) = 31.013, p < 0.001; genotype x trial interaction: F(14, 222) = 4.830, p < 0.001]. Pair-wise comparison shows a difference between *parkin*$^{-/-}$*DJ-1*$^{-/-}$ and wild type [Tukey test, p < 0.001], and between *parkin*$^{-/-}$*DJ-1*$^{-/-}$ and *parkin*$^{-/-}$ [Tukey test, p < 0.001] beginning at the forth trial, while there is no difference between *parkin*$^{-/-}$ and wild type [Tukey test, p = 0.735]. We also tested *DJ-1*$^{-/-}$ mutant mice along with *parkin*$^{-/-}$*DJ-1*$^{-/-}$ and wild type mice at 16 months of age (Figure 2.2B). Previous studies of *DJ-1*$^{-/-}$ mice have

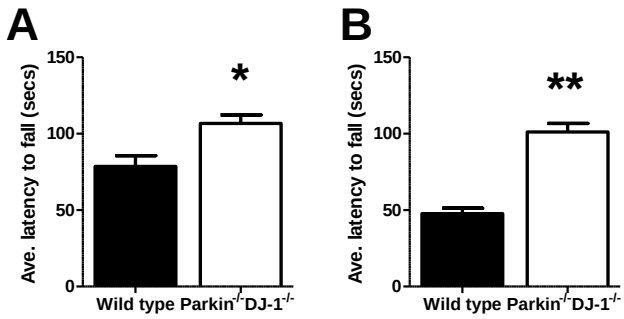


Figure 2.3: Young and aged *parkin*$^{-/-}$*DJ-1*$^{-/-}$ mice consistently perform better than wild type mice on the rotorod test. In 4 month old mice (A), the enhanced performance is not as robust as the difference seen in older mice, age 7-21 months (B). Bars represent mean average latency to fall off the rod across all trials and cohorts ± SEM. * p < 0.05; ** p<0.001

not reported a rotorod phenotype different from wild type, just as we failed to see one in our trials. There is a main effect of genotype [2 way RM ANOVA; main effect of genotype: $F(2, 29) = 7.203$, $p = 0.003$; main effect of trial: $F(7, 202) = 20.476$, $p < 0.001$; genotype x trial interaction: $F(14, 202) = 1.384$, $p = 0.163$]. Pair-wise comparison shows a difference between *parkin*$^{-/-}$*DJ-1*$^{-/-}$ and wild type [Tukey test, $p = 0.006$], and between *parkin*$^{-/-}$*DJ-1*$^{-/-}$ and *DJ-1*$^{-/-}$ [Tukey test, $p = 0.008$], while there is no difference between *DJ-1*$^{-/-}$ and wild type [Tukey test, $p = 0.999$]. Neither the *DJ-1* deficient mice nor the *parkin* deficient mice performed differently from wild type in the rotorod test, however the dKO mice consistently outperform the wild type and single mutant mice in all cohorts. Thus, Parkin and DJ-1 appear to act synergistically to illicit an enhanced rotorod performance in the *parkin*$^{-/-}$*DJ-1*$^{-/-}$ mice.

We pooled the data for wild type and *parkin*$^{-/-}$*DJ-1*$^{-/-}$ mice from preceding trials as there was no effect of age on the average latency to fall, averaged across trials and cohorts, for 7-21 month old or four-month old wild type and *parkin*$^{-/-}$*DJ-1*$^{-/-}$ mice (Figure 2.3). In the older mice, the *parkin*$^{-/-}$*DJ-1*$^{-/-}$ ($n = 56$) stayed on twice as long as the wild type mice ($n = 58$), [$F(1, 112) = 67.193$, $p < 0.001$]. The 4 month old *parkin*$^{-/-}$*DJ-1*$^{-/-}$ ($n = 24$) behaved similarly to the older mice, however the younger wild type mice ($n = 6$) had better performance than the aged wild type, making the difference between genotypes less than in the older mice but still significant, [$F(1, 28) = 4.993$, $p < 0.034$]. We consistently and repeatedly observed an enhanced rotorod performance in the *parkin*$^{-/-}$*DJ-1*$^{-/-}$ mice.

Locomotor behavior is unchanged in mutant mice

The locomotor test is a behavioral test that measures total locomotor activity and can identify mice that are more hypoactive or hyperactive than wild type mice. We hypothesized that our mutant mice would have an age-dependent locomotor deficit that would manifest as reduced ambulatory behavior compared to age matched controls. We found no such locomotor deficit in young or aged mice.

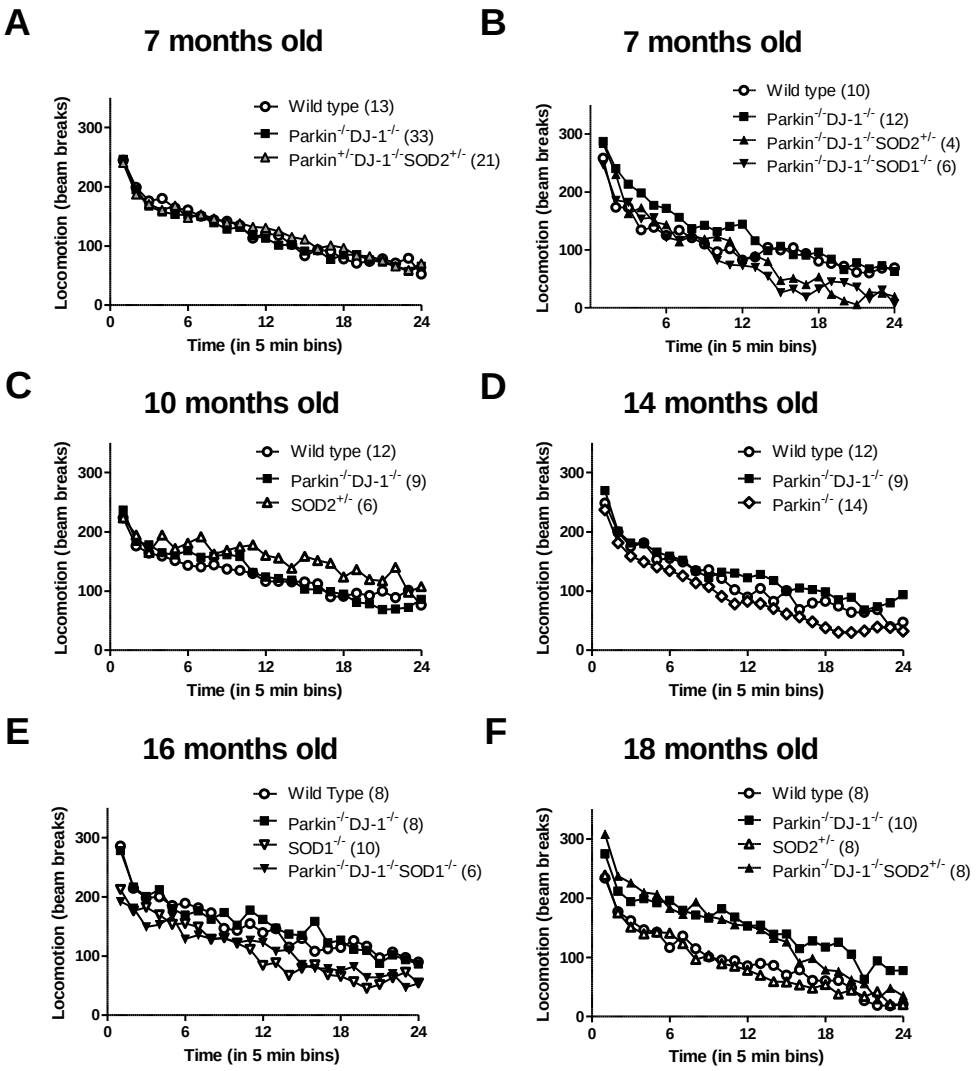


Figure 2.4: Mean total locomotion per five minute bin of time over 2 hours. Young (A, B), middle aged (C, D) and old (E, F) mutant mice were compared to age matched wild type controls. Error bars are not shown for clarity. Sample number is indicated in parentheses.

We measured the locomotor behavior of mice in cohorts with an average age ranging from 7 months to 18 months of age, including wild-type, *parkin*$^{-/-}$, *SOD1*$^{-/-}$, *SOD2*$^{+/-}$, *parkin*$^{-/-}$*DJ-1*$^{-/-}$, *parkin*$^{-/-}$*DJ-1*$^{-/-}$*SOD1*$^{-/-}$, *parkin*$^{+/-}$*DJ-1*$^{-/-}$*SOD2*$^{+/-}$, *parkin*$^{-/-}$*DJ-1*$^{-/-}$*SOD2*$^{+/-}$ mice. We measured total ambulatory movement and fine motor movement, as well as total movement integrating the two movement sub types. Mice were placed individually into a new, plastic mouse cage (18 cm x 28 cm) which was located inside a dark Plexiglas box. Movement was monitored by five photobeams in one dimension (Photobeam Activity System, San Diego Instruments, San Diego, CA) for two hours. The sum total of beam breaks was recorded during each of 24 five minute intervals. All graphs in Figure 4 represent total movement over time. We tested wild type, *parkin*$^{-/-}$*DJ-1*$^{-/-}$, and *parkin*$^{+/-}$*DJ-1*$^{-/-}$*SOD2*$^{+/-}$ mice in a 7 month old cohort to assess locomotion at a young age (Figure 2.4A). When mice are placed in the testing cage, the novelty of the environment causes a high activity level. Over the two hours that the test is conducted, the mice acclimate to their environment as indicated by the decrease in the number of beam breaks per five minute bin. Repeated measures ANOVA showed that all experimental groups had a main effect of bin, indicating that all mice acclimated over time. The young mice in this cohort showed no difference from wild type in activity level or acclimation in this experiment [2 way RM ANOVA; $p = 0.783$]. A separate cohort of 7 month old mice including wild type, *parkin*$^{-/-}$*DJ-1*$^{-/-}$, *parkin*$^{-/-}$*DJ-1*$^{-/-}$*SOD2*$^{+/-}$, and *parkin*$^{-/-}$*DJ-1*$^{-/-}$*SOD1*$^{-/-}$, was tested to determine if the additional loss of one of the SOD proteins in addition to the loss of PD-related genes caused an anticipated decrease in spontaneous locomotion (Figure 2.4B). Consistent with the results from the other 7 month cohort, we did not find a significant difference between the genotypes and all locomotor activity was similar to wild type [2 way RM ANOVA; $p = 0.126$]. Although not statistically significant, the triple mutant mice showed a decreasing trend in locomotion compare

to wild type and double mutant mice, particularly at the later time points, suggesting that either they acclimate to their environment more rapidly than wild type, or their basal activity level is lower than wild type, or both.

In a middle-aged cohort of 7 to 13 month old mice, (average age 10 months), we tested *SOD2*$^{+/-}$ mice together with wild type and *parkin*$^{-/-}$*DJ-1*$^{-/-}$. Neither the *parkin*$^{-/-}$*DJ-1*$^{-/-}$ mice, nor the *SOD2*$^{+/-}$ mice were significantly different from wild type [2 way RM ANOVA; $p = 0.290$] (Figure 2.4C).

We compared 14 months old wild type, *parkin*$^{-/-}$*DJ-1*$^{-/-}$, and *parkin*$^{-/-}$ to determine if there was a difference in locomotor activity in the single *parkin*$^{-/-}$, or if there is an age-dependent phenotype in either *parkin*$^{-/-}$ or *parkin*$^{-/-}$*DJ-1*$^{-/-}$ that would be apparent by 14 months of age (Figure 2.4D). We found a main effect of genotype [2 way RM ANOVA; main effect of genotype: $F(2, 32) = 5.483$, $p = 0.009$; main effect of bin: $F(23, 736) = 77.839$, $p < 0.001$]. Further analysis (Tukey Test) revealed a significant difference between *parkin*$^{-/-}$ and *parkin*$^{-/-}$*DJ-1*$^{-/-}$ mice ($P = 0.008$), but no difference between these groups and wild type ($P > 0.1$). These results are consistent with previously published reports on *parkin*$^{-/-}$ mice which also show no difference from wild type. Locomotor activity in *DJ-1*$^{-/-}$ mice was not assessed in these studies, but previously published reports have shown a small non-progressive decrease in locomotion of *DJ-1*$^{-/-}$ mice (Yamaguchi and Shen 2007; Chandran, Lin et al. 2008).

We tested aged mice to assess any development of a locomotor phenotype in mice deficient for SOD1 or SOD2 antioxidant proteins, Parkin and DJ-1, or all three proteins. In a 16 month old cohort, we assessed voluntarily locomotion in wild type, *parkin*$^{-/-}$*DJ-1*$^{-/-}$, *SOD1*$^{-/-}$, and *parkin*$^{-/-}$*DJ-1*$^{-/-}$*SOD1*$^{-/-}$ mice (Figure 2.4E). There was no difference in locomotor activity between any of the genotypes tested [RM 2-way ANOVA; genotype $F(3, 28) = 1.904$, $p = 0.152$]. Similarly, in a second aged group of 18 month old mice, we measured the locomotor activity of wild type, *parkin*$^{-/-}$*DJ-1*$^{-/-}$, *SOD2*$^{+/-}$, and *parkin*$^{-/-}$*DJ-1*$^{-/-}$*SOD2*$^{+/-}$ mice (Figure 4F). In this experiment, we found a main effect of genotype [RM 2-way ANOVA; main effect of genotype: $F(3, 30) = 5.197$, $p = 0.005$; main effect of bin: $F(23, 690) = 79.698$, $p < 0.001$; genotype x bin interaction: $F(69, 690) = 1.341$, $p = 0.040$]. Further analysis (Tukey Test) revealed a significant and difference between *parkin*$^{-/-}$*DJ-1*$^{-/-}$ mice and wild type ($P = 0.031$), and between *parkin*$^{-/-}$*DJ-1*$^{-/-}$ and *SOD2*$^{+/-}$ ($P = 0.015$). The *parkin*$^{-/-}$*DJ-1*$^{-/-}$ *SOD2*$^{+/-}$ mice were not significantly different from wild type, however their locomotor behavior follows the same trend as *parkin*$^{-/-}$*DJ-1*$^{-/-}$ mice. *SOD2*$^{+/-}$ were not different from wild type in this experiment, nor were double mutant mice different from triple mutant mice.

Anxiety Behaviors are not affected by mutations in Parkin, DJ-1, SOD1 or SOD2

We conducted behavioral tests that are accepted measures of anxiety in mice, including the open field test, the elevated plus maze, and the light/dark test. We postulated that a deregulation of DA could cause changes in anxiety states in our mice. Depression and anxiety are common non-motor symptoms of PD as well as parkinsonism linked to mutations in *parkin* and *DJ-1*.

Open Field Test

The open field test is an accepted behavioral measure of anxiety in mice. It is based upon the instinctual behaviors of mice, which avoid open and brightly illuminated spaces where they could be vulnerable to predators but also instinctually explore novel surroundings. If the mice have high levels of anxiety, they will avoid the center of the testing box and spend more time in the periphery. If anxiety levels are low, they will explore the edges and center of the testing box more equally. This test can also measure hyperactivity or some locomotor defects by monitoring the distance traveled during the test. For two separate cohorts of 6 month old and 9 month old wild-type and mutant mice, Figure 2.5 shows (A,B) the distance traveled by the mice, (C,D) the percent of distance traveled in the periphery, (E,F) the relative time spent in the center, (G,H) and time in spent the periphery during 5 minutes in an open field testing arena. Relative to wild type, the *parkin*$^{-/-}$*DJ-1*$^{-/-}$ mice exhibited no difference in measures of anxiety in this test. We saw a small, but significant decrease in the distance traveled in the periphery (Figure 2.5D) and the time spent in the periphery (Figure 2.5H) in *parkin*$^{+/-}$*DJ-1*$^{-/-}$*SOD2*$^{+/-}$ mice relative to wild-type mice. It is likely that this difference is due to the normal variability of this behavioral test as well as the multiple number of comparisons.

