Elucidating the Role of LRRK2 in the Central Nervous System:

An Examination of Toxin-Induced Neuronal Outcomes

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This thesis is being submitted to the
Faculty of Graduate and Post-Doctoral Studies
in partial fulfillment of the requirements for the
Doctorate in Philosophy degree in Neuroscience.

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Abstract

Parkinson's disease (PD) is the second most common neurodegenerative disorder. Its cause(s) are predominantly unknown; however, a subset of cases has a genetic origin. Mutations in the *leucine-rich repeat kinase 2* (*LRRK2*) gene are the most common genetic cause of PD. Cases are clinically indistinguishable from idiopathic PD and display incomplete penetrance. Thereby, it is predicted that genetic vulnerability combined with environmental factors cause pathogenesis. However, the identity of these factors is unknown. Unfortunately, LRRK2`s native and pathogenic biological function(s) remain to be defined; owing to obstacles including a complex protein structure and the lack of pathological phenotypes in *LRRK2* research models.

To address the knowledge gap in LRRK2 biology, we set out to investigate the role of LRRK2 in the central nervous system (CNS). We generated and characterized a disease-mimicking *D. melanogaster* model of *LRRK2*-linked PD. This system was utilized to perform an *in vivo*, unbiased, high-throughput genetic screen to identify candidate interactors of *LRRK2*. Successful identification of a discrete number of genetic interactors was accomplished and, coupled with published evidence, highlighted the pursuit of subsequent mitochondrial-related investigations of *LRRK2*. These studies were performed using the *M. musculus* model system. Since *LRRK2* murine models lack disease-relevant phenotypes, and LRRK2's incomplete penetrance is predicted to be the result of gene-environment interaction, we employed the mitochondrial-targeting exogenous neurotoxin – MPTP/MPP^+, to investigate neuronal mitochondrial phenotypes and subsequent survival in the context of LRRK2.

Using the pathogenic R1441 GTPase-linked mutation, we did not observe altered neuronal mitochondrial length phenotypes or enhanced CNS sensitization to MPTP/MPP^+-induced death; highlighting that MPTP-mediated, mitochondrial-centered mechanisms of action should be approached cautiously in the context of R1441-LRRK2. Collectively, the work presented herein has unveiled novel targets for the exploration of LRRK2 biological function and encourages the investigation of alternative pathogenic trigger mechanisms in the context of *LRRK2*-linked PD.

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The completion of this dissertation would not have been possible without the contributions of numerous individuals who supported me throughout my studies.

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List of Manuscripts

I. ***Leucine-Rich Repeat Kinase 2*** **interacts with** ***Parkin*****,** ***DJ-1*** **and** ***PINK-1*** **in a** ***Drosophila melanogaster*** **model of Parkinson's disease.**

Venderova K, Kabbach G, Abdel-Messih E**,** Zhang Y, Parks RJ, Imai Y, Gehrke S, Ngsee J, Lavoie MJ, Slack RS, Rao Y, Zhang Z, Lu B, Haque ME, Park DS.

Hum Mol Genet. 2009 Nov 15;18(22):4390-404. doi: 10.1093/hmg/ddp394.

II. **Cellular Responses and Neuronal Survival of R1441-LRRK2 Murine Strains Following MPTP/MPP$^+$-Induced Oxidative Stress.**

Abdel-Messih E, Rousseaux MW, Im DS, Abuaish S, Marcogliese PC, Kulczycki J, Slack RS, Parks RJ, Anisman H, Shen J, Park DS.

Manuscript formatted and in preparation for submission.

Appendix III. Additional Scientific Contributions

III. **Inactivation of Pink1 gene in vivo sensitizes dopamine-producing neurons to 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and can be rescued by autosomal recessive Parkinson disease genes, Parkin or DJ-1.**

Haque ME, Mount MP, Safarpour F, Abdel-Messih E, Callaghan S, Mazerolle C, Kitada T, Slack RS, Wallace V, Shen J, Anisman H, Park DS.

J Biol Chem. 2012 Jun 29;287(27):23162-70. doi: 10.1074/jbc.M112.346437.

IV. Regulation of ischemic neuronal death by E2F4-p130 protein complexes.

Iyirhiaro GO, Zhang Y, Estey C, O'Hare MJ, Safarpour F, Parsanejad M, Wang S, Abdel-Messih E, Callaghan SM, During MJ, Slack RS, Park DS.

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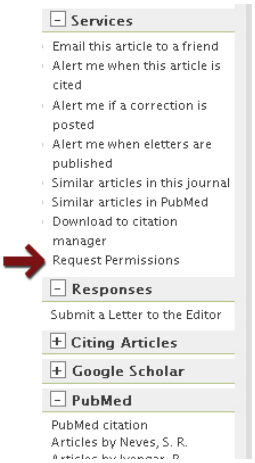

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List of Abbreviations

Abbreviation	Full Meaning
ACh	acetylcholine
°C	degrees celcius
2R	right arm of the second chromosome
5-HT	serotonin
6-OHDA	6-hydroxydopamine
ACh	acetylcholine
AD	Alzheimer's disease
AD	autosomal dominant
AR	autosomal recessive
B	balancer
BAC	bacterial artificial chromosome
BBB	blood-brain barrier
BC	balancer chromosome
BDSC	Bloomington Drosophila Stock Centre
c.c.	corpus callosum
CHIP	c-terminal Hsp70 interacting protein
CMA	chaperone-mediated autophagy
CNS	central nervous system
COR	c-terminal of ROC
COX-2	cyclooxygenase 2
CyO	Curly wing balancer

D1R/D2R	dopamine receptor 1 or 2.
DA	dopamine
DAPK1	death-associated protein kinase 1
DAT	dopamine transporter
DBS	deep brain stimulation
DDC	dopamine decarboxylase
DIP	drug-induced parkinsonism
DIV	days *in vitro*
DNA	deoxyribonucleic acid
DOPAC	3,4-dihydroxyphenylacetic acid
Drp1	dynamin-related protein 1
E	enhancement
EPDA	European Parkinson's Disease Association
ERM	ezrin, radixin, and moesin
ETC	electron transport chain
GAL4	galactosidase 4
GDP	guanosine diphosphate
GH	growth hormone
GMR	glass multiple reporter
GOF	gain of function
GPe/GPi	globus pallidus externa/interna
GSH	glutathione and reduced glutathione
GSK-3β	glycogen synthase kinase 3 beta

GTP	guanosine-5'-triphosphate
GWAS	genome wide associated study
hLRRK2	human leucine-rich repeat kinase 2
HPLC	high performance liquid chromatography
Hsc70-5	heat shock protein cognate 5
HSPA9	heat shock 70kDa protein 9 (mortalin)
i.p.	intraperitoneal
IBD	inflammatory bowel disease
IGF	insulin-like growth factor
IGF1	insulin growth factor 1
IGFALS	insulin-like growth factor binding protein, acid labile subunit protein
IHC	immunohistochemistry
iPD	idiopathic Parkinson's disease
iPSC	induced pluripotent stem cell
kd	knock down
KI	knock in
KO	knock out
L-Dopa	levodopa
LB	Lewy body
LC	locus coeruleus
LDH	lactate dehydrogenase
LN	Lewy neurites

LOF	loss of function
LRR	leucine-rich repeat
LRRK2	leucine-rich repeat kinase 2
LRRK2 KI	R1441C Lrrk2 knock in
LRRK2 KO	Lrrk2 knock out
MAO-B	monoamine oxidase B
MAO-I	monoamine oxidase inhibitors
Mdr50	multi drug resistance 50 (p-glycoprotein)
MFHAS1	malignant fibrous histiocytoma amplified sequence 1
Mfn1/2	mitofusin 1/2
Mg^{2+}	magnesium
MLK	mixed-lineage kinase
MMP	mitochondrial membrane potential
MPP^{+}	1-methyl-4-phenylpyridinium
MPPP	1-methyl-4-phenyl-4-propionoxypiperidine
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
MPTP-(HCl)	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (hydrochloride)
mRNA	messenger ribonucleic acid
mtUPR	mitochondrial unfolded protein response
n.s.	non significant
N/A	not applicable
NA	noradrenaline
NBM	nucleus basalis of Meynert

NC	not completed
ND	no difference
NF-κB	nuclear factor kappa B
NFAT	nuclear factor of activated T-cells
NFT	neurofibrillary tangles
NIH	National Institutes of Health
NIMH	National Institutes of Mental Health
OCD	obsessive compulsive disorders
OMM	outer mitochondrial membrane
Opa1	optic atrophy 1
PBS	phosphate buffer solution
PD	Parkinson's disease
PDF	Parkinson’s Disease Foundation
PET	positron emission topography
PFA	paraformaldehyde
PHAC	Public Health Agency of Canada
PINK1	PTEN-induced kinase 1
PSC	Parkinson Society Canada
QoL	quality of life
qRT-PCR	quantitative real-time polymerase chain reaction
RBD	REM sleep behaviour disorder
REM	rapid eye movement
RG-hLRRK2	R1441G-human leucine-rich repeat kinase 2

RIPK	receptor interacting protein kinases
RN	raphe nuclei
RNAi	ribonucleic acid interference
ROC	ras of complex
ROCO	ROC-COR
ROS	reactive oxygen species
S	suppression
SEM	scanning electron microscopy
SEM	standard error of the mean
SNc	substantia nigra *pars compacta*
SNCA	alpha-synuclein
SNr	sustantia nigra *pars reticulata*
SOD	superoxide dismutase
STN	subthalamic nucleus
TBI	traumatic brain injury
Tg	transgenic
$TH^{(+)}$	tyrosine hydroxylase (positive)
TM6B	tubby humeral balancer
TOM-20	translocase of outer membrane 20
UAS	upstream activating sequence
UPDRS	Unified Parkinson's Disease Rating Scale
VPS35	vacuolar sorting protein 35
VTA	ventral tegmental area

WT	wild type
WT-hLRRK2	wild type human leucine-rich repeat kinase 2
YLD	years lost as a result of a disability

Chapter 1. General Introduction

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1.1. An Introduction to Parkinson's Disease

Parkinson's disease (PD) was named after James Parkinson, the physician who provided one of the first clear descriptions of the disorder by carefully documenting the disease in *An Essay on the Shaking Palsy* – or *Paralysis Agitans*, back in 1817 (Parkinson, 2002). Jean-Martin Charcot, who later renamed the condition "Parkinson's disease," and those who followed, went on to note the distinguishing clinical characteristics of the condition and its vast, at times overlooked, complexity (reviewed in (Goetz, 2011).