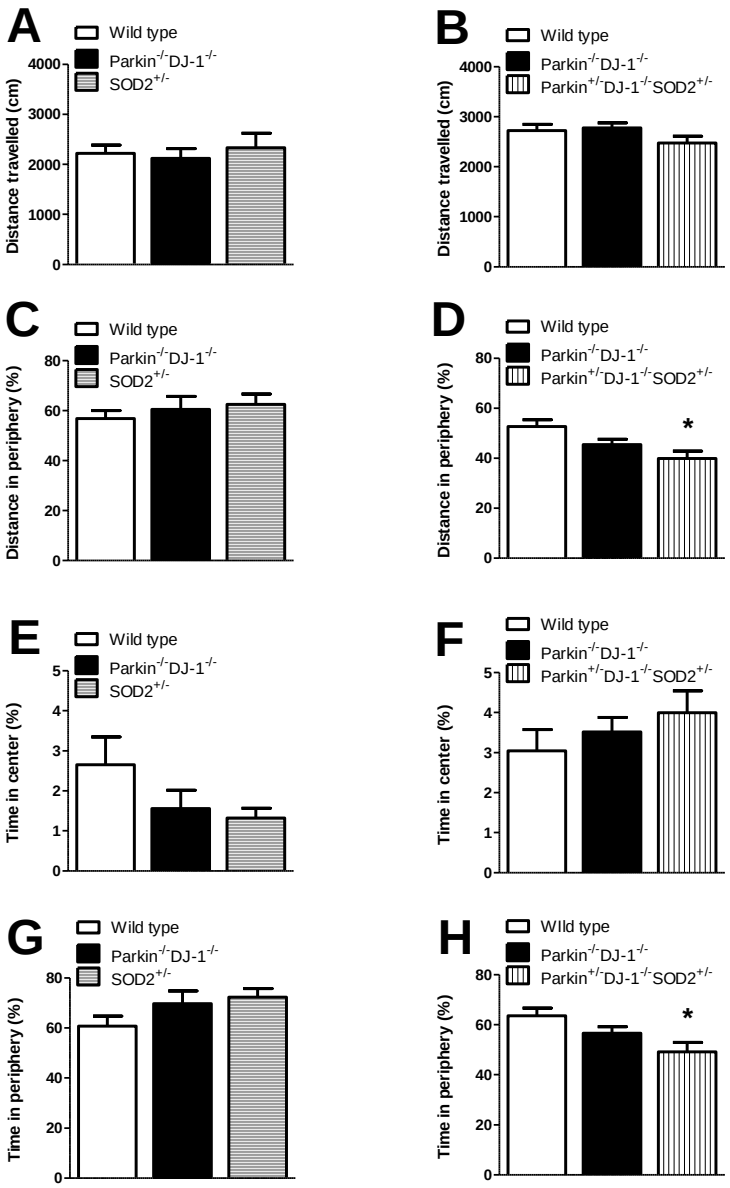


Figure 2.5: Open Field analysis of two cohorts measured *(A,B)* the distance traveled by the mice, *(C,D)* the percent of distance traveled in the periphery, *(E,F)* the relative time spent in the center, *(G,H)* and time in the periphery. The *parkin*$^{-/-}$*DJ-1*$^{-/-}$ mice exhibited no difference in measures of anxiety in this test. Data are presented as mean ± SEM. * $p < 0.05$

Dark/Light Test

The dark/light test is used to assess the relative anxiety status of mice. It is based on the conflict between the innate aversion of mice to brightly illuminated areas and on their spontaneous exploratory behavior in response to mild stressors, such as a novel environment and light. High anxiety levels in mice, as seen in mice treated with anxiogenic compounds, are correlated with a decrease in total exploration of the light side and more time spent in the dark compartment. Mice were placed into a black chamber and allowed to explore for two minutes before a small door was opened allowing them to access the light side of the apparatus for ten minutes. No affect of genotype on light-dark performance was found (Figure 2.6). The first cohort of mice, mean age 10.4 months (ranging 8 to 14 months), showed no difference between wild type (n = 11), *parkin*$^{-/-}$*DJ-1*$^{-/-}$ (n = 9), or *SOD2*$^{+/-}$ mice (n = 5) in the duration of time spent in the light (p = 0.883), the total activity of the mice (p = 0.889), or the number of crosses the mice made between chambers (p = 0.739). An insignificant but evident decrease was observed in the latency of the *SOD2*$^{+/-}$ mice to enter the light compartment (p = 0.587). The second cohort of mice, mean age 6 months (ranging 4 to 9 months), tested the relative anxiety of wild type (n = 12), *parkin*$^{-/-}$*DJ-1*$^{-/-}$ (n = 33), and *parkin*$^{+/-}$*DJ-1*$^{-/-}$*SOD2*$^{+/-}$ mice (n = 19). There was no affect of genotype on the latency to enter the light compartment (p = 0.1), the duration of time spent in the light (p = 0.353), the total activity of the mice (p = 0.931), or the number of crosses the mice made between chambers (p = 0.467). In the dark/light behavioral test, *parkin*$^{-/-}$*DJ-1*$^{-/-}$, *SOD2*$^{+/-}$, *and parkin*$^{+/-}$*DJ-1*$^{-/-}$*SOD2*$^{+/-}$ mice do not exhibit an anxiety phenotype.

Elevated Plus Maze

The Elevate Plus Maze is a behavioral test that was developed to measure levels of anxiety in rats and mice (Pellow, Chopin et al. 1985; Lister 1987). It consists of two open and two closed arms arranged in a plus sign formation and elevated off the ground. Mice are placed in the center of the maze and behavior is recorded for five minutes. The measure of relative anxiety comes from the conflict between the natural tendency of the mouse to approach novel open areas for exploration and the innate fear of dangerous areas (Mongomery KC 1958), specifically heights and open areas. Mice are characterized as more anxious when they spend more time in the closed arms, and less anxious when they spend more time in the open arms of the maze. We analyzed the percent of time the mice spent in the open arms (Figure 2.7A, B), the percent of total entries into open arms (Figure 2.7C, D), the percent of time spent in the closed arms (Figure 2.7E, F), and the total arm entries (Figure 2.7G, H). There were no changes in these measures in the first cohort of mice: wild type (n=12), *parkin*$^{-/-}$*DJ-1*$^{-/-}$ (n=9), and *SOD2*$^{+/-}$ (n=6), average age 10.5 months. The second cohort of mice, average age 5.8 months, consists of wild type (n=13), *parkin*$^{-/-}$*DJ-1*$^{-/-}$ (n=33), and *parkin*$^{+/-}$*DJ-1*$^{-/-}$*SOD2*$^{+/-}$ (n=19). We found a small but significant increase in the percent of time *the parkin*$^{-/-}$*DJ-1*$^{-/-}$ mice spent in open arms compared to *parkin*$^{+/-}$ *DJ-1*$^{-/-}$*SOD2*$^{+/-}$ [$F(2, 62) = 3.636$, $p = 0.032$; Tukey test $p = 0.033$]. The measurements for the *parkin*$^{-/-}$*DJ-1*$^{-/-}$ *and parkin*$^{+/-}$*DJ-1*$^{-/-}$*SOD2*$^{+/-}$ were not statistically different from wild type controls (Tukey: $p = 0.26$, $p = 0.81$). If the mice were hyperactive or hypoactive, we would expect to see a difference in the total number of arm entries which, we did not observe. We conclude that the genotypes tested do not exhibit increase measures of anxiety, nor do they appear to be hyperactive in this test.

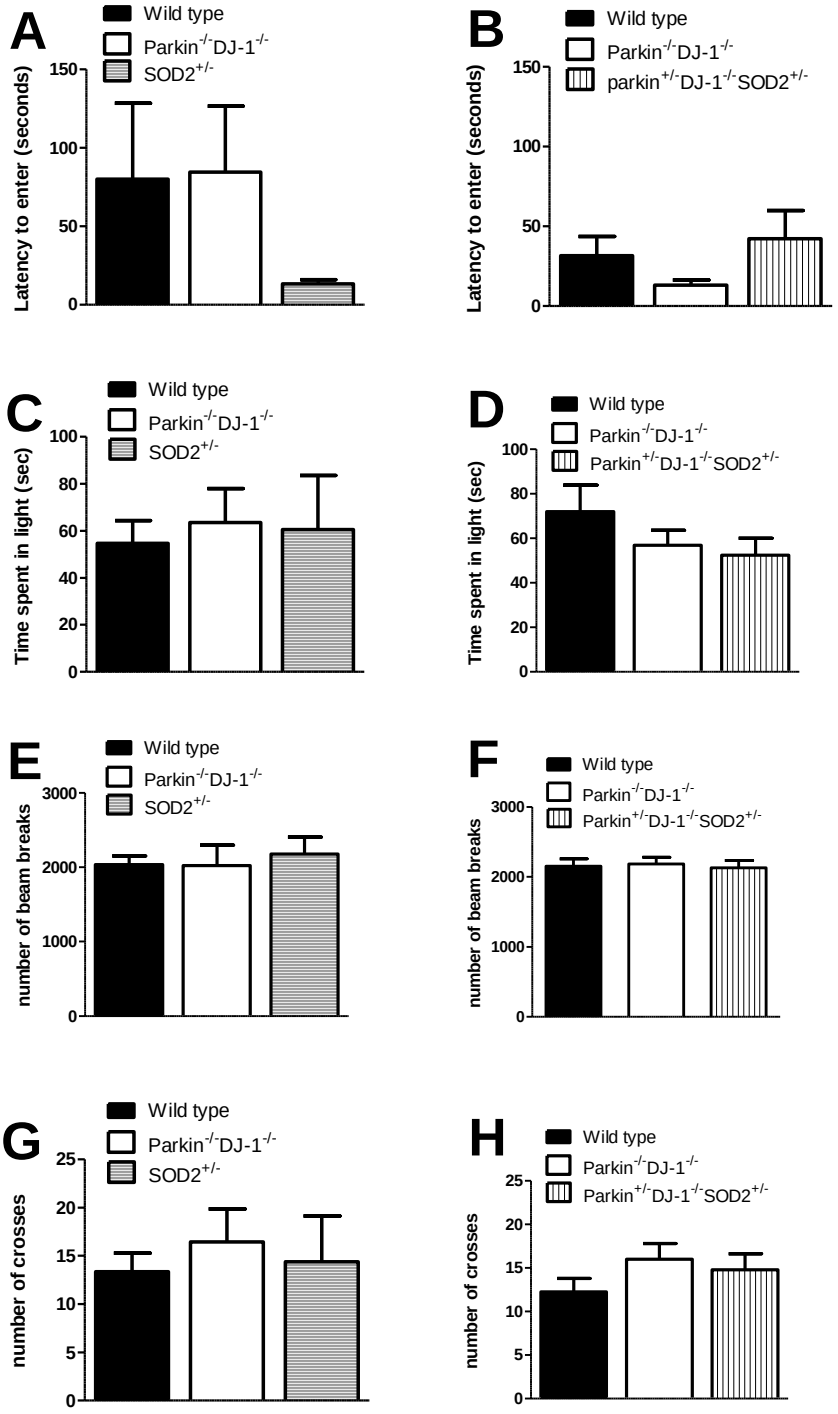


Figure 2.6: Latency to enter the light (A,B), duration of time spent in the light (C, D), total activity (E,F), and number of crosses (G,H) in the Dark/Light behavioral test showed no difference between genotypes. The data are presented as mean ± SEM and compared using one-way ANOVA.

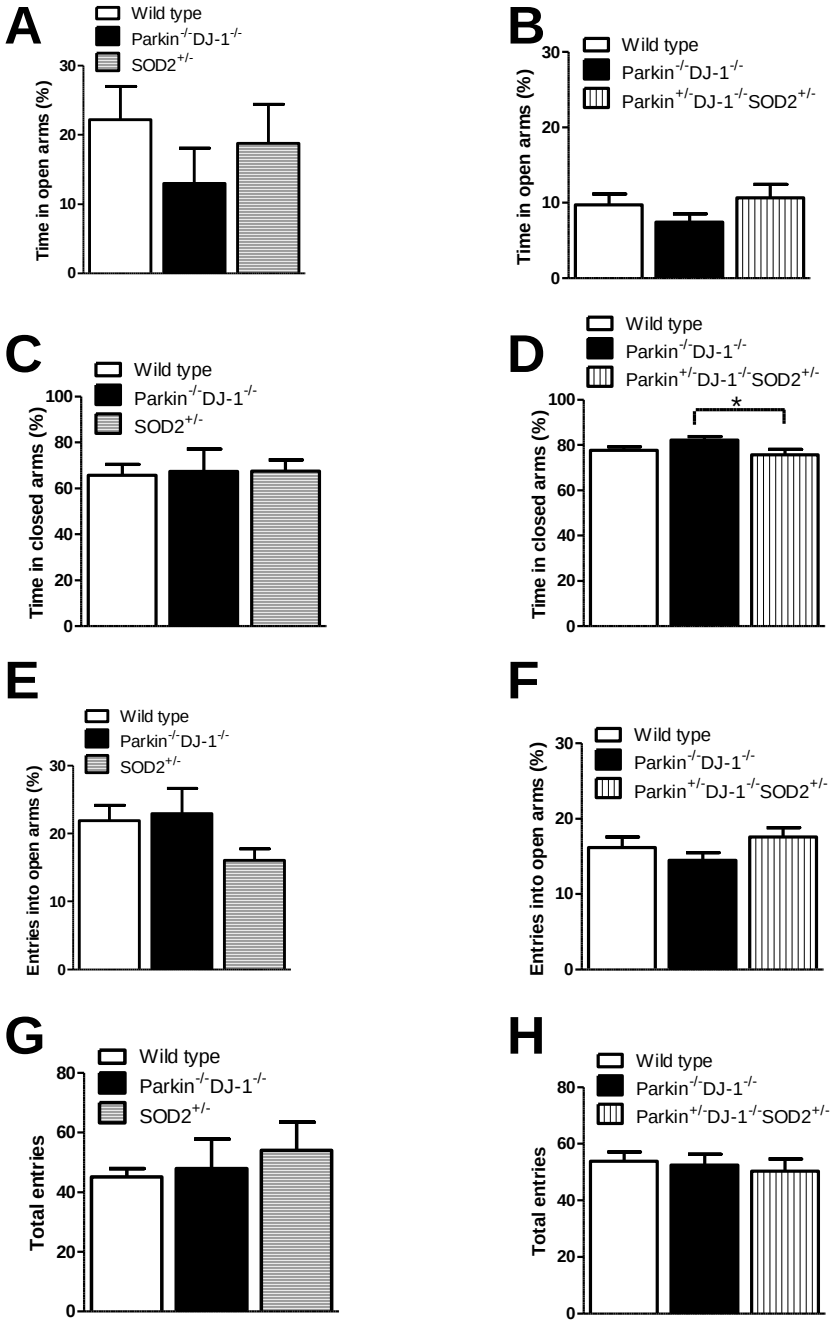


Figure 2.7: The percentage of time spent in open arms (A, B) time in closed arms (C, D), entries into open arms (E, F), and total entries. Cohorts are an 8-14 months cohort (A,C,E,G) of wild type (n=12), *parkin*$^{-/-}$*DJ-1*$^{-/-}$ (n=9), and *SOD2*$^{+/-}$ (n=6), and a 4-9 month old cohort of wild type (n=13), *parkin*$^{-/-}$*DJ-1*$^{-/-}$ (n=33), and *parkin*$^{+/-}$*DJ-1*$^{-/-}$*SOD2*$^{+/-}$ (n=19). Bars represent mean±SEM. *$p<0.05$

Acoustic Startle Response

The acoustic startle test measures the innate reflex of the mouse in response to a sudden and unexpected acute stimulus, in this case a loud noise. The pathway for this reflex begins with the auditory pathway from the ear to the lateral lemniscus nucleus in the brainstem where the motor center in the reticular formation is activated, in turn sending impulses to the lower motor neurons of the limbs causing a fast twitch of the muscles in response to the noise, (Koch 1999) for review. This physical response can be measured by a force transducer under the chamber containing the mouse which records the force with which the mouse jumps in response to the auditory stimulus. Startle can be used as a behavioral tool to assess sensory-motor integration. Additionally, higher startle amplitudes are thought to indicate a relatively higher level of anxiousness. In these experiments, mice were placed into Plexiglas holders and allowed to acclimate to the chamber and background white noise (70 dB) for five minutes. After the acclimation period, startle stimuli (80, 90, 100, 110 and 120 dB, 40 ms, white noise) were presented with an average interstimulus interval of 20 seconds (range 13 - 27 seconds).

We tested the acoustic startle response (ASR) in older (16 months) wild type, *DJ-1*$^{-/-}$, and *parkin*$^{-/-}$*DJ-1*$^{-/-}$ mice and found a main effect of genotype, stimulus intensity, and an interaction [2 way RM ANOVA; main effect of genotype: $F(2, 29) = 4.498$, $p = 0.02$; main effect of stimulus: $F(5, 145) = 63.943$, $p < 0.001$; genotype x stimulus: $F(10, 145) = 3.029$, $p = 0.002$] (Figure 2.8A). Pair-wise comparison shows a difference between *DJ-1*$^{-/-}$ and *parkin*$^{-/-}$*DJ-1*$^{-/-}$ mice [Tukey test, $p < 0.018$], but no difference between *parkin*$^{-/-}$*DJ-1*$^{-/-}$ and wild type, or *DJ-1*$^{-/-}$ and wild type [Tukey test, $p = 0.111$ and $p = 0.579$ respectively]. There is a difference between wild

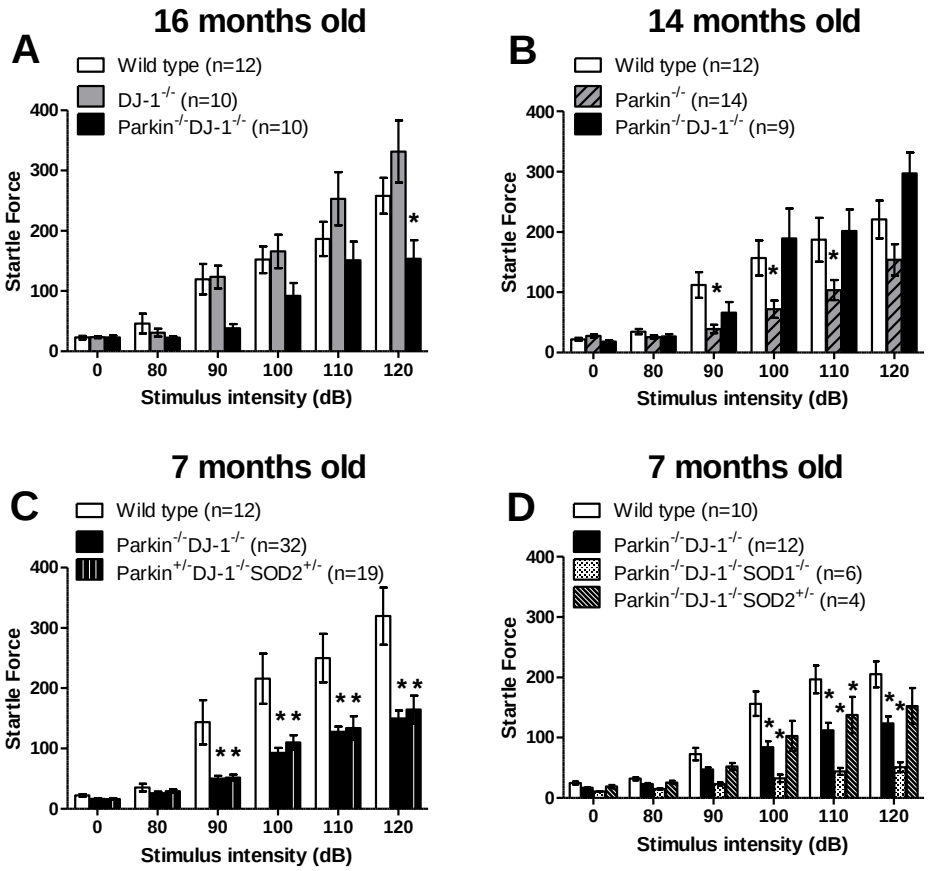


Figure 2.8: Acoustic startle response in mutant mice was assessed at baseline (white noise), 80dB, 90dB, 100dB, 110dB, and 120dB. (A)16 month old wild type, *DJ-1*$^{-/-}$, and *parkin*$^{-/-}$*DJ-1*$^{-/-}$; (B) 14 month old wild type, *parkin*$^{-/-}$, and *parkin*$^{-/-}$*DJ-1*$^{-/-}$; (C) 7 month old wild type, *parkin*$^{-/-}$ *DJ-1*$^{-/-}$, and *parkin*$^{+/-}$*DJ-1*$^{-/-}$*SOD2*$^{+/-}$; (D) 7 month old wild type, *parkin*$^{-/-}$ *DJ-1*$^{-/-}$, and *parkin*$^{-/-}$*DJ-1*$^{-/-}$*SOD1*$^{-/-}$, *parkin*$^{-/-}$*DJ-1*$^{-/-}$*SOD2*$^{+/-}$. Significance by two-way repeated measures ANOVA, * $p < 0.05$ with respect to wild type.

type and *parkin*$^{-/-}$*DJ-1*$^{-/-}$ by pair wise comparison at the 120 dB level. We also conducted the acoustic startle response test in older (14 months) wild type, *parkin*$^{-/-}$, and *parkin*$^{-/-}$*DJ-1*$^{-/-}$ and found a main effect of genotype, stimulus intensity and an interaction [2 way RM ANOVA; main effect of genotype: $F(2, 32) = 5.251$, $p = 0.011$; main effect of stimulus: $F(5, 160) = 60.080$, $p <$

0.001; genotype x stimulus: $F(10, 160) = 3.844$, $p < 0.001$] (Figure 2.8B). Pair-wise comparison shows a difference between *parkin*$^{-/-}$ and wild type [Tukey test, p = 0.038], and between *parkin*$^{-/-}$ and *parkin*$^{-/-}$*DJ-1*$^{-/-}$ [Tukey test, p = 0.019], but no difference between *parkin*$^{-/-}$*DJ-1*$^{-/-}$ and wild type [Tukey test, p = 0.879]. There is a difference between wild type and *parkin*$^{-/-}$ by pair wise comparison at 90dB, 100dB, and 110dB. The lack of significant difference between wild type and *parkin*$^{-/-}$*DJ-1*$^{-/-}$ is consistent with the findings from the preceding test (Figure 2.8A), however the trend in the magnitude of the response is inconsistent, which speaks to the variability of this behavioral test. In a younger cohort of mice, mean age 6.6 months, we found a main effect of genotype, stimulus intensity and an interaction [2 way RM ANOVA; main effect of genotype: $F(2, 60) = 14.356$, $p < 0.001$; main effect of stimulus: $F(5, 300) = 130.597$, $p < 0.001$; genotype x stimulus: $F(10, 160) = 8.204$, $p < 0.001$] (Figure 2.8C). Pair-wise comparison shows a difference between wild type and *parkin*$^{-/-}$*DJ-1*$^{-/-}$ [Tukey test, p < 0.001], and between wild type and *parkin*$^{+/-}$*DJ-1*$^{-/-}$*SOD2*$^{+/-}$ [Tukey test, p < 0.001]. There is no difference between *parkin*$^{-/-}$*DJ-1*$^{-/-}$ and *parkin*$^{+/-}$*DJ-1*$^{-/-}$*SOD2*$^{+/-}$ [Tukey test, p = 0.871]. This decrease in startle response may be age dependent as this cohort is much younger than the other cohorts tested. An additional confound to the ASR is weight. Body weight has a direct effect on the ASR as the force exerted by an animal is directly proportional to weight. We do not have weight data for all the mice tested in the ASR, so we cannot conclusively determine if weight is a factor in these results. Additionally, we cannot determine if sex is a factor in the magnitude of the response, since males are generally larger than female, sex is confounded by weight, however, there has been no published effect of estrous cycle on the ASR. The final cohort we tested included the SOD1 and SOD2 triple mutant lines at a young age, 7 months. In this cohort we also found a main effect of genotype, stimulus intensity and an interaction [2 way RM ANOVA; main effect of genotype:

$F(3, 28) = 11.003$, $p < 0.001$; main effect of stimulus: $F(5, 140) = 123.085$, $p < 0.001$; genotype x stimulus: $F(15, 140) = 10.710$, $p < 0.001$] (Figure 2.8D). Post-hoc comparison revealed a significant difference between wild type and *parkin*$^{-/-}$*DJ-1*$^{-/-}$ [Tukey test, $p = 0.005$], and between wild type and *parkin*$^{-/-}$*DJ-1*$^{-/-}$*SOD1*$^{-/-}$ [Tukey test, $p < 0.001$].

Parkin$^{-/-}$ mice in an older cohort show a decrease in ASR, however, *DJ-1*$^{-/-}$ mice and *parkin*$^{-/-}$*DJ-1*$^{-/-}$ do not show this ASR decrease. It is possible, but not definitive, that the decrease in startle in *parkin*$^{-/-}$*DJ-1*$^{-/-}$ (Figure 2.8A, C, D) is due to a lack of Parkin. This decrease in ASR is also evident in the lines of triple mutant mice we tested at a younger age: *parkin*$^{+/-}$*DJ-1*$^{-/-}$ *SOD2*$^{+/-}$, *parkin*$^{-/-}$*DJ-1*$^{-/-}$*SOD2*$^{+/-}$, *parkin*$^{-/-}$*DJ-1*$^{-/-}$*SOD1*$^{-/-}$. The SOD1 triple mutant mice exhibit a drastic reduction in ASR, however this is likely due to a loss of hearing in mice deficient for SOD1. The cochleae of *SOD1*$^{-/-}$ mice have been shown to have severe spiral ganglion cell degeneration by 7 months of age (Keithley, Canto et al. 2005).

Grip Strength

The unexpected increase in rotorod performance of *parkin*$^{-/-}$*DJ-1*$^{-/-}$ mice prompted us to determine whether these mice have increased ability to grip onto the rotating rod. The grip strength test is highly specific, in that it attempts to measure a single, well-defined

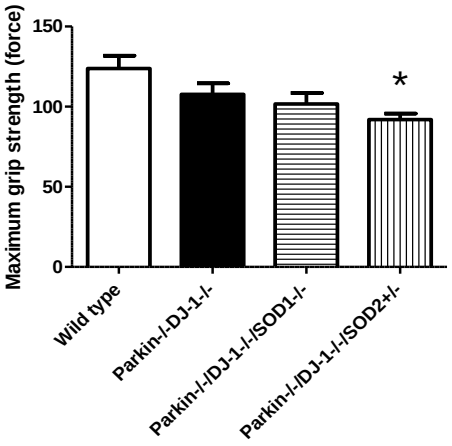


Figure 2.9: Maximum grip strength attained in five trials by wild type (n=10), *parkin*$^{-/-}$*DJ-1*$^{-/-}$ (n=11), *parkin*$^{-/-}$ *DJ-1*$^{-/-}$*SOD1*$^{-/-}$ (n=6), and *parkin*$^{-/-}$*DJ-1*$^{-/-}$*SOD2*$^{+/-}$ triple mutants (n=4). *p=0.032 relative to wild type with Student T-test pair-wise comparison.

aspect of behavior. Grip strength measures the amount of force registered by a mouse gripping a trapeze while constant tension is applied to the tail until the bar is released. Grip strength for each of five trials was recorded as the maximum force before the trapeze was released (Figure 2.9). We analyzed the grip strength of a cohort of 6 month old wild-type (n=10), *parkin*$^{-/-}$*DJ-1*$^{-/-}$ (n=11), *parkin*$^{-/-}$*DJ-1*$^{-/-}$*SOD1*$^{-/-}$ (n=6), and *parkin*$^{-/-}$*DJ-1*$^{-/-}$*SOD2*$^{+/-}$ (n=4) mice. Although there is a strong trend towards reduced grip strength in the mutant mice, there is no effect of genotype by ANOVA [$F(3, 27) = 2.630$; $p=0.07$]. Nevertheless, the *parkin*$^{-/-}$*DJ-1*$^{-/-}$*SOD2*$^{+/-}$ mice have decreased maximum grip strength, (the single maximum force out of five trials), compared to controls, as determined by Student's t-test ($p = 0.032$). Thus the improved rotorod performance of the double and triple mutant mice is not correlated with an ability to stay on the rod via increased grip strength.

Forced swimming test

The forced swimming test (FST) is a behavioral test developed to predict the efficacy of antidepressant drugs (Porsolt, Le Pichon et al. 1977). It is particularly sensitive to compounds acting on the 5-HT and/or NE system. Mice were placed in a beaker of water for 6 minutes and were monitored from the side by a video camera to record when mice were immobile, which is regarded as a surrogate marker for depression within this test. Only the last four minutes of the test were scored for latency to the first immobility and total time spent immobile. Immobility was defined as no body or limb movement other than a minimal forelimb movement required for keeping the head above water. Mice will, after an initial bout of struggling, become immobile when immersed in cold water without means of escape. It is generally thought to reflect a failure

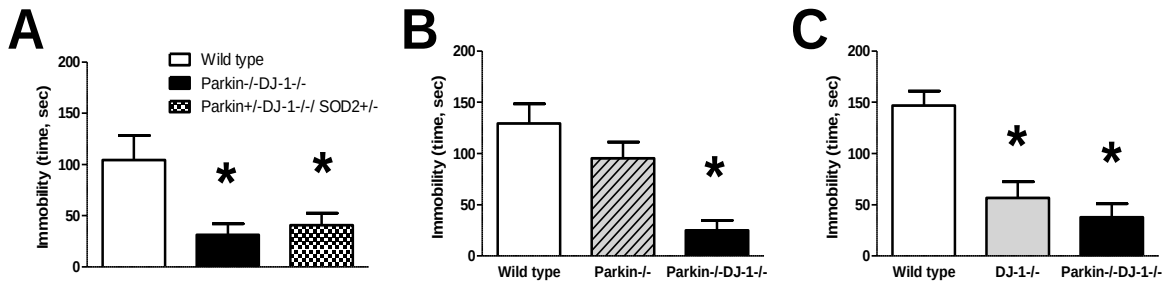


Figure 2.10: The total immobility of mice in the forced swimming test was measured during the last four minutes of the six-minute test. (A) Young 6-10 month old wild type (n=6), *parkin*$^{-/-}$*DJ-1*$^{-/-}$ (n=15), and *parkin*$^{+/-}$*DJ-1*$^{-/-}$*SOD2*$^{+/-}$ (n=9); (B) 14-month old wild type (n=12), *parkin*$^{-/-}$ (n=15), and *parkin*$^{-/-}$*DJ-1*$^{-/-}$ (n=9); and (C) wild type (n=12), *DJ-1*$^{-/--}$ (n=10), and *parkin*$^{-/-}$*DJ-1*$^{-/-}$ (n=10). Significance by one-way ANOVA, $p < 0.03$ with respect to wild type.

of persistence or behavioral despair, not generalized hypoactivity, and is thus considered a model of depression. However, since amphetamine and caffeine have both been showed to reduce immobility in FST (Porsolt, Le Pichon et al. 1977), as well as have a mild antidepressant effect in patients, it is possible that any reduction in immobility is confounded by hyperactivity.

In the first cohort we tested, average age 6.7 months, we found a main effect of genotype [One way ANOVA; main effect of genotype: $F(2, 27) = 6.178$, $p = 0.006$]. Further analysis found a dramatic decrease by *the parkin*$^{-/-}$*DJ-1*$^{-/-}$ and *parkin*$^{+/-}$*DJ-1*$^{-/-}$*SOD2*$^{+/-}$ in the amount of time the mice spent immobile compared to wild type [Tukey Test, $p = 0.005$ and $p = 0.027$ respectively]. Next we tested 14 month old wild type (n=12), *parkin*$^{-/-}$ (n=15), and *parkin*$^{-/-}$*DJ-1*$^{-/-}$ (n=9) to assess the *parkin*$^{-/-}$*DJ-1*$^{-/-}$ phenotype at an older age and also to test for the contribution of the the loss of Parkin in the *parkin*$^{-/-}$*DJ-1*$^{-/-}$ phenotype. We found that there is also a significant difference between the wild type and *parkin*$^{-/-}$*DJ-1*$^{-/-}$ mice [$F(2, 32) = 8.960$, $p < 0.001$; Tukey Test, $p < 0.001$], however, there is no difference between *parkin*$^{-/-}$ and wild type mice, indicating

that loss of Parkin alone is not sufficient for the manifestation of the FST phenotype. We next assessed the phenotype of 16 month old *DJ-1*$^{-/-}$ mice (n=10) as compared to *parkin*$^{-/-}$*DJ-1*$^{-/-}$ (n=10) and wild type mice (n=12). Both *DJ-1*$^{-/-}$ and the *parkin*$^{-/-}$*DJ-1*$^{-/-}$ mice showed an enhanced mobility in this test as compared to wild type [$F(2, 29) = 16.893$, $P < 0.001$; Tukey test $P < 0.001$] (Figure 10C). Together these data indicate that a loss of Parkin alone is insufficient to cause the decreased immobility seen in the *parkin*$^{-/-}$*DJ-1*$^{-/-}$ and *parkin*$^{+/-}$*DJ-1*$^{-/-}$*SOD2*$^{+/-}$ mice, however the loss of DJ-1 alone is sufficient to confer the heightened mobility seen in the FST.