Parkinson's disease is a central nervous system (CNS) disorder that currently afflicts ~1% of the population over the age of 60 and <5% of those over the age of 85 (reviewed in (de Lau and Breteler, 2006)). It is the second most common movement disorder and the second most common neurodegenerative disorder, following Alzheimer's disease (AD) (Adams et al., 2000). Although predominantly characterized by its motor symptoms, there are accompanying non-motor features that when combined provide a complete depiction of the disease (reviewed in (Chaudhuri et al., 2006)). The "classical" clinical and histopathological descriptions of the disease are outlined below; however, it should be noted that atypical forms of PD or *parkinsonism* exist (described in (Lang and Lozano, 1998b, a)). PD itself is not lethal, but renders an individual chronically debilitated. The leading causes of death in patients diagnosed with PD are: pneumonia, cancer and cardiovascular disease (Beyer et al., 2001; D'Amelio et al., 2006; Iwasaki et al., 1990). With the current absence of diagnostic tests and biomarkers, a

suspected clinical diagnosis of PD is validated post-*mortem* - following an autopsy (discussed in (Gelb et al., 1999)).

1.2. Clinical Manifestations of Parkinson's Disease

1.2.1. Primary, Motor-related Symptomology in Parkinson's Disease

PD is clinically diagnosed when either muscular rigidity and/or resting tremor accompany bradykinesia (slowness of movement) (outlined in (Postuma et al., 2015)). A progression in disease severity is usually marked by the change from a unilateral phenotype, present in early stages and often at diagnosis, to a more complex and severe bilateral impairment (Goetz et al., 2008). Some symptoms are commonly perceived to be part of the disease, but are in fact the side effect of treatment, such as L-dopa-induced dyskinesia (Postuma et al., 2015). The severity of the clinical staging is described in the context of the Unified Parkinson's Disease Rating Scale (UPDRS), which has evolved over time and was recently modified to include assessment and scoring of the non-motor symptoms of disease (Goetz et al., 2008).

1.2.2. Non-motor Related Symptomology in Parkinson's Disease

There are wide ranges of non-motor symptoms that commonly accompany the primary, motor symptoms in PD (reviewed in (Chaudhuri et al., 2006)). These symptoms include, but are not limited to: depression, dementia, psychosis, hallucinations (audio and visual), obsessive compulsive disorders (OCD), impairments in gastrointestinal motility, olfactory deficits, orthostatic hypotension, sleep apnea, rapid eye movement (REM) sleep behaviour disorder (RBD), aphagia, and pain among others (reviewed in (Poewe, 2008)).

These impairments are present in the prodromal (preclinical) phases of the disease (Berg et al., 2015). They have been reported to predate the motor symptoms for up to three decades prior to clinical presentation (Schenck et al., 2013; Schenck et al., 1996). These non-motor symptoms drastically affect the severity of the disease by increasing its complexity, which impacts the patient's quality of life (QoL), and the clinical treatment course (reviewed in (Chaudhuri and Schapira, 2009)).

1.2.3. Therapeutic Strategies for the Treatment of Parkinson's Disease

Current therapeutic options target symptom alleviation, primarily of the motor-related impairments. The gold-standard of treatment to this day remains the replenishment of reduced striatal dopamine levels via administration of its precursor, levodopa (L-dopa), which was discovered over 50 years ago (reviewed in (Lees, 1986)). Alternative treatments include administration of dopamine mimetic compounds (dopamine receptor agonists) and compounds that reduced dopamine turnover and metabolism (reviewed in (Connolly and Lang, 2014)). Other non-pharmacological forms of therapy are effective for certain symptoms as well, such as, physiotherapy and speech therapy, but remain under-utilized (Keus et al., 2009; Nijkrake et al., 2009). In severely advanced cases, alternative strategies are possible for a select group of candidates, such as surgical lesions (pallidotomy, thalamotomy) or deep brain stimulation (DBS) therapy (discussed in (Lang and Lozano, 1998b, a) (Bronstein et al., 2011). Exploration of additional alternative avenues, stem cell therapy and vaccines (NCT01885494), remain under investigation (Fu et al., 2015), (NIH, 2015). Unfortunately, all of the current

therapeutic options are unable to slow, halt, alter or reverse the disease progression and trajectory.

1.2.4. Histopathology of Parkinson's Disease

Traditionally, the hallmark pathological findings for a confirmed positive diagnosis at autopsy includes two key features: 1) the loss of the neuromelanin pigmented dopaminergic neurons of the substantia nigra *pars compacta* (SNc) and 2) the presence of proteinaceous inclusions, Lewy bodies (LB), which are found in surviving neurons (Kalia and Lang, 2015). The deficits in voluntary motor initiation are caused by the signaling impairments in the basal ganglia network circuitry as a result of the loss of dopamine released from the degenerating dopaminergic neurons of the SNc area (Cotzias et al., 1967; Sourkes and Poirier, 1965). These dopaminergic (DA) neurons project to the striatum - caudate nucleus and putamen, which signal via direct and indirect pathways the coordinated execution of desired motor movement to the thalamus, then to the motor cortex before transmitting down the brain stem to the body. The lack of striatal dopamine results in a dampened thalamic signal and a subsequent reduced activation of the motor cortex leading to decreased movement output, as illustrated in the schematic in (Figure 1.1) (discussed in (Lanciego, 2012)). Unfortunately, it appears that by the time an individual presents to the clinic over 50% of the dopaminergic producing neurons of the SNc are lost (Agid, 1991). This highlights the urgency to accurately identify prodromal disease states and the development of early stage therapeutic interventions.

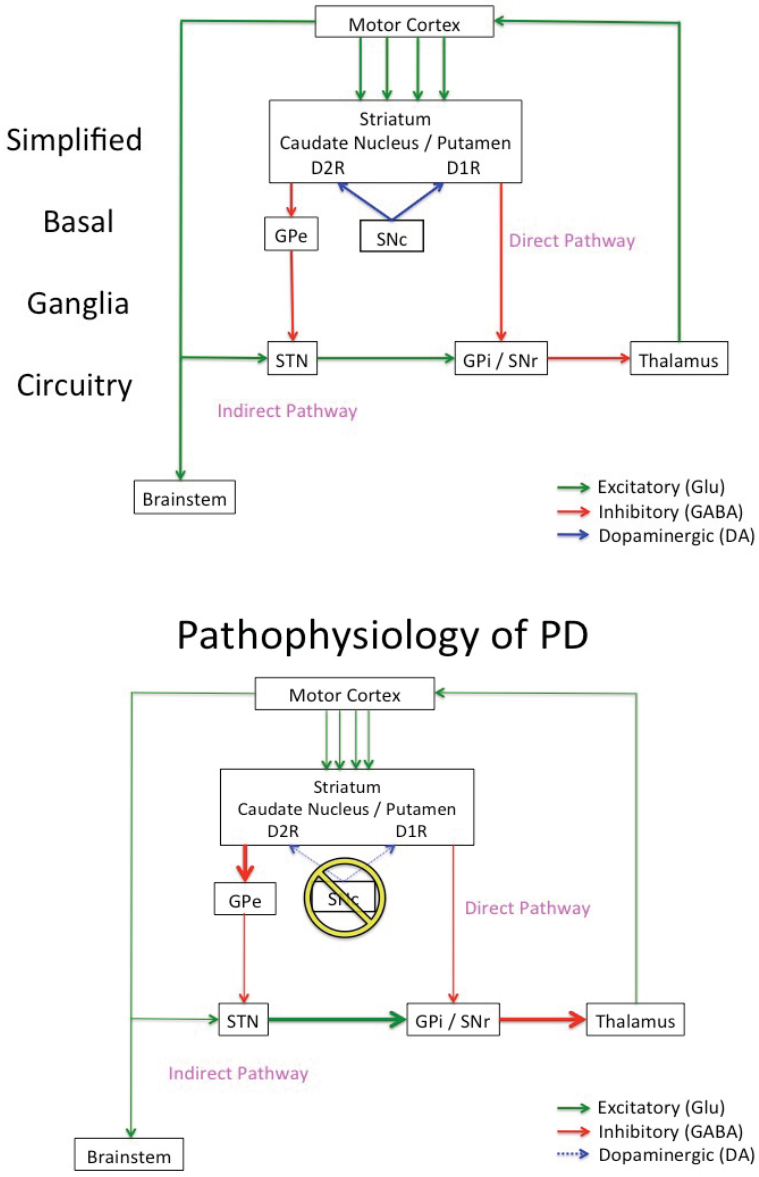

Figure 1.1. Basal ganglia circuitry in healthy and Parkinson's disease states.

Schematic representation of the basal ganglia circuitry in healthy (*top panel*) versus Parkinson's disease pathological conditions (*bottom panel*). Note that the counteracting balancing mechanism mediated by the direct and indirect pathway is lost, resulting in a sustained dampening of the thalamic output to activate the motor cortex. SNc – substantia nigra *pars compacta*, GPe/GPi – globus pallidus externa/interna, STN – subthalamic nucleus, SNr – substantia nigra *pars reticulata*, D1/2R – Dopamine 1 or 2 Receptor.

Analysis of post-*mortem* brain tissue from PD cases, versus healthy age-matched controls, has revealed the robust degeneration of the SNc dopaminergic neurons in PD, while separate dopaminergic clusters, such as the ventral tegmental area (VTA), demonstrate a reduced extent of neuronal loss (discussed in (Perl, 2011)). However, the dopaminergic neuron clusters are not the only regions in the brain that degenerate or have altered physiology. Common regions affected include the locus coeruleus (LC), the raphe nuclei (RN), and the nucleus basalis of Meynert (NBM), which are noradrenergic (NA), serotonergic (5-HT) and acetylcholinergic (ACh) producing regions of the brain, respectively. Degeneration of these nuclei are likely responsible for many of the non-motor symptoms of disease and have also been targeted for the treatment of PD ((Gaspar and Gray, 1984); reviewed in (Dickson, 2012)).