Wheel running

The rotorod paradigm requires forced (involuntary) locomotion of the mice. The locomotor test is a test of voluntary locomotion, however the test duration is only two hours and shows an initial hyperactivity as mice acclimate to the environment. We used a voluntary wheel running paradigm to analyze the voluntary locomotion of 10 wild type and 10 *parkin*$^{-/-}$*DJ-1*$^{-/-}$ five month old mice over a longer period of time: 2 weeks. Additionally, we used a light/dark paradigm to investigate any changes in circadian rhythms in the mutant mice. Mice were placed in individual cages with running wheels attached to a sensor that recorded revolutions over time. Mice were on a normal 12 hour light/dark cycle for 2 weeks and then shifted to a 24 hour dark cycle for an additional 3 weeks. Over the course of the 5 weeks, the *parkin*$^{-/-}$*DJ-1*$^{-/-}$ mice ran on the wheel just as much as

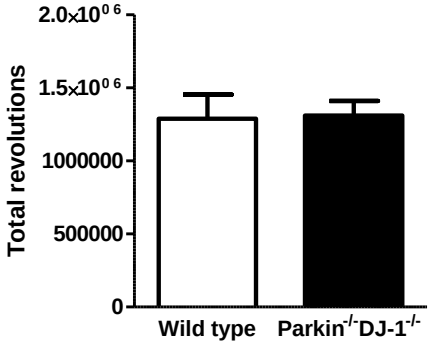


Figure 2.11: Wheel running test shows that *parkin*$^{-/-}$*DJ-1*$^{-/-}$ mice exhibit no difference in total revolutions or revolutions per minute over 5 weeks.

wild type mice. We analyzed the total revolutions run over 5 weeks (Figure 2.11) and found that the number of rotations over time and total rotations is nearly equivalent between the two groups. There was no obvious change in the circadian rhythm shift of *parkin*$^{-/-}$*DJ-1*$^{-/-}$ mice compared to wild type mice during the dark/dark phase.

Muscle, heart, and cerebellar histology appear normal

Given the surprising enhanced rotorod phenotype of the *parkin*$^{-/-}$*DJ-1*$^{-/-}$ mice, we took a deeper look into the histology of other anatomical areas. Parkin is highly expressed in the heart and muscle tissue, as well as most areas of the brain (Kitada, Asakawa et al. 1998). We hypothesized that a loss of Parkin could lead to anatomical changes in the heart or muscle that could account for the enhanced ability to remain on the rotorod. Additionally, the ability to stay on the rotorod is highly dependent on cerebellar function. We therefore sought to determine whether there were visible changes in muscle composition or cerebellar anatomy that might account for the enhanced rotorod ability of *parkin*$^{-/-}$*DJ-1*$^{-/-}$ mice. H&E staining of paraffin sections of hearts from 18 month old wild-type and *parkin*$^{-/-}$*DJ-1*$^{-/-}$ mice showed normal organ structure as well as normal cellular appearance of heart muscle in mice of both genotypes (Figure 2.12A, B). H&E staining of coronal brain sections showed normal anatomical and cellular organization of the cerebellum (Figure 2.12C, D). Muscle tissue was taken from the extensor digitorum longus (EDL) and soleus (SOL) muscles and frozen sections were prepared for H&E staining and metachromatic ATPase staining. H&E staining of the EDL muscle revealed normal muscular organization (Figure 2.12E). The soleus muscle is comprised of a combination of slow twitch type I myofibrils which are rich in mitochondria and fatigue resistant, and fast twitch type II

myofibrils with have faster glycolytic metabolism and fatigue rapidly after rapid bursts of contractions. Classification of type IIa or IIb is determined by the type of myosin heavy chain. Metachromatic ATPase staining makes it possible to view the different muscle fiber types. Type I muscle fibers stain dark blue, whereas Type II fibers stain shades of light blue. We hypothesized we would see a difference in the ratio of fast twitch to slow twitch muscle fiber types, providing the *parkin*$^{-/-}$*DJ-1*$^{-/-}$ mice fatigue-resistant muscles and better stamina on the rotorod. The ratio of Type I and Type II fiber types appeared similar in soleus muscle from *parkin*$^{-/-}$*DJ-1*$^{-/-}$ mice compared to wild type mice (Figure 2.12F), however we did not conduct a quantitative fiber type analysis.

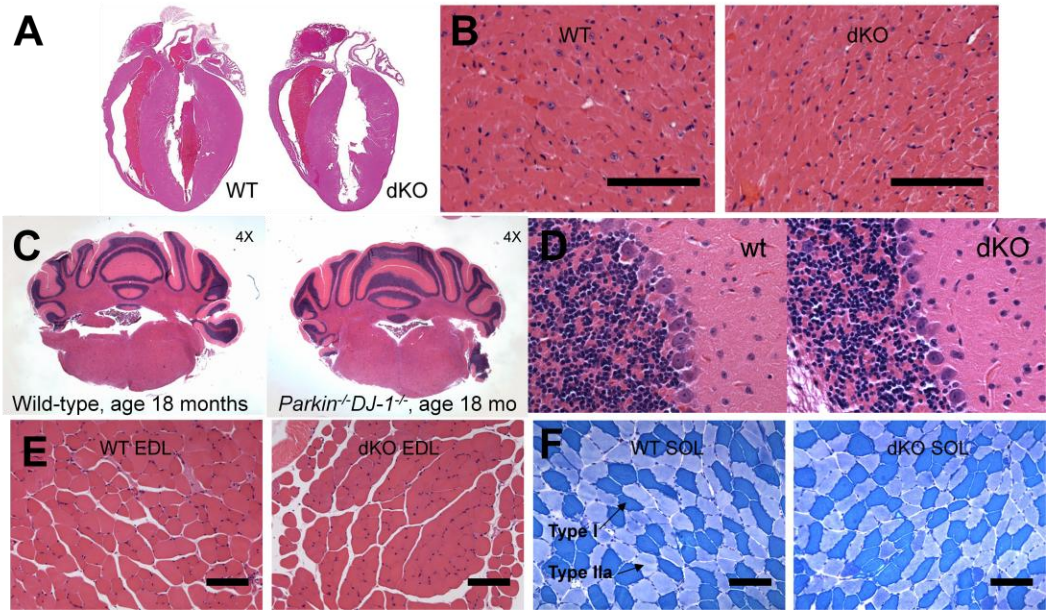


Figure 2.12: Muscle, heart, and cerebellar histology appear normal. H & E staining of the heart (A, B); the cerebellum (C, D); and the EDL muscle (E) revealed normal histology in *parkin*$^{-/-}$*DJ-1*$^{-/-}$ mice (dKO) compared to wild-type mice (WT). Metachromatic ATPase stain (F) showed comparable distribution of dark blue Type I fibers and lighter blue Type II fibers in soleus muscle from wild type and *parkin*$^{-/-}$*DJ-1*$^{-/-}$ mice. Scale bar = 100 μm

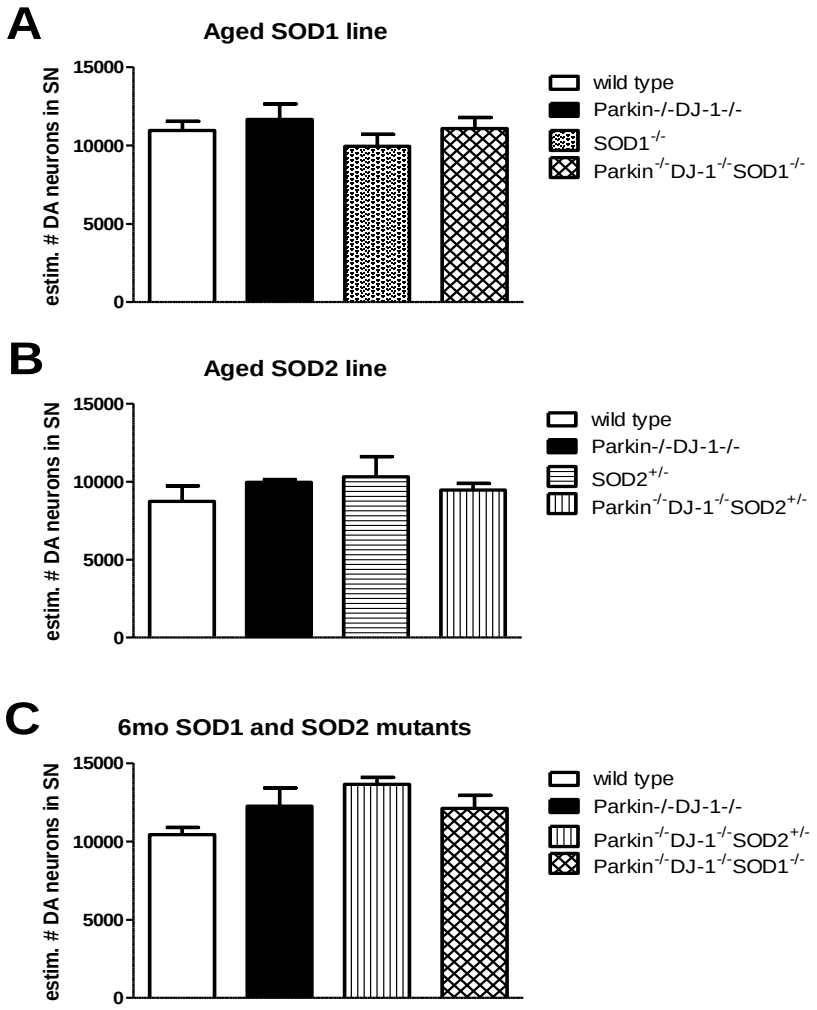


Figure 2.13: Estimated number of DA neurons in the SNpc of the mutant mice tested is not different from wild type in 14 month aged mice (*A, B*) or in 6 month old mice (*C*); (n = 4).

No change in TH-positive neurons in the SNpc of mutant mice

The pathological hallmark of PD is a loss of DA neurons in the substantia nigra pars compacta (SNpc). We postulated that mice with deficiency for Parkin, DJ-1 and major antioxidant proteins would show an age-dependent loss of DA neuron in the SNpc and thereby model human PD

neuropathology. We used rigorous stereology to obtain unbiased estimates of the number of dopaminergic neurons, marked by immunohistochemical staining for tyrosine hydroxylase, in coronal paraffin sections of wild-type and mutant mice. We analyzed two cohorts of mice: age 7 months and 14 months. In the younger cohort (Figure 2.13C), there was a small increase in the estimated number of DA neurons in the SNpc of *parkin*$^{-/-}$*DJ-1*$^{-/-}$*SOD2*$^{+/-}$ mice compare to wild-type mice. In the older cohort, we assessed DA neuron number in the SNpc of the *SOD1*$^{-/-}$ (Figure 2.13A) and *SOD2*$^{+/-}$ (Figure 2.13B) single mutant mice in addition to *parkin*$^{-/-}$*DJ-1*$^{-/-}$, *parkin*$^{-/-}$*DJ-1*$^{-/-}$*SOD1*$^{-/-}$, and *parkin*$^{-/-}$*DJ-1*$^{-/-}$*SOD2*$^{+/-}$. There was no statistical difference between the wild type and any of the mutant mice at 14 months of age ($p = 0.483$ and $p = 0.598$); (Figure 13, n=4 for each genotype in each age group). We postulate that the slight increase in the estimated number of DA neurons seen in the younger *parkin*$^{-/-}$*DJ-1*$^{-/-}$*SOD2*$^{+/-}$ group is solely due to the small sample size ($n = 4$).

Dopamine levels are elevated in the striatum of SOD triple mutant mice

One of the hallmarks of PD is a decrease in DA in the striatum as a result of the loss of DA producing neurons, which project to the dorsal striatum from the substantia nigra pars compacta. Following the hypothesis that mice with deficiency for Parkin, DJ-1 and major antioxidant proteins would model PD, we measured levels of DA, serotonin and their metabolites in the striata of these mice and expected to see a decrease in DA compared to wild type age matched controls. Loss of dopaminergic terminals in the striatum could be expected to precede loss of nigral cell bodies. We used an HPLC with electrochemical detection to assess the levels of DA and its metabolites in the striatum of our wild-type and mutant mice. We dissected out the dorsal

striatum, homogenized the tissue, and ran the samples on an HPLC with electrochemical detection on DA, 5-HT and their metabolites. Surprisingly, we found a consistent and significant increase in DA in the *parkin*$^{-/-}$*DJ-1*$^{-/-}$*SOD1*$^{-/-}$ mice compared to wild type mice.

In a younger cohort of mice, 7 months (Figure 2.14), we compared wild type mice to *parkin*$^{-/-}$*DJ-1*$^{-/-}$, *parkin*$^{-/-}$*DJ-1*$^{-/-}$*SOD2*$^{+/-}$ and *parkin*$^{-/-}$*DJ-1*$^{-/-}$*SOD1*$^{-/-}$ mice. Levels of DA in the striatum of *parkin*$^{-/-}$*DJ-1*$^{-/-}$*SOD1*$^{-/-}$ mice were significantly higher than control levels [$F(3, 25) = 16.068$, $p < 0.001$; Tukey Test, $p < 0.001$]. In the same group, DA turnover was decreased for SOD1 triple mutant mice, but not SOD2 triple mutants [$F(3, 25) = 9.125$, $p < 0.001$; Tukey Test, $p < 0.001$]. Levels of DOPAC, HVA, 3-MT, 5HIAA, and 5-HT were not significantly different among genotypes.

In 16 month old aged wild type, *parkin*$^{-/-}$*DJ-1*$^{-/-}$, *SOD2*$^{+/-}$, and *parkin*$^{-/-}$*DJ-1*$^{-/-}$ *SOD2*$^{+/-}$ mice, we observed a trend towards increase DA in the triple mutant, but it was not statistically significant by ANOVA [$F(3, 30) = 2.265$, $p < 0.101$] (Figure 2.15). In the 18 month group of wild type, *parkin*$^{-/-}$*DJ-1*$^{-/-}$, *SOD1*$^{-/-}$, and *parkin*$^{-/-}$*DJ-1*$^{-/-}$ *SOD1*$^{-/-}$ mice (Figure 2.16), there was a significant increase in DA between the triple mutant and wild type [$F(3, 31) = 8.031$, $p < 0.001$; Tukey Test, $p < 0.001$]. There was also a significant increase in HVA, 5HIAA, and 5HT.