In addition to cell death, there are pathological markers of cellular dysfunction, which are found in surviving neurons throughout the CNS. This phenomenon manifests itself in the form of spherical LB or the string-like Lewy neurites (LN) that are scattered throughout the CNS (reviewed in – (Shults, 2006)). These LB/LNs are aggregates composed mainly of α-synuclein and other ubiquitinated protein products (Goedert et al., 2013; Spillantini et al., 1998). Tau-positive protein aggregate inclusions, such as neurofibrillary tangles (NFT), have also been observed in PD (Gaspar and Gray, 1984; Irwin et al., 2012). These broad-range, protein aggregate-related pathologies (synucleinopathies and tauopathies) lend support to the notion that neurological disorders can at times overlap.

Based on the observations from post-mortem pathological specimens, the relationship between neurological pathology and disease progression was explored by

Braak and colleagues (Braak et al., 2003). According to the Braak Staging Hypothesis of PD, classic LB body pathology is detected early in disease stages and in non-centralized areas of the body (Braak et al., 2003). Cross-referencing pathology to clinical disease progression indicates that pathology travels centrally in a retrograde fashion, since it appears to progress from zones that are at the interface of the body and the environment, such as those of the olfactory bulb and gastrointestinal system (Braak et al., 2003). From there it progresses to the midbrain region and continues to spread up into the higher order cortical structures of the brain (Braak et al., 2002; Braak et al., 2003). Based on these correlative observations, *Braak* and colleagues developed the "Dual Hit hypothesis" which proposes that not yet identified harmful agents enter the system directly via the nasal passages (olfactory system) or are ingested, either directly or indirectly, and enter via the gastrointestinal tract (enteric system) (Hawkes et al., 2007). The nasal cavity provides multiple CNS entry sites: diffusion through the epithelium and rapid uptake into the blood stream, direct passage via uptake by the olfactory receptor cells, or via transport by the trigeminal nerve (Doty, 2008). The gastrointestinal system provides a route of entry into the CNS by breaching of the gut mucosal lining, circulatory uptake and retrograde transport up the vagus nerve and into the CNS (Hawkes et al., 2007), described in (Bergin and Witzmann, 2013).

1.3. Societal and Economic Impact of Parkinson's Disease

The impact of PD on an individual, a family network, and society can be drastic. There are an estimated six to ten million PD cases worldwide and although certain areas and cultures appear to display an increased frequency, its occurrence is generally

widespread and nondiscriminatory (EPDA, 2016; PDF, 2016) (Muangpaisan et al., 2011; Pringsheim et al., 2014). In Canada, there are an estimated 100,000 individuals living with PD; this value is reflective of diagnosed cases, and is thought to be an underestimate due to the presumable large number of un- or misdiagnosed individuals (PSC, 2003). The burden of PD on the community is primarily in the form of impact to quality of life (QoL) for the patient, caregiver(s) and family; for society it is years of lost productivity, or years lost as a result of a disability (yld), and the associated healthcare costs (WHO, 2006). Although economics is not the primary concern of healthcare providers and researchers, it provides a quantitative measure of disease impact. The estimated Canadian healthcare costs associated with PD, according to the Public Health Agency of Canada (PHAC), were $446.8M in 2000-2001 (PSC, 2003). Unfortunately, the number of PD cases globally is predicted to double by 2030 with the ageing population (Dorsey et al., 2007). The burden of neurological disorders will be staggering, given the current age demographics (Andreev et al., 2013). Therefore, understanding disease origin and progression is critical for the development of cause directed therapies, disease prevention and management of care.

1.4. Understanding the Etiology of Parkinson's Disease

1.4.1. Etiology of Parkinson's Disease – Risk Factors

Unfortunately, the etiology (cause) of PD is largely unknown. The identification of a clear, disease-causing agent is lacking in the majority of cases; therefore classical PD is often referred to as idiopathic, meaning of unknown origin, and is denoted as "iPD."

This is not to be confused with sporadic PD, which technically refers to the irregular frequency or unpredictability of occurrence. Therefore, PD cases can be both sporadic and idiopathic in nature, or the two can remain mutually exclusive. Accordingly, PD is believed to be a "complex disease," wherein a combination of genetic risk factor(s) and environmental trigger(s) are needed to manifest disease (discussed in (Kitada et al., 2012)). In fact, there is a subset of cases (~10%) that are linked to genetic factors (i.e. have been identified to segregate in family pedigrees) and/or have been detected in genome wide association studies (GWAS) (reviewed in (Klein and Westenberger, 2012; Trinh and Farrer, 2013)). The genetic causes of PD will be discussed below.

The lack of a clear cause in the majority of PD cases makes studying the disease exceptionally difficult. Basic scientific research attempts to replicate the human condition in model systems and the majority of models are based on our current understanding of PD-related risk factors and genetics. There are certain risk factors that correlate with disease manifestation. The strongest risk factor known is the ageing process; wherein on average 41 in every 100,000 individuals aged 40-49 have PD and this steadily increases to 1903 in individuals 80 years of age and older (Pringsheim et al., 2014). Other associated risk factors reported include: sex, as the average weighted male to female ratio of PD has been reported to range between ~1.50-2.0 (Taylor et al., 2007; Van Den Eeden et al., 2003; Wooten et al., 2004); exposure to: pesticides (Ascherio et al., 2006; Wan and Lin, 2015), chemical compounds/solvents (Goldman et al., 2012), high levels of certain heavy metals (Gorell et al., 1999; Guilarte, 2010); and head trauma, specifically traumatic brain injury (TBI) (Gardner et al., 2015), among others. Drug-induced Parkinsonism (DIP) - as a result of certain prescribed medications (neuroleptic antipsychotics and

others) is reported to be the second most common cause of parkinsonism (Barbosa et al., 2006; Benito-Leon et al., 2003; Errea-Abad et al., 1998; Hall et al., 1956; Vazquez-Alen et al., 2000; Wenning et al., 2005) reviewed in (Lopez-Sendon et al., 2013). Conversely, a few studies have demonstrated that certain factors are correlated with a negative risk of developing PD, such as: smoking (Hernan et al., 2002) and caffeine intake (Liu et al., 2012). Although the definite cause(s) of PD remain largely unknown, hypothesis can be *proposed* based on pathological, epidemiological and correlative data. However, one thing remains clear; PD manifestation is complex.

1.4.2. Etiology of Parkinson's Disease – Environmental Risk Factors

Although a variety of factors have been correlated with an increased risk of developing PD, I was particularly interested in the environmental factors, such as exposure to pesticides and herbicides. Early studies demonstrated that incidence of PD overlapped with "market farming" geographic regions, which suggested a correlation with environmental toxins (Barbeau et al., 1987). Numerous studies have explored this topic and have report positive data for single compounds (paraquat) (Wan and Lin, 2015), compounding evidence (paraquat + maneb) (Costello et al., 2009), or no correlation to PD risk (discussed in (Wirdefeldt et al., 2011)). Although there is epidemiological data to support this hypothesis (reviewed in (Kamel, 2013)), the discovery of the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) compound provided convincing evidence that parkinsonism could be caused by an exogenous toxic insult (Langston et al., 1983). MPTP itself is not a pesticide/herbicide; however, its active metabolite 1-methyl-4-phenylpyridinium (MPP^+) was used as an herbicide and was sold under the commercial

name Cyperquat (CID:39484) (NCBI, 2016). With the identification of the MPTP/MPP^+ neurotoxin, scientists searched for toxic candidates that had similar structure and mechanism of action. Many of these compounds are the basis of the toxin-induced models of parkinsonism and will be discussed in the toxin-induced models of parkinsonism section.

1.4.3. Genetic Forms of Parkinson's Disease

1.4.3.1. General Introduction to Parkinson's-linked Genetics

Early studies rejected the hypothesis that PD had genetic or familial forms largely due to the discordant results obtained from early monozygotic twin studies (Duvoisin et al., 1981; Gudmundsson, 1967; Pembrey, 1972). A few early studies provided evidence of heredity and concordance, however, the small sample size or presence of non-*classical* descriptions of the disease made the findings unconvincing to the field (Kissel and Andre, 1976). It was not until 1997 that this viewpoint changed with the discovery of an Italian kindred with linkage to mutations in the *SNCA* gene (Polymeropoulos et al., 1997). To date there are approximately 18 loci that are generally accepted to be associated with the development of PD, and of those loci there are 6 genes (summarized in Table 1.1.) that have been directly linked to the development of PD (reviewed in (Klein and Westenberger, 2012)). There are a limited number of pure monogenic (100% penetrant) forms, but these make up a minor (3-5%) percentage of the entire population and ~30% of the genetically inherited or "familial" forms of PD, as presented in (Klein and Westenberger, 2012).

Table 1.1. General summary and comparison of the 6 confirmed genes linked to PD.

Gene / Locus	Inheritance	Age of Onset	Gene Alteration	Protein Function
SNCA (PARK1/4)	Autosomal Dominant (AD)	Early Onset	Missense Mutations / Multiplications	Synaptic Transmission
LRRK2 (PARK8)	Autosomal Dominant (AD)	Late Onset	Missense Mutations	Various Functions Proposed (*Please refer to Section 1.5*)
VPS35 (PARK17)	Autosomal Dominant (AD)	Late Onset	Missense Mutations	Retromer Complex, Vesicular Sorting
PARKIN (PARK2)	Autosomal Recessive (AR)	Early Onset	Missense Mutations	E3 Ligase – Protein Degradation
PINK1 (PARK6)	Autosomal Recessive (AR)	Early Onset	Missense Mutations / Truncations	Mitochondrial Function
DJ-1 (PARK7)	Autosomal Recessive (AR)	Early Onset	Missense / Truncations	Mitochondrial, Chaperone Function and Oxidative Stress

1.4.3.2. Autosomal Recessive (Monogenic) Forms of Parkinson's Disease

Of the autosomal recessive genes, the most commonly studied are those bearing mutations in the: Parkin (Park2) (Kitada et al., 1998), Pink1 (Park6) (Valente et al., 2004) and DJ-1 (Park7) (Bonifati et al., 2003) genes. These penetrant monogenic cases typically present in the form of early-onset parkinsonism, appear relatively motor-dominant and at the pathological level, are reported to display nigral degeneration (overviewed in (Doherty and Hardy, 2013)). LB pathology has been reported in Pink1-linked cases and has also been noted in Parkin-linked PD, with the latter commonly reported as rare (Doherty and Hardy, 2013; Samaranch et al., 2010). To date, there have been no reported cases of DJ-1-linked disease pathology, which have come to autopsy. Mutations in these genes produce loss of function phenotypes (reviewed in (Klein and Westenberger, 2012)). Thereby the majority of data for biological function has been derived from gene knock down (kd) or knock out (KO) models versus wild-type (WT) protein, and are supplemented with ectopic expression of pathogenic mutant forms (Dave et al., 2014; Haque et al., 2012; Itier et al., 2003; Kim et al., 2005a; Kitada et al., 2007). All three proteins appear to mediate neuroprotection and share a common biological function related to mitochondrial function and quality control (Bouman et al., 2011; Clark et al., 2006; Greene et al., 2012; Haque et al., 2012; Irrcher et al., 2010; Joselin et al., 2012; Narendra et al., 2008; Narendra et al., 2010; Parsanejad et al., 2014b; Poole et al., 2008). However, it should be noted that additional non-mitochondrial functions have been proposed for some genes as well (Aleyasin et al., 2010).