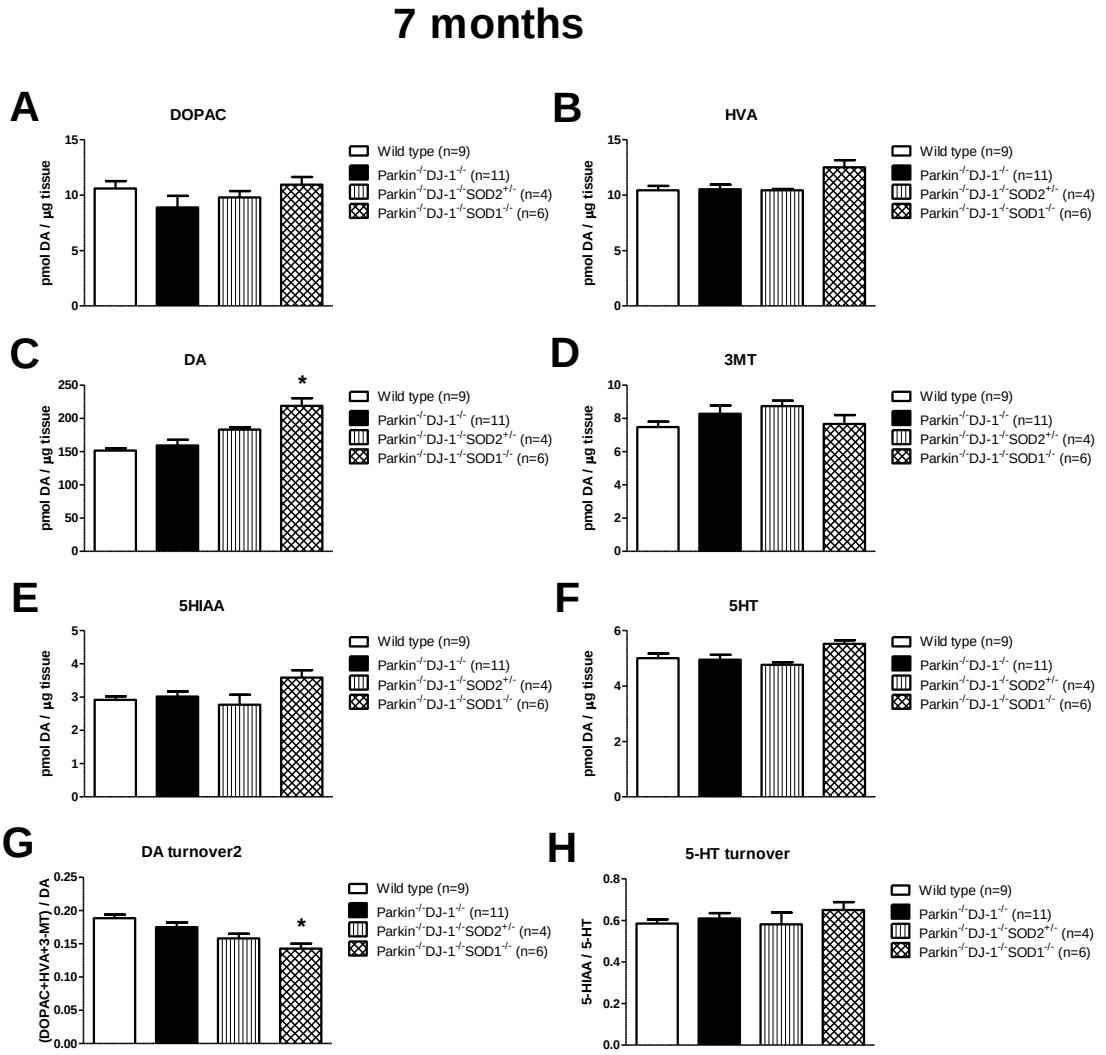


Figure 2.14: Levels of dopamine and serotonin, precursors and metabolites in the striatum of 7 month old mutant mice measured by HPLC. DA is elevated in *parkin*$^{-/-}$*DJ-1*$^{-/-}$*SOD1*$^{-/-}$ mice while DA turnover is decreased.

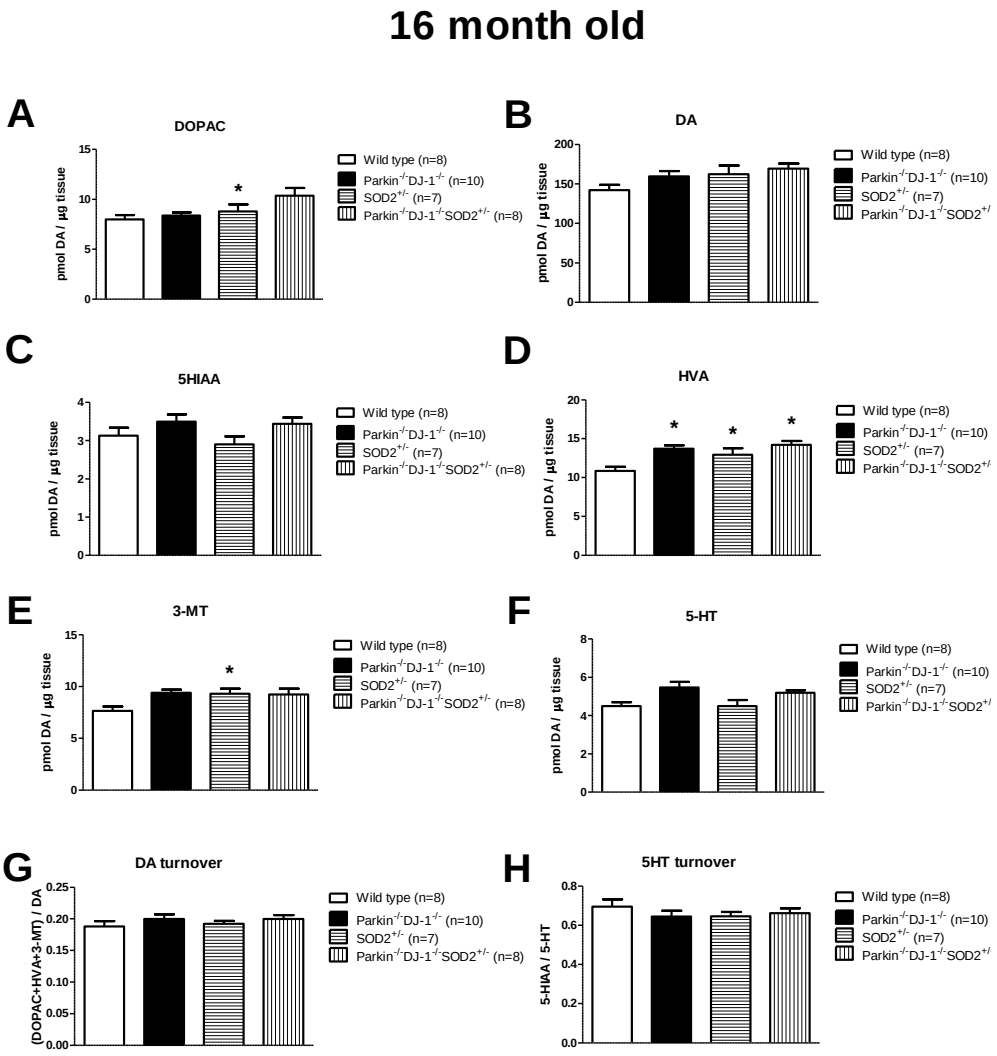


Figure 2.15: Levels of dopamine, serotonin, precursors and metabolites in the striatum of 16 month old wild type, *parkin*$^{-/-}$*DJ-1*$^{-/-}$, *SOD2*$^{+/-}$, and *parkin*$^{-/-}$*DJ-1*$^{-/-}$ *SOD2*$^{+/-}$ mice as measured by HPLC.

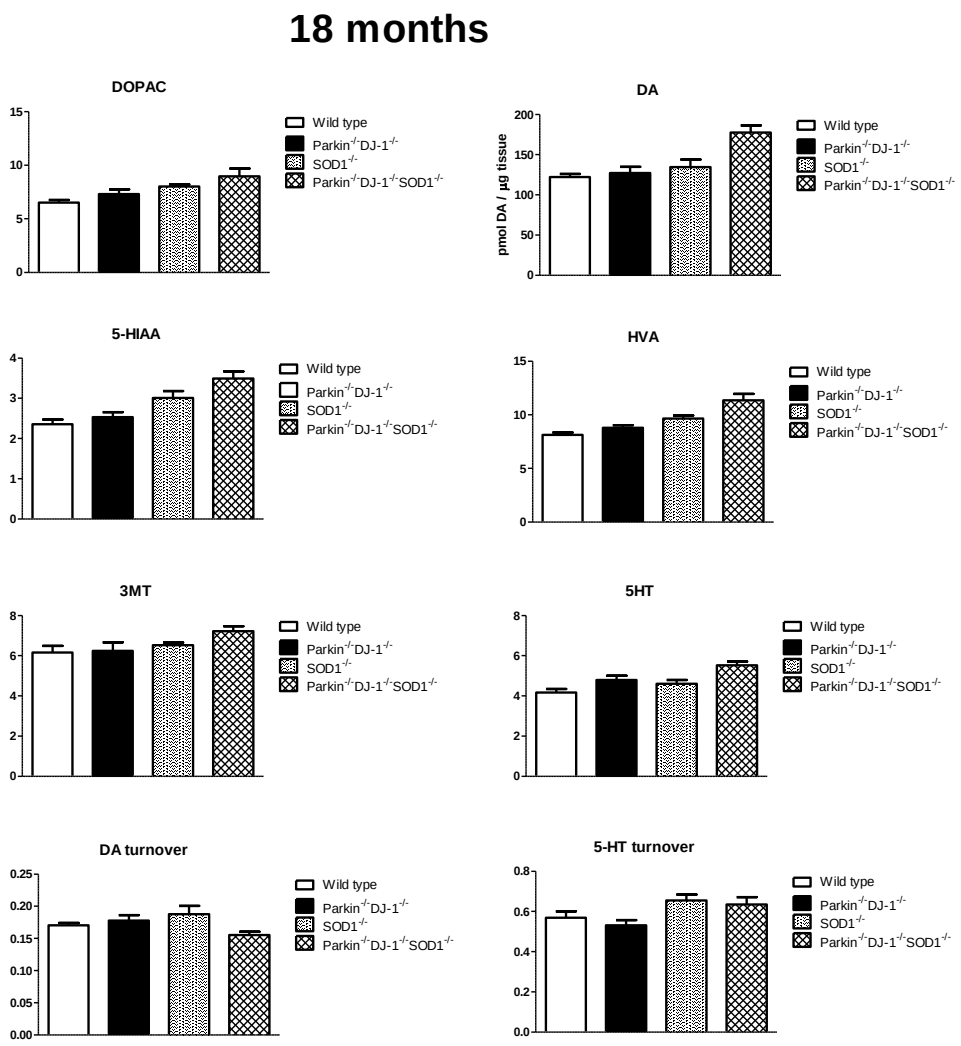


Figure 2.16: Levels of dopamine, serotonin, precursors and metabolites in the striatum of 18 month old mutant mice measured by HPLC.

Levels of D1 and D2-like receptor density in the striatum are similar to wild type

The significant increase in DA levels in the lines of triple mutant mice prompted us to measure levels of DA receptors in the striatum. We would expect to see a compensatory change in DA receptor density in response to elevated DA levels in *parkin$^{-/-}$DJ-1$^{-/-}$SOD1$^{-/-}$* mice. Using

radiolabeled DA ligands, we quantified the surface expression of D1-like and D2-like dopamine receptors in the striatum in fresh frozen sections. Contrary to the expected results, we saw no statistical change in the binding of radiolabeled DA ligand to D1 or D2 receptors (Figure 2.17). Although there is a trend towards increased D1-like receptor density in the *parkin*$^{-/-}$*DJ-1*$^{-/-}$*SOD1*$^{-/-}$ mice, it is not significant. It is possible that there is not a significant difference due to the small number of samples (n=4).

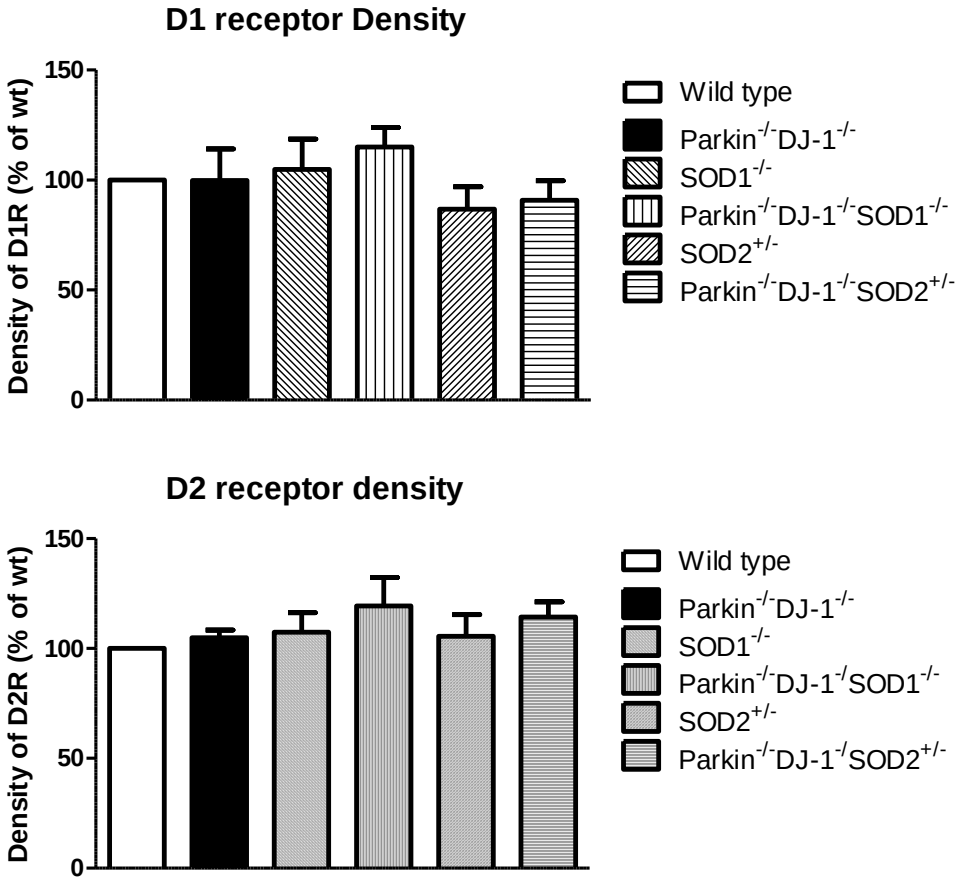


Figure 2.17: Levels of dopamine receptors density in the striatum of mutant mice measured by autoradiography. D1 and D2 receptor density is not statistically different from wild type in any of the mutant mice.

Additional preliminary analyses

Digigait

I utilized the Digigait system to objectively quantify movement and gait dynamics in wild type and mutant mice. Other mouse models of movement disorders, such as HD, have a pronounced phenotype. Mice were set to walk on a treadmill with increasing speed until they reached the setting of 10. Video of the mice was collected from below the translucent treadmill. About two minutes of video was recorded for analysis. From the video, we isolated a clip of ten sequential steps in a straight line where the mice did not veer from the center, rear up, or groom. One of the issues we encountered was the inability to measure all the mice at the same speed due to age-related degeneration of the *SOD1*$^{-/-}$ mice. When the speed was decreased, it became difficult to find 10 sequential steps where the more able mice did not pause or veer off course. We did not see obvious gait changes, but we did note that the *parkin*$^{-/-}$*DJ-1*$^{-/-}$ mice were more attentive to the walking task and spent less time turning around and grooming.

Oxyblot and Western Blot analysis of Anti-oxidant Proteins

We postulated that there may be an upregulation or compensation of other antioxidant proteins in the mutant mice that could account for the lack of DAergic neurodegeneration. First, I analyzed by western blot the levels of SOD1 protein in heart and brain lysate using a sheep anti-human SOD1 antibody. The results showed no change in SOD1 levels in wild type, *parkin*$^{+/-}$*DJ-1*$^{+/-}$ *SOD2*$^{+/-}$, *Gpx1* heterozygous or knockout mice. SOD1 levels were decreased in *SOD1*$^{+/-}$ mice

and absent in *SOD1*$^{-/-}$ mice. It is possible there is a slight upregulation of SOD1 in the mutant mice, but further experiments are necessary. Blotting with a SOD2 antibody (rabbit anti-mouse SOD2, Upstate catalog #06-984, 1μg/ml) and the Gpx1 antibody yielded inconclusive results. I analyzed levels of oxidative damage using the OxyBlot Protein Detection Kit (Millipore) in cortical brain lysate (cytosolic and mitochondrial fractions) from wild type, *parkin*$^{-/-}$*DJ-1*$^{-/-}$, *parkin*$^{-/-}$*DJ-1*$^{-/-}$*SOD1*$^{-/-}$, and *parkin*$^{-/-}$*DJ-1*$^{-/-}$*SOD2*$^{+/-}$. The OxyBlot detects carbonyl groups introduced into proteins by oxidative reactions with ozone, nitrogen oxides, or metal catalyzed oxidation. The blots had a significant amount of background immunoreaction, so I was unable to quantify the data. I also blotted for 3-nitrotyrosine (1 μg/ml, Upstate catalog # 06-284), but was unable to visualize the bands with the antibody used.

In a preliminary experiment with wild type, *parkin*$^{-/-}$, *DJ-1*$^{-/-}$, *SOD1*$^{-/-}$, *SOD2*$^{+/-}$, *Gpx*$^{+/-}$, and *Gpx*$^{-/-}$ mouse brain mitochondria and cytosol, it appears that there may be an upregulation of Parkin in the *SOD1*$^{-/-}$ and *SOD2*$^{+/-}$ mice. DJ-1 protein levels did not appear to be altered in the mutant mice. These experiments need to be repeated for validation, however if there is an upregulation of Parkin, it may implicate a compensatory mechanism in the Parkin pathway to protect cells from increased ROS and oxidative damage. In another western I blotted for levels of TFAM protein. Preliminary results suggest a modest increase in TFAM in DJ-1-/- mice, contrary to what we expect. Parkin can increase TFAM levels, so we would expect a decrease in TFAM in *parkin*$^{-/-}$ mice, and DJ-1 stabilizes Nrf2 which can increase TFAM levels, so we would expect a decrease in TFAM in the *DJ-1*$^{-/-}$ as well. Further analysis is required to confirm the significance of the preliminary data.

In further analysis, we propose an immunoprecipitation assay to pull down mtDNA with anti-TFAM to assess the mtDNA copy number as a marker of mitochondrial abundance. Measuring levels of mRNA levels of specific genes in the mutant mice will help elucidate if there is compensatory upregulation of other genes encoding Parkin, DJ-1, Gpx1, Gpx2, SOD1, SOD2, NQO1, Nrf2, TFAM, iNOS, catalase and cyclophilin, TH, MAO, and post-synaptic DA receptors.

Excised Muscle Fatigue Testing

With assistance in the Kamm lab, EDL and soleus muscles were isolated from fully anesthetized mice, mounted on Grass FTO3.C force transducers and continuously gassed with 95% O_2/5% CO_2 while immersed in PSS containing 120.5 mM NaCl, 4.8 mM KCl, 1.2 mM $MgSO_4$, 1.5 mM $CaCl_2$, 1.2 mM Na_2PO_4, 20.4 mM $NaHCO_3$, 10.0 mM dextrose and 1.0 mM pyruvate at 30°C and pH 7.6. Muscles were subjected to electrical stimulation at 30 Hz until the muscle fatigued. I calculated the time it took the muscle to become 70% fatigued. Preliminary results suggest that

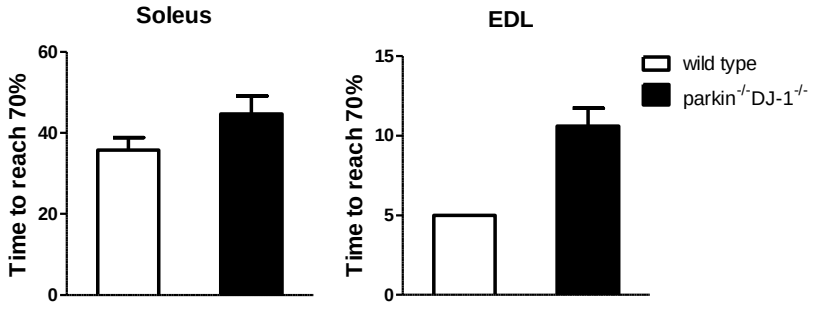


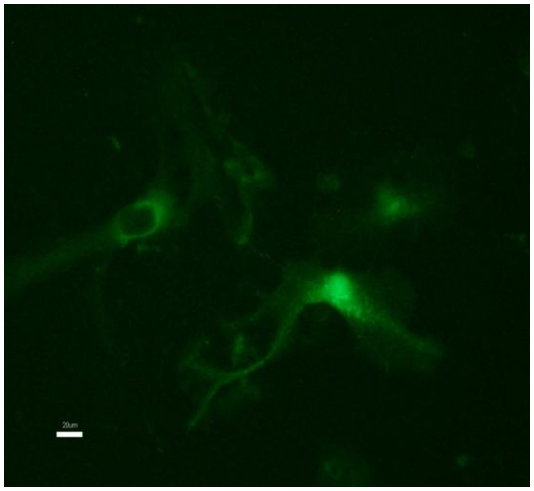
Figure 2.18 Preliminary results from excised muscle suggest that *parkin*$^{-/-}$*DJ-1*$^{-/-}$ mouse muscle does not fatigue as rapidly as wild type. (n=2; wt EDL, n=1)

parkin$^{-/-}$*DJ-1*$^{-/-}$ mouse muscles do not fatigue as quickly as wild type, but sample size is not large enough for statistical power.

Mitochondrial Imaging

I used primary cortical neuron cultures from wild type, *parkin*$^{-/-}$, and *DJ-1*$^{-/-}$ P1 mouse pups. The cultures were treated with FCCP to explore the hypothesis that Parkin targets damaged mitochondria for autophagy and that a loss of Parkin will result in aberrant mitochondrial morphology. FCCP causes depolarization of the mitochondrial membrane, which in turn causes Parkin and DJ-1 to translocate to the mitochondria from the cytosol. We infected neurons with an LC3-GFP virus and treated the cultures with mitotracker to image mitochondria on the confocal microscope (Figure 2.19). The drawbacks to this study were the difficulty maintaining healthy primary neuronal cultures, the preferential infection of glial cells by AAV-LC3-GFP, and difficulty imaging mitochondria in neurons. We were unable to draw conclusions from these experiments.

A



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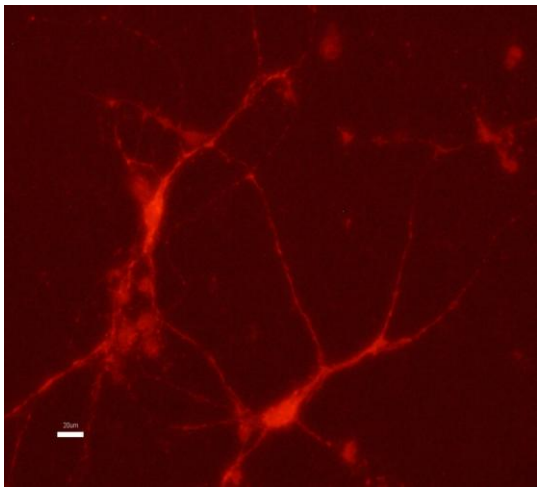

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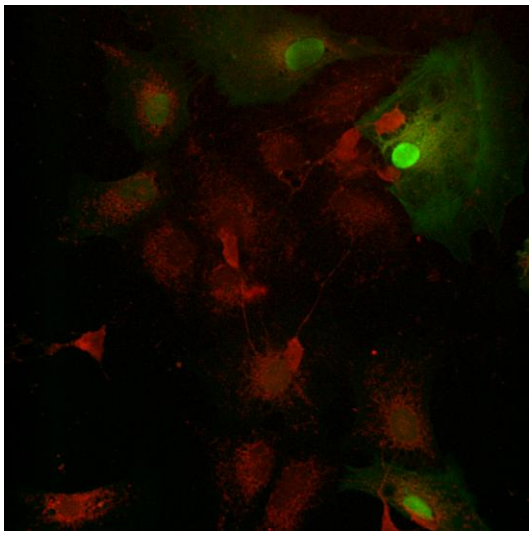

Figure 2.19: Primary postnatal mouse cortical cultures infected with LC3-GFP as a marker for mitophagy (A), and stained with mitotracker to visualize mitochondrial morphology (B). Overlapping channels for LC3-GFP (green) and mitotracker (red) shows the expression of LC3-GFP in glia and highlights the ease of visualizing mitochondria and mitophagy in glia but not neurons (C).

CHAPTER 3

DISCUSSION

Parkinson's disease affects over one million Americans every year. Patients suffer a debilitating progressive degeneration of dopamine neurons leading to severe motor dysfunction, depression and dementia. Although treatments for this disease are available, none are able to halt or reverse the progression of neurodegeneration. Advances in drug discovery for PD are limited by our knowledge of its pathophysiology and the lack of an accepted PD animal model with progressive nigral cell loss for research.

We proposed to generate a genetic animal model of PD that recapitulates the behavioral and pathological symptoms of the disease. Despite the extensive research on PD *in vitro* and *in vivo* in animal models, applying conclusions from non-representative animal models to human therapeutics is inherently problematic. Studies of human subjects would yield the most accurate data on novel treatment options for PD, however the scope of research that can be conducted in patients is limited. The animal models discussed in Chapter 1 are useful in many ways, however in most cases they lack progressive DA neuron degeneration. In the literature, several factors have been identified that have expanded our knowledge of the etiology of PD. Ultimately understanding the pathophysiology of PD in humans through extensive research will contribute to finding better treatment and prevention options for PD.

The pathological hall mark of PD is progressive nigral DA neuron degeneration. DA replacement therapy is the common treatment for PD, but it cannot reverse the pathological changes or stop the disease progression. If scientists are able to identify the pathogenesis of the disease, particularly by studying the disease in a pre-symptomatic model, there may be a means to prevent it in the future.

There are a number of etiological factors contributing to PD including genetic predisposition and exposure to neurotoxins and oxidative stressors. Efforts in drug discovery are limited by the inability to replicate the disease process in laboratory conditions. Some widely accepted animal models exhibit DA neuron degeneration, such as the 6-OHDA and MPTP models, however the neuron loss in these models is chemically induced and acute which is in contrast to the chronic and progressive pathology of the disease in patients. Such animal models replicate the late phase of the disease, but do not allow for investigation into the organic mechanisms behind neuronal degeneration.

Most clinical cases of PD are idiopathic, though about 10% of cases can be causally linked to heritable mutations in genes such as *parkin*, *DJ-1*, *PINK1* and *LRRK2*. Surprisingly, none of published studies on mouse models lacking these genes have reported any progressive nigral cell loss or severe motor dysfunction. This suggests that genetic mutations are not the sole causative agent in PD, or that the mouse genome may possess compensatory mechanisms which impart resistance to a PD-like condition. Current research in the field supports the theory of cumulative oxidative damage and mitochondrial dysfunction leading to autophagy and DA cell death.

In this study, an effort has been made to produce a progressive PD mouse model. Cumulative oxidative damage and mitochondrial dysfunction have been presumed to play a key role in idiopathic and inherited PD. Loss of the gene *parkin* or *DJ-1* is sufficient to cause PD in patients, but *DJ-1*$^{-/-}$ and *parkin*$^{-/-}$ mice do not exhibit DA neuron degeneration or depleted striatal DA, perhaps due to their inherent antioxidant defenses and limited life span. Our strategy was to lower the antioxidant defenses in mice with combined loss-of-function *parkin* and *DJ-1* mutations. To start, we crossed *parkin*$^{-/-}$ and *DJ-1*$^{-/-}$ mice to create a mouse deficient for both proteins, speculating that two disease causing mutations may be sufficient to induce a PD-like phenotype in mice. This has precedence in neurodegenerative models as one of the most widely accepted model for AD is the triple transgenic mouse carrying the $PS1_{M146V}$, APP_{Swe}, and tau_{P301L} transgenes (Oddo, Caccamo et al. 2003). Even still, some scientists find that APP transgenic mice fail to completely recapitulate the pathology of AD and may be considered only as a pre-symptomatic model (Ashe and Zahs 2010). Given that the major cytoplasmic and mitochondrial antioxidant enzymes in the brain are SOD1 and SOD2, we crossed our *parkin*$^{-/-}$ *DJ-1*$^{-/-}$ mice with *SOD*$^{-/-}$ and *SOD2*$^{+/-}$ mice respectively. These crosses result in Parkin, DJ-1 and superoxide dismutase deficient progenies that should be more sensitive to oxidative stress leading to DA neuron degeneration.

We collected data for a number of behavioral paradigms, quantified striatal DA levels, DA receptor density, and nigral cell loss. The battery of established behavioral tests we conducted included rotorod performance, locomotor activity, open field activity, elevated plus maze behavior, dark/light activity, acoustic startle response, and forced swim test mobility. We hypothesized that the combinatorial mutations would result in age-dependent PD-like pathology

and symptoms, however our data revealed rather startling results which belied our hypothesis and expectations. Since age is the greatest risk factor for developing PD, we conducted our studies in young and old mice (4 months to 18 months). The mice that underwent behavioral testing were sacrificed for immunohistochemistry, HPLC and DA receptor autoradiography to identify pathological changes.

One of our most surprising results, contrary to our initial hypothesis was the enhanced rotorod performance of the *parkin*$^{-/-}$*DJ-1*$^{-/-}$ mutant mice. The rotorod performance of wild type, *parkin*$^{-/-}$, *DJ-1*$^{-/-}$, *parkin*$^{-/-}$*DJ-1*$^{-/-}$, *SOD1*$^{-/-}$, *SOD2*$^{+/-}$, *parkin*$^{-/-}$*DJ-1*$^{-/-}$*SOD1*$^{-/-}$, *parkin*$^{+/-}$*DJ-1*$^{-/-}$ *SOD2*$^{+/-}$, and *parkin*$^{-/-}$*DJ-1*$^{-/-}$*SOD2*$^{+/-}$ at various ages revealed that the *parkin*$^{-/-}$*DJ-1*$^{-/-}$ mice had enhanced performance over wild type mice instead of the expected impaired performance ($p = 0.004$). There was no difference from wild type in the single *parkin*$^{-/-}$, *DJ-1*$^{-/-}$, *SOD1*$^{-/-}$, or *SOD2*$^{+/-}$ mice, indicating that the enhanced performance seen in the *parkin*$^{-/-}$*DJ-1*$^{-/-}$ mice is due to a synergistic effect of the loss of both Parkin and DJ-1. The effect was apparent in all age groups (6 month, 9 month, 15 months, and 17 months) and became more significant and pronounced with age. The increasing significance over time may be due to an age-dependent mechanism, but it may also be confounded by weight as there is a trend towards more weight gain in wild type than *parkin*$^{-/-}$*DJ-1*$^{-/-}$ mice over time. We assed weight in 4, 8, and 8-12 month old mice. It would also be informative to collect this data for an older 15 month cohort across all mutant genotypes. It is unlikely that the subtle difference in weight between wild type and *parkin*$^{-/-}$*DJ-1*$^{-/-}$ mice could account entirely for the drastic change in rotorod performance, especially given that the trend is not significant.

There are a number of possible mechanisms for the enhanced rotorod performance of the *parkin*$^{-/-}$*DJ-1*$^{-/-}$ mice which all warrant further investigation. While conducting the Digigait experiments, I observed that the *parkin*$^{-/-}$*DJ-1*$^{-/-}$ mice were more task-oriented and less distracted by their environment than the other genotypes while walking on the treadmill. Attention is regulated by a DA pathway different from the nigrostriatal pathway. The DA neurons in the meso-cortical pathway project from the VTA to the dorsolateral prefrontal cortex (PFC) and regulate attention, initiative, motivation, planning, decision making, working memory, and other higher cognitive functions. Preliminary studies in our lab show an increased attentiveness of *parkin*$^{-/-}$*DJ-1*$^{-/-}$ mice compared to wild type, *DJ-1*$^{-/-}$, and *parkin*$^{-/-}$ mice as measured by a decrease in time spent distracted while performing the rotorod task (data not shown). Other means of measuring attention, such as the nose poke test, are confounded by the ability of the mouse to learn the task. An increase in DA levels in the prefrontal cortex could enhance attention in *parkin*$^{-/-}$*DJ-1*$^{-/-}$ mice. This increase may be observed as an increase in DA terminals, DA release, DA receptor density, or a change in DA metabolism or clearance. DA is cleared slower in the PFC than in the striatum due to a decrease in the density of DAT in the synaptic terminals. Metabolism of DA and clearance by NET are the likely means of alternative clearance. It is possible that loss of Parkin and DJ-1 leads to a down-regulation of NET, particularly in the prefrontal cortex, leading to elevated DA and enhanced attention. NET protein levels in the PFC could be assessed by a protein immunoblot.

Very few studies report an enhanced rotorod performance in mice. The DA receptor D_4 (DRD4) knockout mouse has an enhanced rotorod phenotype and elevated L-DOPA accumulation in the caudate putamen (Rubinstein, Phillips et al. 1997). The D_4 receptor is a D_2-

like receptor in which the activated receptor inhibits the enzyme adenylate cyclase, thereby reducing the intracellular concentration of the second messenger cyclic AMP. If the enhanced rotorod phenotype in the *parkin*$^{-/-}$*DJ-1*$^{-/-}$ mice is due to a similar mechanism, we could expect to see a decrease in the density of D_4 receptors, but not necessarily all D_2-like receptors. Contrary to *DRD4*$^{-/-}$ mice, D_2 DA receptor knockout mice have age-dependent motor and learning deficits (Fowler, Zarcone et al. ; Fetsko, Xu et al. 2005). On the contrary, D_1 receptor knockout mice have a decreased rotorod performance, indicating that D_1 receptor signaling is required for rotorod performance and spatial learning (Karasinska, George et al. 2000).