1.4.3.3. Autosomal Dominant Forms of Parkinson's Disease

Of the autosomal dominant forms of PD, there are genes that have been linked to disease pathogenesis: the α-synuclein (*SNCA*) gene (Polymeropoulos et al., 1997); the Leucine-Rich Repeat Kinase 2 (*LRRK2*) gene (Paisan-Ruiz et al., 2004; Zimprich et al., 2004); and more recently the Vacuolar Sorting Protein 35 (*VPS35*) gene (Vilarino-Guell et al., 2011; Zimprich et al., 2011). As LRRK2 is the focus of this dissertation, it will be covered in more detail in its own section (below). With regards to *VPS35*, it has been included here since a genetic interplay between it and LRRK2 has been published (Linhart et al., 2014). A brief overview of *SNCA* is provided due to its inherent pathological link to PD and its proposed correlation with LRRK2 (Lin et al., 2009).

A mutation in the *SNCA* gene (OMIM:163890) was the first identified genetic cause of PD (Polymeropoulos et al., 1997). PD cases that are linked to *SNCA* mutations include point mutations and gene multiplications (reviewed in (Petrucci et al., 2016)). The *SNCA* gene product, α-synuclein, is the major constituent of LBs (Spillantini et al., 1998). Thereby, although the genetic *SNCA* PD-linked cases are relatively rare, α-synuclein is classically dysregulated in iPD; whether causative or consequential remains unknown. Understanding how and why the native protein takes on a toxic nature is not fully understood. α-Synuclein is prone to misfolding, transitioning from its native monomeric state to a presumed toxic oligomeric and subsequent fibril form (reviewed in (Lashuel et al., 2013)). It is the fibril species that is found in LB (Spillantini et al., 1998; Vilar et al., 2008). As such, it is debated whether the formation of the LB is an attempt to scavenge toxic material and promote neuroprotection (Schulz-Schaeffer, 2015).

1.5. *Leucine-Rich Repeat Kinase 2* (*LRRK2*) in Parkinson's Disease

1.5.1. Clinical *LRRK2*-linked Parkinson's Disease

The complexity of PD disease etiology is illustrated in cases involving mutations in the *Leucine-Rich Repeat Kinase 2* (*LRRK2*) gene (OMIM: 609007). In 2002, the PARK8 locus was identified to be the region linking a familial form of PD to a family in the Japanese population (Funayama et al., 2002). Mutations in this gene are inherited in an autosomal dominant fashion (Funayama et al., 2002; Paisan-Ruiz et al., 2004; Zimprich et al., 2004). PARK8 was described as having an incomplete penetrance and the authors suggested that additional genetic or environmental contributions might contribute to the development of PD in this family (Funayama et al., 2002). The *LRRK2* gene was later identified as the culprit in the PARK8 locus in separate families (Paisan-Ruiz et al., 2004; Zimprich et al., 2004). Since then, *LRRK2* mutations have been associated with both familial and sporadic PD cases (Gilks et al., 2005; Nichols et al., 2005). It is currently considered the most common cause of genetic PD and its frequency is exceptionally high (≤40%) in specific ethnic populations (Hassin-Baer et al., 2009; Healy et al., 2008; Lesage et al., 2005).

LRRK2-linked PD cases typically display as a late onset, slow-progressive form of PD with a reported incomplete (~30%) lifetime penetrance (Hernandez et al., 2005; Ozelius et al., 2006). However, there is an age-dependent increase in penetrance associate with *LRRK2*, reaching up to 74% at age 79 for the G2019S mutation and a reported 83.4% at 80 years for the R1441G mutation (Healy et al., 2008; Ruiz-Martinez et al., 2010). Interestingly, there are reported cases of G2019S carriers that at advanced ages

(89 and 91) did not present with any clinical indications of disease (Gaig et al., 2006; Kay et al., 2005). Generally, *LRRK2*-linked PD's average age of onset has been described as late (~60 years) (Hassin-Baer et al., 2009; Ruiz-Martinez et al., 2010). However, younger ages of onset have been reported (Nuytemans et al., 2008). *LRRK2*-linked PD is strikingly similar to clinical idiopathic PD (Hulihan et al., 2008). It also displays a neurochemical profile (PET scans) similar to iPD (Adams et al., 2005). Symptomatically, dementia was noted as uncommon in *LRRK2*-linked PD cases (Saunders-Pullman et al., 2006). Patients have been reported to present with early lower limb tremor and generally have good initial L-dopa treatment responsiveness (Alcalay et al., 2013; Hernandez et al., 2005). However, at the histopathological level *LRRK2* becomes increasingly more complex (Alcalay et al., 2013). Overall, there is typical pathology: neuronal loss (SNc and LC) and LB pathology; however, there is considerable heterogeneity reported within histopathological profiles (Ross et al., 2006; Zimprich et al., 2004). For example 4 members of one pedigree carrying the same mutation displayed: nigral loss, LB and LN pathology; nigral loss tau and ubiquitin inclusions; nigral loss, LB a NFT; and the fourth displayed purely nigral degeneration (Wszolek et al., 2004). Due to the frequency and clinical similarities between *LRRK2*-linked and iPD it has become a central figure in PD research.

1.5.2. *LRRK2* - The Gene

The human *LRRK2* gene (OMIM:609007) is found on chromosome 12 (12p11.23-q13.1) (Funayama et al., 2002). The gene spans a 144kb region that consists of 51 exons, producing a 9kb transcript encoding a 2527 amino acid protein that travels at

approximately 280 kDa (West et al., 2005; Zimprich et al., 2004). This highly complex protein contains multiple domains including: a leucine-rich repeat (LRR) domain, a GTPase consisting of ras of complex (ROC) and c-terminal of ROC (COR) domains, a kinase domain resembling the mixed-lineage kinase (MLK) and more recently - receptor interacting protein kinases (RIPK), and a WD-40 domain (West et al., 2005). In addition, there are armadillo and ankyrin domains at the N-terminus of mammalian *LRRK2* (Marin, 2008). *LRRK2* homologues exist across a broad spectrum of species (Marin, 2006, 2008). There is a single Drosophila Lrrk (dLrrk) homologue (CG5483) in the invertebrate (*D. melanogaster*) system (Attrill et al., 2016). Comparatively, both LRRK2 and a LRRK1 paralogue are present in mammalian systems (*M. musculus and H. sapiens*) (Marin, 2006). For the purpose of this dissertation, the focus will remain on the aforementioned species.

1.5.3. *LRRK2*-linked Pathogenicity

There are a total of 6 missense mutations that meet pathogenic criteria and are linked to PD: the common G2019S (Di Fonzo et al., 2005; Ferreira et al., 2007; Kachergus et al., 2005; Nichols et al., 2005; Zabetian et al., 2005) and its neighbouring I2020T (Zimprich et al., 2004) in the kinase domain; the R1441 site which has been linked to multiple substitutions C/G/H (Ferreira et al., 2007; Mata et al., 2005a; Mata et al., 2005b; Ross et al., 2009; Spanaki et al., 2006; Zabetian et al., 2005; Zimprich et al., 2004); and the Y1699C mutation (Khan et al., 2005; Zimprich et al., 2004). An additional N1437H mutation has been identified, although its independent replication remains to be reported (Aasly et al., 2010). There are mutations (G2385R, R1628P) that have been

noted to increase risk susceptibility, albeit at times restricted within certain geographical regions (Di Fonzo et al., 2006; Ross et al., 2008; Tan et al., 2007; Yu et al., 2009). Notably, mutations in *LRRK2* have also been implicated as a susceptibility gene in both Crohn's disease and leprosy (Barrett et al., 2008; Franke et al., 2010; Wang et al., 2015; Zhang et al., 2009). As the pathogenic G2019S has been reported to be the most common mutation, much of the data generated focuses on carriers and analysis of this specific mutation (Healy et al., 2008).

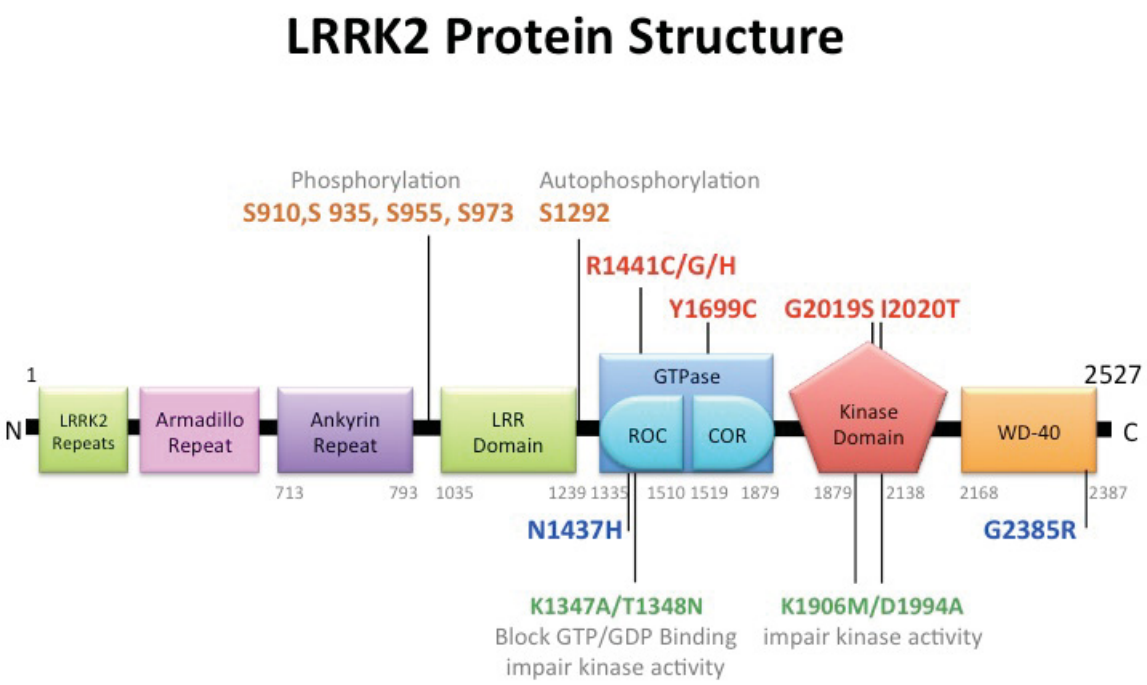

Figure 1.2. Schematic illustration of the LRRK2 protein structure

This schematic illustrates the large and complex, multi-domain structure of the LRRK2 protein. Bearing two catalytic domains (GTPase and kinase) and numerous protein-protein interaction domains (LRR, WD-40, Ankyrin and Armadillo regions), this structure highlights the inherent complexity in fully delineating the biological(s) role of LRRK2. Pathogenic substitutions are indicated in red and risk variants in blue.