We considered the possibility that *parkin*$^{-/-}$*DJ-1*$^{-/-}$ mice stayed on the rotorod longer due to increased anxiety over falling off the rotorod. Since anxiety and depression affect about 40% of PD patients, we utilized accepted behavioral tests of anxiety and depression to assess these phenotypes in our mice. Anxiety-like behaviors can be measured effectively in mice by using the elevated plus maze (EPM), open field testing, and dark/light exploration. We saw no particular effect on anxiety due to genetic mutations in 9 month old *parkin*$^{-/-}$*DJ-1*$^{-/-}$, *SOD2*$^{+/-}$ and *parkin*$^{+/-}$ *DJ-1*$^{-/-}$*SOD2*$^{+/-}$ mice. The parameters evaluated in the open field test were the total distance travelled, percentage of distance travelled in the periphery (thigmotaxis), and the relative time spent in the center. Although the slight decrease in the distance travelled in the periphery and the time spent in the periphery by the *parkin*$^{+/-}$*DJ-1*$^{-/-}$*SOD2*$^{+/-}$ mice was significant, we considered the difference may be an aberration due to the inherent variability of this test. Further testing is warranted to determine the significance of this result. No anxiety phenotype has been reported for the Goldberg *parkin*$^{-/-}$ mice, but the *parkin*$^{-/-}$ mouse with an exon 3 deletion reportedly has increased thigmotaxic behavior (Zhu, Maskri et al. 2007). *DJ-1*$^{-/-}$ mice are reported to be

hypoactive in the open field test, which may account for some of the decreased activity in the *parkin*$^{+/-}$*DJ-1*$^{-/-}$*SOD2*$^{+/-}$ mice (Goldberg, Pisani et al. 2005). Similar results were obtained when the dark/light test was used to detect differences in anxiety behavior of the mutant mice. Data collected for the duration of time spent in the light, total activity and the number of crosses between the light and dark chambers did not reveal any behavioral differences between the tested genotypes. No results have been reported for the dark/light test with the *parkin*$^{-/-}$ or *DJ-1*$^{-/-}$ mice used in our studies. In the elevated plus maze, *parkin*$^{-/-}$*DJ-1*$^{-/-}$ mice spent significantly more time in the open arms compared to *parkin*$^{+/-}$*DJ-1*$^{-/-}$*SOD2*$^{+/-}$ mice ($p=0.032$), but the time spent in the open arms was not significantly different from wild type mice. The parameters evaluated were the percent of time spent in the open arms, the percent of total entries into open and closed arms.

Acoustic startle response (ASR) measures the involuntary reaction to a sudden loud noise. The output was measured through a transducer which records the force the mouse exerts when it jumps in response to the acoustic stimulus. As higher startle amplitude is related to a higher predisposition for anxiety, this test can be used to evaluate anxiety levels. We tested the ASR in older (16 months old) wild type, *DJ-1*$^{-/-}$, and *parkin*$^{-/-}$*DJ-1*$^{-/-}$ mice as well as 14 month old wild type, *parkin*$^{-/-}$, and *parkin*$^{-/-}$*DJ-1*$^{-/-}$ mice. There was a statistical reduction in the ASR of *parkin*$^{-/-}$ mice that was not seen in *DJ-1*$^{-/-}$ or *parkin*$^{-/-}$*DJ-1*$^{-/-}$ mice at this age. This agrees with previously published data showing no change in ASR in *DJ-1*$^{-/-}$ mice, but a reduced ASR in the *parkin*$^{-/-}$ mice, the *A53T α-synuclein* overexpressing mice, and the *parkin/A53T* double transgenic mice (Von Coelln, Thomas et al. 2004; von Coelln, Thomas et al. 2006). In younger mice (7 months old), a reduction of ASR is observed in the *parkin*$^{-/-}$*DJ-1*$^{-/-}$ mice.

In younger cohorts (7 months old) there were significant differences in the magnitude of the startle response between wild type and *parkin*$^{-/-}$*DJ-1*$^{-/-}$ (Tukey test, $p < 0.001$) as well as between wild type and *parkin*$^{+/-}$*DJ-1*$^{-/-}$*SOD2*$^{+/-}$ (Tukey test, $p < 0.001$) but there were no differences between the two genotypes when compared with each other. Additionally, we found a significant difference between wild type and *parkin*$^{-/-}$*DJ-1*$^{-/-}$*SOD1*$^{-/-}$ mice, which is likely due to deafness in *SOD1*$^{-/-}$ mice. Confounding factors in the ASR test included the age of the mice as well as sex and weight. The estrous cycle in female mice has no effect on ASR amplitude, while males tend to have a higher ASR due to overall elevated anxiety (Plappert, Rodenbücher et al. 2005). Lack of Parkin may be the main contributing factor in the decrease of ASR in *parkin*$^{-/-}$*DJ-1*$^{-/-}$ and *parkin*$^{+/-}$*DJ-1*$^{-/-}$*SOD2*$^{+/-}$ mice. The mechanism behind the involvement of Parkin in this phenotype requires further investigation.

Grip strength measures a single and well defined behavioral aspect. When the grip strength of *parkin*$^{-/-}$*DJ-1*$^{-/-}$, *parkin*$^{-/-}$*DJ-1*$^{-/-}$*SOD1*$^{-/-}$, and *parkin*$^{-/-}$*DJ-1*$^{-/-}$*SOD2*$^{+/-}$ mice was compared with those of wild type, the general trend was a weakened grip in the mutants although no effect of genotype was statistically significant by ANOVA. It is necessary to take into consideration possible operator error in the grip strength test. Since the mouse is pulled back manually by the tail, there may be variability of the speed and amount of force used to pull the mouse back from the apparatus. The *parkin*$^{-/-}$*DJ-1*$^{-/-}$*SOD2*$^{+/-}$ genotype had the least grip strength of wild type, *parkin*$^{-/-}$*DJ-1*$^{-/-}$ and *parkin*$^{-/-}$*DJ-1*$^{-/-}$*SOD1*$^{-/-}$. It would be important to measure the grip strength in these mice at an older time point since aged *DJ-1*$^{-/-}$ mice have reported decreased grip strength that is not seen in younger mice (Chandran, Lin et al. 2008). I would speculate that this is a direct readout of mitochondrial function in these mice. It has been well-documented that

Parkin and DJ-1 play a role in maintaining mitochondrial function and that loss of these proteins can lead to mitochondrial dysfunction, fragmentation, mitophagy, and cell death. Neurons and muscles contain a high concentration of mitochondria due to their high energy demand. If the *parkin*$^{-/-}$*DJ-1*$^{-/-}$, *parkin*$^{-/-}$*DJ-1*$^{-/-}$*SOD1*$^{-/-}$, and *parkin*$^{-/-}$*DJ-1*$^{-/-}$*SOD2*$^{+/-}$ mice have decreased functional mitochondria, then we would expect less efficient energy production in the cell, which would lead to decreased muscle strength.

In the forced swimming test (FST), where the end points are quantification of swimming mobility and the total time spent in the immobile position, the time spent immobile was dramatically decreased in the *parkin*$^{-/-}$*DJ-1*$^{-/-}$ and *parkin*$^{+/-}$*DJ-1*$^{-/-}$*SOD2*$^{+/-}$ genotypes as compared to wild type in the first cohort tested (7 months old). In older 14 and 16 month old mice, *parkin*$^{-/-}$ *DJ-1*$^{-/-}$ still presented with decrease immobility which can be attributed to the loss of DJ-1. Decreased immobility was seen in *DJ-1*$^{-/-}$ mice but not *parkin*$^{-/-}$ mice.

In order to determine whether any histological changes that could explain the surprising rotorod phenotype occur in the *parkin*$^{-/-}$*DJ-1*$^{-/-}$ mice, heart, muscle and cerebellar tissue samples were obtained and subjected to H&E staining. Microscopic observation revealed that there were no morphological abnormalities in the stained tissue. The myofibrils obtained from the extensor digitorum longus (EDL) and soleus (SOL) revealed a similar qualitative ratio of Type I and Type II muscle fibers as revealed by metachromatic ATPase staining. We did not conduct a quantitative analysis of the fiber type abundance. No morphological changes of the heart, muscle or cerebellum have been previously reported in *parkin*$^{-/-}$ or *DJ-1*$^{-/-}$ mice.

The pathological hallmark of PD is progressive loss of DA neurons in the SNpc. We expected to see a loss of DA neurons in the SNpc of our mutant mice, but when fixed, paraffin embedded brain sections were subjected to rigorous stereology, they revealed a startlingly modest increase in DA neurons in 7 month old *parkin*$^{-/-}$*DJ-1*$^{-/-}$*SOD2*$^{+/-}$ mice. This increase was significant by t-test, but not significant by ANOVA. In the 14 month old cohort, we assessed DA neuron number in the SNpc of the *SOD1*$^{-/-}$ and *SOD2*$^{+/-}$ single mutant mice in addition to *parkin*$^{-/-}$*DJ-1*$^{-/-}$, *parkin*$^{-/-}$*DJ-1*$^{-/-}$*SOD1*$^{-/-}$, and *parkin*$^{-/-}$*DJ-1*$^{-/-}$*SOD2*$^{+/-}$ mice. There was no statistical difference between wild type and mutant mice at 14 months of age ($p = 0.483$ and $p = 0.598$). The small sample size (n=4) could contribute to the aberrant observation from the younger cohort. Alternatively, the *parkin*$^{-/-}$*DJ-1*$^{-/-}$*SOD2*$^{+/-}$ mice could have an elevated number of nigral neurons at a younger age that are later pruned. A trend towards elevated DA in the striatum of young mice is seen in *parkin*$^{-/-}$*DJ-1*$^{-/-}$*SOD2*$^{+/-}$ mice which is significant at an older age. DA receptor activation can promote neurogenesis and may contribute to an increase in DA neurons in this model due to excessive DA levels in the striatum (Winner, Desplats et al. 2009). These neurons may be pruned later due to increased oxidative stress, mitochondrial damage, or apoptosis. This would be a novel and significant finding that could justify the use of these mice as a model for pre-symptomatic PD.

The other most surprising and significant finding in our study is the elevated level of striatal DA in the triple mutant mice. In a PD model, we expected a decrease in the number of DA neurons in the SNpc and depletion of striatal DA, however, there was an increase in DA neurons and a significant increase in DA levels in the striatum of *parkin*$^{-/-}$*DJ-1*$^{-/-}$*SOD1*$^{-/-}$ mice across age groups. The fact that we do not see this increase in *parkin*$^{-/-}$*DJ-1*$^{-/-}$ mice leads us to conclude that the enhanced rotorod phenotype is independent of elevated striatal DA and that SOD1 plays a critical role in

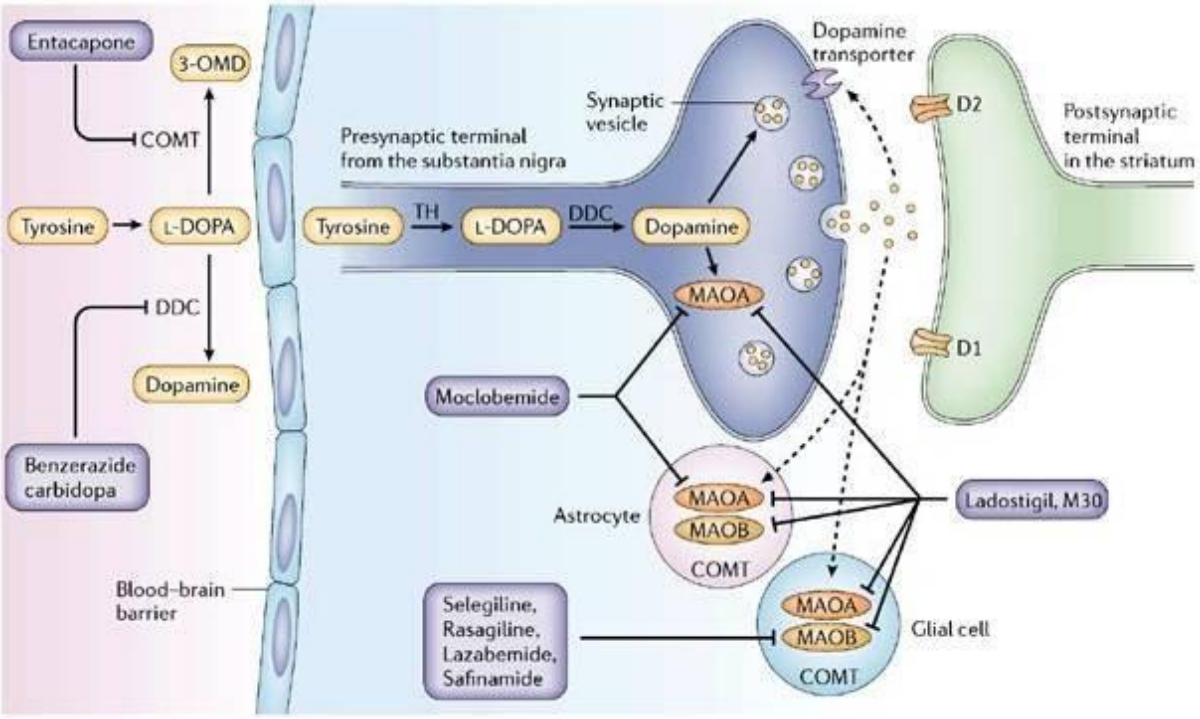

Figure 3.1: The pathway of dopamine synthesis proceeds from tyrosine via tyrosine hydroxylase (TH) catalysis to levodopa (L-DOPA), and subsequent decarboxylation by dopa decarboxylase (DDC) to dopamine. DA is metabolized by intraneuronal monoamine oxidase A (MAOA), and by glial and astrocytic MAOA and MAOB. Selective inhibitors of MAOA (for example, moclobemide) and MAOB (selegiline, rasagiline and safinamide) do not alter the steady-state striatal DA levels, although chronic treatment with these drugs does enhance DA release. Non-selective MAOA/B inhibitors (such as ladostigil) induce highly significant increases in the levels of DA in the striatum and other regions. Inhibitors of catechol-*O*-methyltransferase (COMT), such as entacapone, also enhance L-DOPA availability and prevent the inactivation of DA by COMT.

maintaining regulated DA neurotransmission. It is important to explore some of the potential mechanisms for the elevation of DA in the triple mutant mice. Previously published triple mutant mice deficient for PINK1, Parkin, and DJ-1 have elevated DA in 24 month old mice, but not in 16 month old mice (Kitada, Tong et al. 2009). Upregulation of DA could be due to an increase in synthesis of DA. It has been shown that human DJ-1 activates transcription of the *tyrosine*

hydroxylase gene, suggesting that an increase in TH could lead to an increase in DA synthesis (Ishikawa, Taira et al. 2010). This is contrary to what we have observed in that the increase in striatal DA occurs in the absence of DJ-1, so TH would theoretically be downregulated in *DJ-1*$^{-/-}$ mice, leading to decreased DA synthesis. We could evaluate the mRNA and protein levels for TH to determine if an upregulation in TH correlates with increased striatal DA. In the study by Kitada et al., they evaluated TH activity in their triple mutant mice and found there was no change in TH activity, however, they only measured activity in 2-5 month old mice, not the 24 month old affected population. We could also measure TH activity in vivo in our mutant mice by monitoring L-DOPA formation. Mice would be injected intraperitoneally with a selective AACD inhibitor. Thirty minutes after injection, the mice would be sacrificed, the striata dissected out, placed in 0.1 N perchloric acid and sonicated. The levels of DOPA can be determined by HPLC.

Another cause of increased striatal DA could be the vesicular storage, repackaging, or release of DA at the synaptic cleft. Synaptotagmin XI is a member of the synaptotagmin family that is well characterized in its importance for vesicle formation and docking. The interaction between Parkin and synaptotagmin XI suggests a role for Parkin in the regulation of the synaptic vesicle pool and in vesicle release. Loss of Parkin could thus affect multiple proteins controlling vesicle pools, docking and release and explain the deficits in dopaminergic function seen in patients with *parkin* mutations (Huynh, Scoles et al. 2003). In addition to DA synthesis, there could be a decrease in DA metabolism or turnover. DA is taken up from the synaptic cleft by neuronal and glial DAT and NET where is can be repackaged into vesicles or degraded by COMT and MAO (see Figure 3.1). It has been shown that DA turnover increases in carriers of asymptomatic *LRRK2* mutations (Sossi et al., 2010). We could measure levels of NE and

epinephrine in the striatum as an additional indicator of DA metabolism since DA is a precursor to these neurotransmitters.