1.5.4. LRRK2 Tissue Expression Profile

Based on assessment of human tissue, evidence of *LRRK2* mRNA has been detected in various organs, but at higher levels in lungs and heart versus brain (Zimprich et al., 2004). In the brain, its expression is more pronounced in areas such as the cortex, hippocampus and striatum (Biskup et al., 2006; Han et al., 2008; West et al., 2014; Zimprich et al., 2004). LRRK2 expression is reported as either absent or with low signal in human SNc tissue (Galter et al., 2006). Within animal studies, SNc Lrrk2 expression has been reported as low (qRT-PCR) or absent (*in situ* hybridization) (Melrose et al., 2006). There are reports indicating Lrrk2 midbrain detection, with higher SNc expression compared to the SN *pars reticulata* (SNr) and ventral tegmental area (VTA) (Han et al., 2008; Simon-Sanchez et al., 2006). Using animal tissue analysis, *LRRK2* mRNA is detected as early as embryonic gestational day 11.5 (E11.5), and transcript and protein expression gradually increase with development and continue to peak post-natal (Biskup et al., 2007). Post-natal mRNA expression profiles are high in the kidney, lung, heart and whole brain (Biskup et al., 2007). In adult tissue, this mRNA expression profile is maintained (Biskup et al., 2007). However, one report indicated that with age, there is a steady decrease in the levels of Lrrk2 mRNA in the spleen, whereas other organ profiles assessed were unaltered (Maekawa et al., 2010). Interestingly, there is both complementary and partial redundancy in mammalian tissue expression between *LRRK2* and its homologue *LRRK1* (Biskup et al., 2007; Westerlund et al., 2008).

1.5.5. LRRK2 Protein Structure

LRRK2 is from a family of proteins known as the ROCO family whose members include LRRK1 and LRRK2, death-associated protein kinase 1 (DAPK1), and malignant fibrous histiocytoma amplified sequence 1 (MFHAS1) ((Marin et al., 2008)). The characteristic of the ROCO family is the ROC-COR GTPase that is composed of a ras of complex (ROC) domain and a c-terminal of ROC (COR) domain (Bosgraaf and Van Haastert, 2003). The numerous protein-protein interaction domains suggest that LRRK2 has the potential to bind and interact with various partners. Since LRRK2 contains dual catalytic domains (GTPase and kinase) and various protein-protein interaction domains, an assembly or scaffolding role has been proposed (Cookson, 2010). Additionally, LRRK2 can exist as a monomer and a homo or heterodimer (with LRRK1), thus increasing the magnitude of the structure and its corresponding complexity (Klein et al., 2009).

1.5.6. LRRK2 Biology and Protein Function

The combination of protein interaction domains, a GTPase and a kinase, within one protein structure may be an evolutionary method of hosting key regulation factors in concert, rather than requiring individual proteins to perform a task (discussed in (Li and Beal, 2005)). What we discern about the protein's interaction and catalytic entity will shed light on the function of this multi-domain structure. Looking first at the GTPase domain, its ability to bind both GDP and GTP has been demonstrated, and it is reported to do so with equal affinity (Ito et al., 2007). Pathogenic mutations do not appear to affect GDP/GTP affinity and binding, however, mutations such as the R1441C/G and the

Y1699C have been reported to reduce GTP hydrolysis rates (Daniels et al., 2011; Lewis et al., 2007; Li et al., 2007). This is believed to mean that the protein remains in an active state longer than usual (reviewed in (Cookson, 2015; Taymans, 2012)). It was originally proposed that dimerization is destabilized by the R1441C mutation; however, more recently it appears that the COR domain is critical for dimer interaction (Daniels et al., 2011).

The second catalytic domain is the serine/threonine kinase domain, which is predicted to be related to the mixed lineage kinase (MLK) family and receptor-interacting protein kinases (RIPK) (Bae and Lee, 2015; West et al., 2005). This domain has garnered much attention in the field, likely due to the commonality of the G2019S mutation and the urgency to find an exploitable therapeutic target (such as modulating kinase activities) (Sridhar et al., 2000). The G2019S mutation consistently demonstrates increased kinase activity, while others report negative (Y1699C) or inconsistent (R1441C/I2020T) results (Gloeckner et al., 2006; Greggio et al., 2006; Jaleel et al., 2007; West et al., 2007). The G2019S mutation is predicted to be in the magnesium (Mg^{2+}) binding site of the activation loop that is required for ATP hydrolysis. This mutation is predicted to unhinge the loop, changing its conformation so that it is constitutively active or accessible by substrates, and hence results in a hyper-kinase activity (West et al., 2005). As a result of this hyper-kinase function, the field generally assumes that this pathogenic phenotype is linked to a toxic gain of function (GOF) behaviour (West et al., 2005). In addition to targeted phosphorylation at specific sites, such as S910 and S935, LRRK2 demonstrates a capacity for autophosphorylation at various proposed sites, with Ser1292 detected *in vivo*

(Dzamko et al., 2010; Dzamko et al., 2012; Gloeckner et al., 2010; Greggio et al., 2009; Kamikawaji et al., 2009; Sheng et al., 2012; Webber et al., 2011).

Importantly, there is a debate in the field over whether the undisputed (G2019S) hyper-kinase phenotype translates to a protein GOF. However, data generated from Lrrk2 KO mice demonstrate that in the kidney, where Lrrk2 expression is high and levels of Lrrk1 are low, there are bi-phasic, age-dependent alterations in autophagy resulting in the accumulation of α-synuclein pathology and cell death (Tong et al., 2012; Tong et al., 2010). Renal dysfunction has also been described in the Lrrk2 KO rats, although in a different capacity (Ness et al., 2013). However, incidence of kidney disease in PD patients has not been reported. There are indications of lung problems (respiratory infections) in PD patients, typically believed to be a result of sedentary lifestyle that may in fact have an underlying disease-related etiology (Iwasaki et al., 1990; Mehanna and Jankovic, 2010). In addition, Lrrk2 KO models or systemic exposure to LRRK2 inhibitors has been reported to cause kidney and lung pathology, demonstrating that silencing or blocking LRRK2 kinase activity can be harmful (Fuji et al., 2015; Tong et al., 2010). Collectively, these findings support the notion that a LRRK2 LOF is toxic, and that perhaps mutations resemble LOF alterations or are dominant negative in nature (Adams et al., 2000).

The answer to the GOF/LOF debate lies in our ability to discern the true targets and outcome of kinase and GTPase activity, and more specifically, how they work in concert. There is evidence to suggest that the GTPase and kinase domains share a bi-directional relationship, and likely function in concert for the protein's catalytic output (Cookson, 2015; Liu et al., 2016). Specifically, reports indicate that the presence of the

ROC domain and C-terminal regions are required for kinase function (Jaleel et al., 2007), and that sites required for GDP/GTP binding (K1347 and T1348) are required for proper kinase function (Ito et al., 2007; Taymans et al., 2011). Similarly, there is an apparent relationship between kinase function (autophosphorylation) and the rate of GTP activity (hydrolysis) (Liu et al., 2014; Liu et al., 2016). Delineating catalytic synchrony and physiological targets, if present, is critical in our understanding of LRRK2 biological function.

1.5.7. LRRK2 Cellular Localization

At the subcellular level, LRRK2 is predominantly found in the cytosol (Smith et al., 2005). LRRK2 is reported to localize to various membranous and vesicular structures, such as: mitochondria, lysosomal and endosomal vesicles, endoplasmic reticulum, golgi apparatus and associated transport vesicles, and microtubule network-associated vesicles where is appears to associate with lipid rafts (Biskup et al., 2006; Hatano et al., 2007). The membrane associated LRRK2 maintains a dimeric form, which has been reported to coincide with increased catalytic activity (Berger et al., 2010; Sen et al., 2009).

1.5.8. LRRK2 Toxicity

Early publications reiterated a common theme; LRRK2 mutations confer deregulated kinase activity and result in neuronal toxicity (Gloeckner et al., 2006; Greggio et al., 2006; West et al., 2005), the formation of cytoplasmic and perinuclear inclusion bodies (Greggio et al., 2006) and shortened neurite process morphology

(MacLeod et al., 2006; Plowey et al., 2008). Although the notion that mutant LRRK2 induces cell death is widely accepted, the mechanism responsible remains at large. A few papers have made various conclusions as to the manner in which LRRK2 is mediating its cell death. One publication demonstrated that LRRK2 was mediating its cell toxicity primarily via apoptotic mechanisms (Iaccarino et al., 2007). Another indicated that the mechanism responsible for the shortened neurite phenotype observed in the LRRK2 expressing neurons was the result of the activation of the autophagic mechanisms (MacLeod et al., 2006; Plowey et al., 2008). This suggests that LRRK2 toxicity may be mediated by the autophagy programmed cell death pathway (Cherra and Chu, 2008). Interestingly, there is evidence that autophagy precedes apoptosis and sequentially contributes to cell death outcomes (Marino et al., 2014).

1.5.9. LRRK2 Pathophysiology and Interactions

Many publications have identified interactors and substrates of LRRK2, however, validation of a physiological and disease-relevant mechanism(s) of action remains lacking (reviewed in (Cookson, 2015; Taymans and Cookson, 2010)). A very brief overview of some of the main "umbrella" categorized cellular pathways of proposed LRRK2 function(s) and related interactors is included. Although presented categorically, certain functions and roles converge or overlap in some aspects. The goal is to highlight the vast array of data produced in the field. Notably, this list is not comprehensive – it is merely a partial reflection of the current literature reports.

1.5.9.1. Cytoskeletal and Microtubule-Related Dynamics and Trafficking

Among the proposed cellular roles of LRRK2 is its function in cytoskeletal related functions and dynamics, as supported by its phenotypic outcomes on neurite process morphology (Dachsel et al., 2010a; MacLeod et al., 2006; Plowey et al., 2008; Winner et al., 2011). This phenotype may be mediated by its reported association interaction and phosphorylation of certain proteins of the ERM family (Jaleel et al., 2007; Parisiadou et al., 2009). It is reported to interact with microtubule-related proteins, such as: β-tubulin (Gandhi et al., 2008; Gillardon, 2009; Law et al., 2014); it reportedly phosphorylates tubulin-associated Tau (Kawakami et al., 2012); increases levels of phosphorylated Tau, via GSK-3β (Kawakami et al., 2014); interacts with Wnt signalling (Berwick and Harvey, 2012; Sancho et al., 2009); and more recently interacts with PAK6 (Civiero et al., 2015) - all of which may affect neurite extension, vesicular transport and even cell migration (Caesar et al., 2013).

1.5.9.2. Axonal Transport, Vesicular Dynamics and Trafficking

Related to the material discussed above, this category covers a broad range of cellular functions and pathways, which all share the common aspect of shuttling membranous vesicles in the cell. There are clear indications of phenotypes, specifically at the synaptic terminals with, when modulating LRRK2 forms (Migheli et al., 2013; Miklavc et al., 2014; Piccoli et al., 2011). Cellular trafficking is thought to be a key role of LRRK2, as has been demonstrated in reports indicating interaction proteins such as: Rab5b (Heo et al., 2010; Shin et al., 2008; Yun et al., 2015) RAB7L1 (MacLeod et al., 2013); or with 14-3-3 proteins (Dzamko et al., 2010; Lavalley et al., 2016; Muda et al., 2014; Nichols et

al., 2010; Reyniers et al., 2014); and indirectly with VPS35 (Linhart et al., 2014). LRRK2 is reported to directly associate and bind to vesicles (Piccoli et al., 2014). Another type of membranous body that LRRK2 associates with is the mitochondria (West et al., 2005). Evidence suggests that it relates to an interaction between LRRK2 and dynamin-related proteins Drp1 (Niu et al., 2012; Su and Qi, 2013; Wang et al., 2012b) and Mfn1 among others (Stafa et al., 2014). This axonal and vesicular transport also likely has some effects on the autophagy phenotypes noted with LRRK2 (Orenstein et al., 2013; Tong et al., 2012; Tong et al., 2010).

1.5.9.3. Immune Related and Additional Functions

This is a more recent and emerging role for LRRK2 that is predicted to produce exciting data. This area of research is related to immune function in cells of both the central and peripheral systems (Gardet et al., 2010; Gillardon et al., 2012; Hakimi et al., 2011; Moehle et al., 2012). Currently, there are some indications of the involvement of master transcriptional regulatory pathways, such as reported with NFAT (Liu et al., 2011) and NF-κB (Russo et al., 2015), and an effect on COX-2 (Lopez de Maturana et al., 2014). LRRK2 has also been reported to have effects on development neurogenesis and differentiation capacities, wherein the G2019S mutation has negative effects on these hippocampal related processes (Winner et al., 2011).

1.6. Hypothesis of Parkinson's Disease-related Pathogenic Mechanisms

1.6.1. Proposed Hypotheses of Parkinson's Disease Pathogenesis

Based on the pathological data, several hypotheses have been proposed to explain the mechanism by which PD pathogenesis is occurring and/or is potentiated. The main hypotheses include the protein degradation hypothesis (reviewed in (Cook et al., 2012a)), the prion hypothesis (reviewed in (Chu and Kordower, 2015)), the inflammatory hypothesis (reviewed in (McGeer and McGeer, 2004)) and the oxidative stress hypothesis, discussed in more detail below. Although not all of the hypotheses are being presented in detail, one thing remains common among them - each hypothesis presents supporting evidence, yet not a single one successfully points to a direct cause or trigger mechanism of disease. However, the summation of evidence supports that PD has a complex disease etiology, wherein genetic factors may render individuals susceptible, but confounding environmental or exogenous factors are needed to manifest disease profiles (reviewed in (Kitada et al., 2012)). This concept will be central to the discussion moving forward.

1.6.2. Oxidative Stress Hypothesis of Parkinson's Disease

This hypothesis originates from the notion that naturally occurring cellular metabolism generates oxygen containing species that can produced free-radical species or reactive oxygen species (ROS); these species, when not managed properly, can cause cellular damage and contribute to cell death (review in (Fahn and Cohen, 1992)). However, exogenous factors can also influence internal oxidant status; this is of

particular relevance to the work presented in my thesis, given that the mitochondrial-targeting agent (MPTP/MPP^+) can induce oxidative stress (Mizuno et al., 1987; Nicklas et al., 1985). The results of dysregulated oxidative stress can present themselves in various forms, such as: 1) lipid alterations, which affect signaling cascades and membranes via alterations in: viscosity, thermodynamics, conductivity and phospholipid exchange; 2) protein and enzymatic alterations in the form of: activation/deactivation of signalling mechanisms (pro-survival or pro-apoptotic), inflammatory responses, and protein degradation pathways; and 3) via DNA damage which can result in: ageing, mutations, dysfunction, death and cancer (reviewed in (Lin and Beal, 2006; Uttara et al., 2009)). The evidence and sources from where these apparently toxic oxidants may be derived in the context of PD are described below.

1.6.2.1. Pathological Evidence:

Post-mortem analyses have provided evidence that the SNc has lower levels of antioxidant enzymes (glutathione and reduced glutathione (GSH)) when compared to other brain regions and that this reduction is exacerbated in PD samples (Perry et al., 1982; Perry and Yong, 1986). In addition, increases in the levels of superoxide dismutase (SOD), as well as increased levels of certain elements (iron) that can alter the redox status of cells, have been reported (Brion and Saffar, 1979; Saggu et al., 1989; Sofic et al., 1988). There is evidence of reduced complex I subunits of the electron transport chain (ETC) along with a corresponding reduction in complex I activity in the SNc region from PD samples (Mizuno et al., 1989; Schapira et al., 1990a; Schapira et al., 1989; Schapira et al., 1990b). Moreover, evidence of ROS-induced damage, in the form of lipid

peroxidation is reported in SNc tissue samples (Dexter et al., 1989). Together, these findings support a cellular environment wherein defensive antioxidant mechanisms are dampened, while sources from where ROS can be derived are heightened, as are their detrimental outcomes.

1.6.2.2. Dopamine-derived Oxidative Stress:

Dopamine can undergo autoxidation and has the ability to create toxic quinone-derivatives upon degradation (Graham, 1978). These species produce free radicals in the form of reactive oxygen species (ROS) and ultimately produce the neuromelanin pigment found within these neuronal populations (Fahn and Cohen, 1992; Graham, 1978). This would somewhat explain why pigmented nuclei (SNc, LC) appear to be vulnerable in PD (Hirsch et al., 1989). This is also the rationale behind the use of the 6-hydroxydopamine (6-OHDA) model of parkinsonism (not further discussed herein) (reviewed in (Tieu, 2011)).

1.6.2.3. Mitochondrial-derived Oxidative Stress Hypothesis:

Another key source of oxidative stress in neurons is the mitochondria and their associated intermediary metabolism (Lin and Beal, 2006). Neurons are highly dependent on oxidative phosphorylation, given their high metabolic demand (Hall et al., 2012). Recent evidence suggested that the SNc neurons are innately prone to such damage, likely due to the high metabolic demand of this specific cell population and their reliance on L-type calcium channel-mediated pace-making firing capacity (Chan et al., 2007; Putzier et al., 2009). Notably, there was the recent link in function to the PD-linked DJ-1

gene (Guzman et al., 2010). Of interest to my project, certain environmental toxins (MPTP, rotenone) can target the mitochondria and induce cell death via oxidative stress (Tieu, 2011). This will be overviewed in the toxin-induced models of PD section.

1.6.3. Genetic-Environment Interplay in the Etiology of Parkinson's Disease

As reviewed above, the majority of PD is idiopathic in nature and likely requires a combination of factors to elicit a disease manifesting phenotype. Even in the monogenic forms of the disease, it is debated whether this is Parkinson's disease or parkinsonism (Puschmann, 2013). This complex etiology is supported by three general findings: 1) the presence of idiopathic cases that are not linked to any specific cause(s), 2) evidence of genetic risk factors that increase susceptibility, but are not directly linked to disease, and 3) the presence of genetically-linked PD cases that have an incomplete penetrance and variable age of onset (Puschmann, 2013). Even in cases when *SNCA* mutations are aggressive, there is an incomplete (~85%) penetrance (Klein and Westenberger, 2012). LRRK2 mutations only display an overall 30% penetrance (Hakimi et al., 2011). Even monozygotic and dizygotic twins display complex familial disease manifestations, and they share genetic commonalities and early rearing environments (Tanner et al., 1999). Specifically the identity and permutation of disease causing factor(s) needed to manifest PD remains to be solved. Until then, the field has directed attention at combinatorial efforts in the hopes of developing better models for the understanding of pathogenesis and the development and testing of therapeutic interventions.