Another possibility is an increase in synaptic terminals in the striatum. We have seen with our data that there is no significant change in DA neuron number in the substantia nigra, but we could see an increase in DA terminals by IHC for TH or DAT. Since DAT can also be expressed in glia, we would colocalize DAT with a synaptic protein such as synaptophysin. Aging mice selectively lacking DJ-1 and the GDNF receptor Ret under the DAT promoter display an accelerated and progressive loss of SN cell bodies, but not axons, and pronounced glial activation. Additionally, Ret transcription and translation is regulated by DJ-1 and a loss of DJ-1 results in decreased Ret expression. The absence of Ret signaling in DA neurons results in progressive and late degeneration of the nigrostriatal system. This indicates that DJ-1 promotes survival of aging DA neurons by inducing Ret expression necessary for GDNF trophic support of DA neurons. (Aron, Klein et al. 2010; Foti, Zucchelli et al. 2010).

The elevated striatal DA could be a precursor to DA neuron degeneration. Elevated tonic extracellular DA concentration and altered DA modulation of synaptic activity precede DA loss in the striatum of mice overexpressing human α-synuclein (Lam, Wu et al. 2011). Additionally, a subset of *DAT*$^{-/-}$ mice, which have chronically elevated striatal DA, develop degeneration of GABAergic neurons in the striatum (Cyr, Beaulieu et al. 2003). In vitro data suggests that chronic stimulation of postsynaptic D_1 DA receptors induces nitric-oxide synthase activation and cytotoxicity (Chen, Wersinger et al. 2003). It is unclear why DA neurons are the susceptible

neuronal population in PD, but it has been shown that mitochondria mass is low in mouse SN DA neurons, which may contribute to the selective vulnerability of these neurons in PD (Liang, Wang et al. 2007).

We measured DA receptor binding levels for D_1-like and D_2-like DA receptors. We did not observe any significant difference in the density of D_1-like or D_2-like DA receptors in the striatum of wild type, *SOD1*$^{-/-}$, *SOD2*$^{+/-}$, *parkin*$^{-/-}$*DJ-1*$^{-/-}$, *parkin*$^{-/-}$*DJ-1*$^{-/-}$*SOD1*$^{-/-}$, and *parkin*$^{-/-}$*DJ-1*$^{-/-}$*SOD2*$^{+/-}$ mice. There was a trend toward elevated D_1-like receptor levels in *parkin*$^{-/-}$*DJ-1*$^{-/-}$ *SOD1*$^{-/-}$ mice. We expected to see compensatory changes in response to the elevated striatal DA, particularly in D_2-like DA receptors. These trends may become a significant difference with analysis of a larger sample size. Previously published results show there is an upregulation of D_2 receptors in the striatum of drug-naïve PD patients with *parkin* mutations which is down-regulated following levadopa therapy (Scherfler, Khan et al. 2006). These results are specific to *parkin* mutations and are not seen in idiopathic PD, suggesting interplay between Parkin and regulation of DA receptors that warrants further investigation.

Since increased oxidative stress and oxidative damage may be causative factors in PD etiology, we crossed SOD deficient mice with *parkin*$^{-/-}$*DJ-1*$^{-/-}$ mice. We would expect elevated levels of oxidative damage resulting in nigral neuron degeneration. We did not observe DA neuron degeneration, but it would be of interest to extensively measure levels of oxidative damage in our mutant mice. Increased levels of protein carbonyls and 3-nitrotyrosine have been reported as markers of oxidative damage in PD (Alam, Jenner et al. 1997; Beal 2002). Oxidative

damage to DJ-1 has been linked to sporadic cases of PD and AD (Choi, Sullards et al. 2006). Additionally, aged *parkin*$^{-/-}$ mice have been reported to have increased oxidative damage to protein carbonyls and mitochondrial dysfunction in the brain compared to wild type mice (Palacino, Sagi et al. 2004). Leakage of electrons from the mitochondrial electron transport chain is the primary cellular source of free radicals that damage proteins, lipids and DNA. We hypothesize that Parkin deficiency leads to accumulation of dysfunctional mitochondria and increased oxidative damage. Antioxidant response elements (ARE) and Nrf2 (an antioxidant transcriptional regulator) are known to regulate expression of NAD(P)H:quinone oxidoreductase (NQO1). DJ-1 stabilizes Nrf2 by preventing association with Keap1, thereby preventing ubiquitination of Nrf2. Loss of DJ-1 leads to a decrease in NQO1 due to a loss in Nrf2. (Clements, McNally et al. 2006). Indirectly, Nrf2 can increase TFAM expression which may lead to increased mitochondrial biogenesis. We could measure mRNA NQO1 levels in wild-type, *parkin*$^{-/-}$, *DJ-1*$^{-/-}$, and *parkin*$^{-/-}$*DJ-1*$^{-/-}$ mice by qPCR and compare mRNA levels to Nrf2, TFAM, and cyclophilin levels, as well as iNOS, catalase, Gpx1, Gpx2, SOD1, and SOD2 levels. Preliminary data in collaboration with Joyce Repa showed decreased NQO1 expression in muscle tissue from *parkin*$^{-/-}$*DJ-1*$^{-/-}$ mice compared to wild-type mice (data not shown). Protein levels of antioxidants by western blot of SOD1, SOD2, Gpx1, and catalase in wild type and mutant brains would be expected to change, but preliminary data does not show a significant difference. It is possible that a significant difference in antioxidant protein levels or oxidative damage is only evident in DA neurons and this effect may disappear when is averaged over the whole brain.

There are many published reports highlighted in Chapter 1 that support the theory that mitochondrial dysfunction is a causative mechanism of PD, especially since the genes linked to PD, AD and HD, associate with the mitochondria under distress. There is reason to presume that glia play a large and under-appreciated role in the pathogenesis of PD. Interestingly, *α-synuclein* and *parkin* expression are predominantly neuronal, however, *parkin* can be upregulated in glia in response to unfolded protein stress (Ledesma, Galvan et al. 2002). DJ-1 is predominantly located in the cytoplasm and nucleus of glial cells in the human brain, and *in vitro* it is concentrated in hippocampal neurons and astrocytes (Bandopadhyay, Kingsbury et al. 2004). We could knockout *parkin* or *DJ-1* under the GFAP promoter to assess if loss of Parkin and/or DJ-1 in glia is sufficient to confer increased sensitivity to MPTP or recapitulate the rotorod phenotype. Gpx1 is one of the major antioxidants in the brain. Gpx1 immunoreactivity is seen exclusively in glial cells surrounding DA neurons in PD, and an increase in Gpx1-positive cells correlates with more severe DA neuron loss (Damier et al., 1993). And finally, DA induces mRNA transcription of certain genes in primary rat astrocytes, like *Gpx1*, the Ubiquitin ligase *Nedd4*, and *NQO1* which suggest we could identify numerous local changes in the striatum as a result of elevated DA (Shi, Cai et al. 2001).

As mentioned previously, the glia to neuron ratio in the SN is the lowest ratio in the brain. Since glia acts to clear and metabolize DA from the synaptic cleft, a decrease in glia could cause an upregulation of DA, assuming no compensatory changes in DAT or metabolic enzymes. This can easily be tested by immunohistochemitry for GFAP and stereological quantification of glia in the SN. Additionally, glia from the striatum of mutant mice can be cultured and tested *in vitro* for DAT uptake of ^{3}H-DA.

Although we do not yet have mechanistic answers for the enhanced rotorod performance and elevated striatal DA in our mutant mice, we have the tools available to understand these phenotypes and better understand the role Parkin, DJ-1, SOD1, and SOD2 play in PD pathogenesis. Data from current literature support a hypothesis of mitochondrial dysfunction in PD and in our mice with PD-related gene deletions. Mitochondrial dysfunction is also implicated in AD and ALS since neurofibrilary tangles inhibit complex I and mutant SOD1 blocks mitochondrial protein transport. Therapeutics that improve mitochondrial health may be beneficial for treatment of many neurodegenerative diseases.

Mouse models for other neurodegenerative diseases reproduce many of the same key pathologies that occur in humans. AD is the most common neurodegenerative disease, characterized by senile plaques of aggregated amyloid-β (Aβ) peptide, interneuronal neurofibrillary tangles containing hyperphosphorylated tau protein, and severe cortical neurodegeneration and atrophy. Transgenic mice overexpressing human mutant amyloid precursor protein (APP) exhibit Aβ deposition and neuritic plaques, dystrophic neurites and synaptic loss. These changes are first evident at 3 months (Games, Adams et al. 1995). Transgenic mice with the APP Swedish mutation also develop Aβ plaques and memory deficits by 9 months, but there is no neurodegeneration in either model (Hsiao, Chapman et al. 1996; Kawarabayashi, Younkin et al. 2001). Although many of the AD models present with key pathologies such as Aβ plaques and neurofibrillary tangles in an age-dependent manner, they do not develop cortical neurodegeneration.

HD is a progressive neurodegenerative disorder caused by a triplet CAG repeat expansion encoding a polyQ expansion of the *huntingtin* (*Htt*) gene. The hallmark pathology of HD is the selective and progressive loss of medium spiny neurons in the caudate putamen resulting in chorea, dystonia, and cognitive deficits. Transgenic *Htt* mice have a progressive development of the full HD phenotype with onset ranging from 8 weeks to 54 weeks depending on the transgene (Trancikova, Ramonet et al. 2011). Despite mouse models that reproduce the pathology of HD, there are still no treatments available.

ALS is a rare motor neuron disease that affects both upper and lower motor neurons. The disease onset is in adulthood and is characterized by progressive weakness, spasticity, paralysis and death within 3-5 years of the diagnosis. Only about 10% of ALS cases are caused by heritable mutations. Most familial cases are caused by genetic mutations in *SOD1*. Transgenic mice expressing human *SOD1* with the G93A mutation have a rapid and robust ALS phenotype (Gurney, Pu et al. 1994). The onset and progression of the ALS phenotype in mice depends on the mutation in *SOD1*. *SOD1* transgenic mice are useful to understand the molecular basis of ALS since it reproduces the clinical symptoms and specific neurodegeneration seen in patients.

The HD and ALS mouse models available reproduce the onset, progression, pathology, and symptoms seen in human patients. Contrary to this, AD mouse models reproduce some pathological hallmarks of the human disease, but critically lack neurodegeneration. The models available have helped scientists understand APP processing and Aβ production, but do not model the disease progression. Mouse models of PD are lacking robust disease-relevant phenotypes,

particularly progressive DAergic neurodegeneration. It is possible that our genetic model is representative of pre-symptomatic PD, but an appropriate endogenous or exogenous stressor will need to be identified to exacerbate the disease phenotype and induce late-onset progressive nigral cell loss.

CHAPTER 4

CONCLUSIONS AND FUTURE DIRECTIONS

Conclusions

Our goal in this research was to develop a genetic mouse model of PD that possessed progressive DA neurodegeneration in the SN. Such a model would provide the field with a valuable tool to study the progression of PD and develop new therapeutics. Although these mice do not lack nigral cell loss and are not an acceptable model of PD based on the criteria outlined in Chapter 1, they are a valuable tool to investigate how a systemic loss of Parkin or DJ-1 causes PD. These mice are a valuable tool to research the role of Parkin and DJ-1 in maintaining regulation of the DA system in the context of elevated oxidative stress and may serve as a model of pre-symptomatic PD.

Future Directions

We have two very interesting phenotypes that warrant further investigation. The first is the enhanced rotorod performance in *parkin*$^{-/-}$*DJ-1*$^{-/-}$ mice, and the second is the elevated DA in the striatum of the *parkin*$^{-/-}$*DJ-1*$^{-/-}$*SOD1*$^{-/-}$ mice. The first necessary analysis is the evaluation of striatal DA levels and rotorod performance in the double mutant controls we did not have available: *parkin*$^{-/-}$*SOD1*$^{-/-}$, *parkin*$^{-/-}$*SOD2*$^{+/-}$, *DJ-1*$^{-/-}$*SOD1*$^{-/-}$, and *DJ-1*$^{-/-}$*SOD2*$^{+/-}$ mice. It is possible that loss of Parkin is sufficient to increase striatal DA levels in the absence of SOD1, indicating a role for Parkin but not DJ-1 in regulation of DA transmission in the absence of SOD1, or vice versa. Additionally, we could measure levels of DA in the pre-frontal cortex to

correlate with increased attention in our mutant lines. I would predict an increase in DA in the PFC of the *parkin*$^{-/-}$*DJ-1*$^{-/-}$ mice. It would be interesting and informative to measure ROS production and oxidative damage in the DA neurons in the SNpc. I would expect to see elevated levels of oxidative damage in DA neurons of aged double and triple transgenic mice that we may not be significant if it is averaged over all brain regions.

Parkin and DJ-1 are involved in mitochondrial regulation and degradation. Parkin is an E3-ubiquitin ligase that mediates proteasome-dependent protein degradation. Parkin is localized to the mitochondria under oxidative stress or mitochondrial membrane depolarization and can cause the rupture of the mitochondrial outer membrane (Narendra, Tanaka et al. 2008; Narendra, Jin et al. 2010; Yoshii, Kishi et al. 2011). DJ-1 deficiency leads to loss of mitochondrial polarization, fragmentation of mitochondria and accumulation of markers of autophagy (Thomas, McCoy et al. 2011). There is no data on the combined effect of Parkin and DJ-1 deficiency on mitochondrial function. To compliment the data on *parkin*$^{-/-}$ and *DJ-1*$^{-/-}$ mice, our double and triple transgenic mice allow us the ability to study the interaction of Parkin and DJ-1 in cells from a constitutive knock out, eliminating the caveats of working with siRNA. Oxidative stress due to normal calcium influx in SN DA neurons causes mitochondrial depolarization, suggesting that over-stimulation, by elevated DA for example, may lead to mitophagy and cell death. Loss of DJ-1 causes increased oxidation of the mitochondrial matrix following depolarization (Guzman, Sanchez-Padilla et al. 2010). Damaged mitochondrial genomes are known to accumulate with age. This is especially true in PD patients. If Parkin deficiency causes an accumulation of damaged mitochondrial DNA, we could measure this by HPLC for 8-hydroxydeoxyguanosine (8-OHdG), the most widely used marker for oxidative damage to DNA.

The trend toward decreased grip strength in our young double and triple mutant mice could be due to mitochondrial dysfunction in the muscle. It would be interesting to study the mitochondrial involvement of Parkin and DJ-1 in muscle, glia, and DA specific neurons.

The Pael receptor (Pael-R) has been identified as one of the substrates of Parkin. When Parkin is inactivated, unfolded Pael-R accumulates in the endoplasmic reticulum and results in neuronal death by unfolded protein stress. In Pael-R-deficient mice the striatal DA level was only 60% of that in normal mice, while in *Pael-R* transgenic (tg) mice, striatal DOPAC as well as vesicular DA content increased, suggesting that the Pael-R signal regulates the amount of DA in the dopaminergic neurons and excessive Pael-R expression renders DAergic neurons susceptible to chronic DA toxicity (Imai, Inoue et al. 2007). In our *parkin*$^{-/-}$ mice we could expect to see an increase in Pael-R accumulation. Excessive DA release in the striatum may be a precursor to DA neurodegeneration. To point, *parkin*$^{-/-}$*Pael-R(tg)* mice exhibited early and progressive loss of dopaminergic as well as noradrenergic neurons without formation of inclusion bodies (Wang, Imai et al. 2008). Additionally, sustained extracellular elevation of DA in the DAT KO mice can cause selective degeneration of postsynaptic striatal neurons without any loss of DA neurons in the SN. This pathology occurs in a subset of aged DAT KO mice, suggesting an additional oxidative stress is required to induce degeneration (Cyr, Beaulieu et al. 2003).

Nigrostriatal DA neurons have the smallest glial/neuron ratios in the brain, therefore the consequences of glial dysfunction could be more critical in the nigrostriatal pathway than in

other brain regions. Abnormal glial function is critical in *parkin* mutations, and its role increases with age (Ledesma, Galvan et al. 2002; Solano, Casarejos et al. 2008). Understanding the putative role of Parkin in glial function could explain a selectivity of Parkin dysfunction in DAergic neurons. DJ-1 is required for GDNF expression in glia and is also upregulated under oxidative stress (Foti, Zucchelli et al. 2010). Additionally, DA neuron grafts in PD patients have shown development of Lewy bodies many years following transplant, indicating that the local environment has a significant effect on DA neuron health independent of genetic background. *Parkin*$^{-/-}$, *DJ*$^{-/-}$, and the triple mutant mice may present with glial dysfunction and give us insights into our elevated striatal DA levels or the pathogenesis of PD in the absence of Parkin or DJ-1.

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