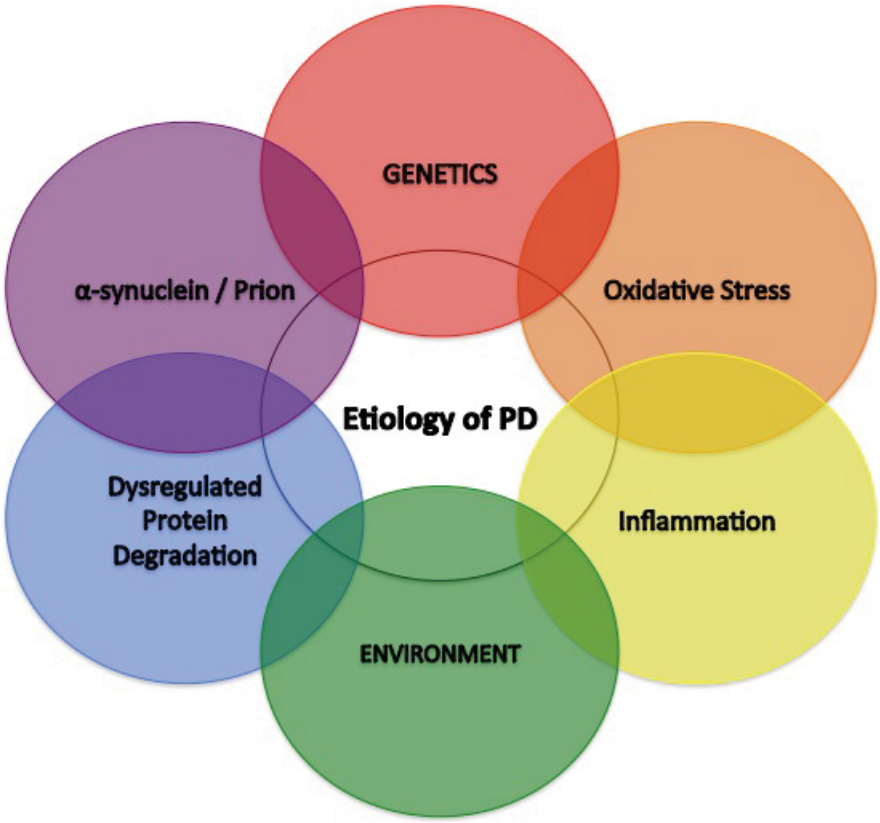

Figure 1.3. The complex etiology of Parkinson's disease

The lack of a single, disease-causing factor(s) has made the study of PD a challenge. Based on epidemiological data, pathology and genetic analysis, a few key hypothesis have been proposed. However, it appears that a combination of multiple elements dictates disease manifestation and/or age-of-onset. Understanding these elements and how they work in concert is critical.

1.7. Modeling Parkinson's Disease for Research

1.7.1. The Importance of Modeling Disease in Basic Research Settings

Human studies provide vital information on: clinical manifestation, symptoms, human-relevant biomarkers, epidemiology studies, links to causal factors, genetics, etc. However, to complement and to allow for more comprehensive in-depth basic research, science employs model systems, which attempt to mimic the human condition (Adams et al., 2000). The more accurately the model recapitulates the human condition, the more likely the results obtained through its usage will contribute positive knowledge that can be cautiously extrapolated to the clinical condition (Adams et al., 2000; Denayer et al., 2014; McGonigle and Ruggeri, 2014). However, in the case of PD, the disease origins are unknown, and even the known causes when employed do not recapitulate most of the disease conditions, which makes it extremely difficult to model and investigate (Antony et al., 2011). Historically, the toxin-induced models of Parkinsonism dominated the field, but with the more recent genetic linkages, a wave of genetic models of PD has emerged (Blesa et al., 2012). However, independently neither approach completely replicates the hallmark features of the disease (Antony et al., 2011). Therefore, the field in general, and my project included, began to pursue combinatorial approaches.

1.7.2. Neurotoxin-Induced Models of Parkinsonism

While several models have been utilized historically (as reviewed in (Tieu, 2011)), those most commonly employed, environment-related, neurotoxic-induced models of Parkinsonism will be overviewed.

1.7.2.1. The Paraquat-Induced Model of Parkinsonism

Paraquat is a commercially available synthetic herbicide that crosses the BBB and appears to selectively affect the DA system (Brooks et al., 1999; McCormack et al., 2002; Shimizu et al., 2001). Once inside cells, paraquat produces superoxide radicals and generates ROS (Bus et al., 1974). Its ability to confer DA cell death is present (inconsistently), however, it does not significantly reduced striatal DA levels (Cicchetti et al., 2005; McCormack et al., 2002). Interestingly, paraquat induces increases in the levels of α-synuclein aggregation and has been reported to increase protein aggregation in animals (Manning-Bog et al., 2002). However, due to its inability to significantly reduced striatal dopamine levels and its relatively limited and inconsistent cell death phenotype (unless coupled to another agent), it is usually not considered the model of choice (Cicchetti et al., 2005; McCormack et al., 2002; Tieu, 2011).

1.7.2.2. The Rotenone-induced Model of Parkinsonism

Rotenone is a naturally occurring substance, produced by certain legume family plants, and has been used as an "organic" pesticide and piscicide (Adams et al., 2000; Metcalf, 1948). Historically it was used to model PD, and only recently did an NIH study positively correlate rotenone to an increased risk of developing PD based on statistics from patients and controls in the southern USA (Tanner et al., 2011). Rotenone is lipophilic and crosses the blood-brain barrier (BBB) where it inhibits complex I of the mitochondrial ETC (Tieu, 2011). It is non-selective and generally produces broad range damage (Ferrante et al., 1997; Lapointe et al., 2004).

1.7.2.3. The 1-Methyl-4-Phenyl-1,2,3,6-Tetrahydropyridine (MPTP) Model of Parkinsonism

MPTP revolutionized how science could more accurately produce parkinsonism in animal models, and has been referred to as the gold standard for preclinical modeling (Dauer and Przedborski, 2003). Illicit MPTP-linked parkinsonism first appeared, albeit unknowingly, in the 1970's (Davis et al., 1979) and then again in the 1980's (Ballard et al., 1985; Langston et al., 1983), when drug users injected a synthetic opioid contaminated with what was later discovered to be MPTP. The latter incident brought MPTP to the forefront attention of neurology, law enforcement, and science (Langston and Palfreman, 1995).

1.7.2.3.1. Human MPTP Exposure: Clinical and Neuropathological Findings

The reported human cases, which were said to have occurred by direct intravenous injection, led to immobility and symptoms of advanced Parkinson's disease within days in relatively young individuals (Ballard et al., 1985). Initially, patients responded well to levodopa treatment, which confirmed the hypothesis of a parkinsonian-like condition, but wearing off was rapidly observed (Langston and Ballard, 1984). When an autopsy was performed on the first known case of exposure to MPTP, the report indicated severe degeneration of the SNc and evidence of LB pathology (Davis et al., 1979). However, as this individual had a long-standing history of drug abuse, not all assessments found in CNS tissue can be attributed solely to MPTP (Davis et al., 1979). Pathology from the latter occurrence of exposures noted SNc degeneration lacking LB pathology (Langston et al., 1999).

1.7.2.3.2. The MPTP Mechanism of Action

MPTP is a highly lipophilic compound that readily crosses the BBB (Dauer and Przedborski, 2003; Langston et al., 1984d; Langston et al., 1984a). There it is taken up by astrocytes and/or serotonergic cells and via subsequent reactions (mediated by the enzyme monoamine oxidase B (MAO-B) and what is believed to be spontaneous oxidation) is converted into its toxic metabolite 1-methyl-4-phenylpyridinium (MPP^+) (Dauer and Przedborski, 2003; Irwin and Langston, 1985; Langston et al., 1984c). MPP^+ is then expelled from astrocytes and has a high affinity for the dopamine transporter (DAT). Once taken up by DA neurons, MPP^+ targets the mitochondria and inhibits complex I of the electron transport chain (ETC) (Nicklas et al., 1985). This results in decreased ATP synthesis and increased mitochondrial-derived ROS, which ultimately leads to cell death, as summarized in Figure 1.4 (as reviewed in (Tieu, 2011))**.** The MPTP model has historically been viewed as the "gold standard" for modeling parkinsonism due to its: ease of delivery, well-defined mechanism of action, SNc death selectivity and reproducibility (Dauer and Przedborski, 2003). Although, extrapolation of the relevance of data produced using this model to clinical PD is now being approached with more caution (Narabayashi, 1987). It is often employed in combination with genetic models to 1) determine whether gene-environment interplay can produce any PD-relevant phenotypes, and 2) to define the role of PD-linked genes in the context of mitochondrial driven dopaminergic cell death outcomes. These points are the basis of the results present in Chapter 3 of this study.

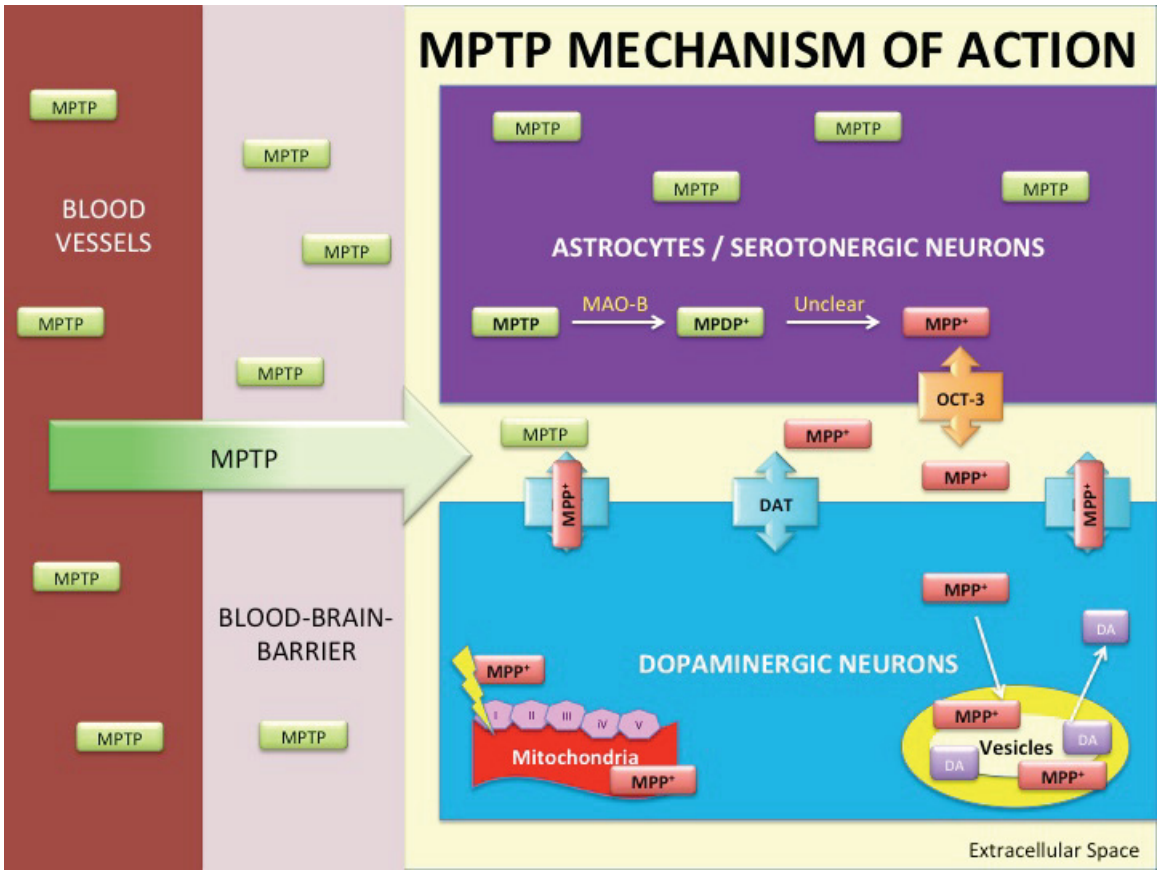

Figure 1.4. Schematic representation of the MPTP/MPP$^+$ mechanism of action

MPTP readily crosses the BBB where it is taken up by astrocytes and/or serotonergic cells. There it is converted to its active metabolite, MPP$^+$, via two sequential reactions, before it is released into the extracellular space where it has a high affinity for the DAT transporter and is taken up into DA neurons. Once in the cytosol, MPP$^+$ targets and inhibits complex I of the mitochondrial ETC, alongside other functions, and increases the oxidative status of cells resulting in toxicity.

1.7.2.3.3. MPTP Exposure in Primates – Behavioural and Neuropathological Findings

In primates, MPTP reacts similarly to what is demonstrated in human subjects exposed to the neurotoxin. Exposure provides rapid loss of motor activity, leaving primates immobile, stiff and expressionless (Langston et al., 1984d). Upon administration of L-dopa, primates recover their functional movement (Burns et al., 1983; Jenner et al., 1984). Administration of MAO inhibitors (MAO-I) halts the metabolism of MPTP and blocks its toxicity (Langston et al., 1984b). There is clear evidence of nigral degeneration, with additional areas also reported to be affected (VTA, LC) following injection with MPTP (Langston et al., 1984d; Mitchell et al., 1985, 1986). As is the case with many models – LB pathology is lacking; however, alterations in α-synuclein expression are observed (Purisai et al., 2005). Interestingly, primate brain analyses years following exposure to MPTP demonstrate high levels of neuroinflammation and activated microglia (Barcia et al., 2004; McGeer et al., 2003). This suggests that acute events can have long-lasting and potentially detrimental effects on our brain.

1.7.2.3.4. Rodents – Behavioural and Neuropathological Findings

MPTP does not have the same effects in rodents. Rats are resistant to MPTP, whereas in mice, MPTP has a more pronounced effect and will be the focus of the subsequent discussion (Giovanni et al., 1994). MPTP injections in mice produce behavioural deficits in the following tests: locomotor activity as assessed by the open field test and the rotarod test, among others (Meredith and Rademacher, 2011). SNc neuronal death has been confirmed following a variety of administration paradigms

introduced via various routes, such as: subcutaneous (s.c.) (Hallman et al., 1985); intraperitoneal (i.p.) (Kim et al., 2005a); and intranasal (i.n.) (Prediger et al., 2010). The most common systemic method of administration is via i.p. injection, as employed by our lab (Crocker et al., 2003a; Crocker et al., 2003b; Haque et al., 2012; Haque et al., 2008; Huang et al., 2010; Kim et al., 2005a; Lira et al., 2011; Mount et al., 2007; Mount et al., 2013; Smith et al., 2003). It is important to note that a wide range of treatment paradigms has been reported: ranging from chronic to acute (Alvarez-Fischer et al., 2008; Jackson-Lewis and Przedborski, 2007). The extent of impairment is dependent on the treatment paradigm (Jackson-Lewis and Przedborski, 2007; Sedelis et al., 2001).

Although widely accepted, there are considerations that should be highlighted: 1) The MPTP model of parkinsonism produces an acute and, relative to iPD, rapid onset of dopaminergic cell death; 2) The MPTP model, alone, lacks the ability to produce Lewy body (LB) or Lewy neurite (LN) pathology in mice; 3) MPTP behavioral deficits are reported to recover with time; and 4) It is well equipped for use in non-human primates and mice, but not for use in rat models (reviewed in (Dauer and Przedborski, 2003)). Although the model has limitations and is not perfect, it is still consider the best pre-clinical model that exists, although extrapolation and clinical relevance of findings should be approached with caution (Narabayashi, 1987; Porras et al., 2012).

1.7.3 Model Species to Study Parkinson's disease in the Laboratory

1.7.3.1. Different Disease Modeling Species in Parkinson's Research

Modeling PD has been attempted in various model organisms. Some of these include, but are not limited to: *Caenorhabditis elegans* (*C. elegans*), *Drosophila melanogaster* (*D. melanogaster*), *Danio rerio* (zebrafish), *Mus musculus* (mice) *Rattus norvegicus* (rats), and various primates: *Macaca mulatta* (rhesus), *Macaca fascicularis* (cynomolgus), and *Saimiri sciureus* (squirrel monkey). Considerations for specific model organisms include: purpose/nature of research, available facilities and cost of maintenance. Although the latter two significantly impact the ability to perform studies, it is the nature and purpose of the research that dictates the type of model organisms (Adams et al., 2000). For the sake of this project, I will very briefly discuss strengths and limitations of the two model systems employed herein: *D. melanogaster* and *M. Musculus.*

1.7.3.2. The *Drosophila melanogaster* model of Parkinson's disease

The employment of Drosophila to study human related disease has proven invaluable (Hirth, 2010; Pandey and Nichols, 2011). When comparing an assortment of human disease-related genes, it was noted that the fruit fly has orthologues for ~60% of these genes (Rubin et al., 2000). The strength of the *D. melanogaster* (fruit fly) model lies in its use as a genetic tool (Bier, 2005). The fruit fly has a total of four chromosome pairs (3 autosome and 1 sex chromosome) and its genome has been mapped (Adams et al., 2000). Genetic manipulations can be performed, using either gene down-regulation

(knock down or knock out/deletion), or gene up-regulation (enhanced promoter) or transgenic (over-expression) systems (Venken and Bellen, 2005). Employing the yeast-derived UAS-Gal4 bipartite system allows for both spatial and temporal control of gene expression (Duffy, 2002). For our purposes, the transgene of interest is placed under the control of an upstream activating sequence (UAS), which requires and is modulated by binding of the yeast Gal4 transcription factor. When flies expressing the UAS-transgene insert are bred with "driver" fly lines carrying tissue specific expression of Gal4, the resulting progeny will display controlled transgene expression (Busson and Pret, 2007). In addition, the presence and copy number of the gene can easily be monitored using visual phenotypic markers that exploit the use of "balancer" chromosomes. Balancer chromosomes are genomic regions that do not allow for homologous recombination, have a phenotypic marker and are homozygous lethal (Adams et al., 2000).

In the context of neurodegenerative disease, the fruit fly presents a powerful model system given: 1) Drosophila have a relatively rapid life cycle that is temperature-sensitive; 2) Large progenies and populations can be produced and analyzed simultaneously in a reasonable time span; 3) Genetic crosses can be performed readily since female virgins and balancer chromosomes are easily identified using visual body markers; 4) Commercially available stock lines are extremely diverse and can be obtained at a fraction of the cost; and 5) Maintenance of fly stocks, food, housing and experiment-related equipment are relatively inexpensive (Bier, 2005).

For the purpose of PD research, manipulating the genetics or exposing fruit flies to parkinsonian-related toxic compounds, studies have demonstrated that the fruit fly: loses the appropriate corresponding tyrosine hydroxylase (TH) positive staining neuronal

clusters in their CNS; display pathological protein aggregation phenotypes; demonstrate locomotor impairments; and when challenged with environmental factors, have a reported susceptibility (Liu et al., 2008; Venderova et al., 2009). Cumulatively, this is more representative of the clinical condition than the alterations seen in mouse models of PD (Dawson et al., 2010; Gubellini and Kachidian, 2015). Moreover, this system can be employed for large scale, genome saturated, high-throughput, functional, *in vivo,* genetic-interaction screens (St Johnston, 2002). This was a key objective outcome from my work presented in Chapter 2.

1.7.3.3. The *Mus musculus* model of Parkinson's Disease

Using the mouse model system also allows for genetic manipulation (van der Weyden et al., 2011). As a mammalian species, the genetic similarity generally better resembles that of humans (Yue et al., 2014). However, in the majority of the genetic alterations associated with PD-linked genes (knock out (KO), knock in (KI), or transgenic over-expression), none or at best one of the disease-related phenotypes exist (Dawson et al., 2010). Genetics alone cannot produce cell death in the nigral regions (Dawson et al., 2010). Commonly the genetically modified strain must be challenged with an additional stressor – such as MPTP, and even so only certain models (DJ-1 KO and Pink1 KO) display sensitized death-related phenotypes (Haque et al., 2012; Kim et al., 2005a). The DJ1-C57 KO model was the first and only mouse to display PD-relevant phenotypes in the absence of an insult (Rousseaux et al., 2012). Secondly, most do not develop alterations in protein aggregation pathology, with the exception of the mutant SNCA over-expressing transgenic mice (Masliah et al., 2005; Sommer et al., 2000; van der

Putten et al., 2000). Behavioural phenotypes have been noted in certain models, but phenotypic penetrance is said to diminish with time (Li et al., 2009). The results of employing these dual-acting model systems (genetic and toxin-based) for PD-related research will be the focus of Chapter 3.

1.8. Objectives of the Research Endeavours

This research program was tailored to develop systems that would enable us to explore the role of LRRK2 in the CNS. The field is also lacking an understanding of LRRK2's contribution to PD vulnerability. Therefore, our goal was to develop a model system wherein we could investigate genetic interactors of LRRK2 in an unbiased manner, to unveil candidate pathways or proteins that may be related to its function. Additionally, we were interested in exploring the nature of the hypothesized gene-environment interplay that appears to be important in *LRRK2*-linked PD. Having a few indications of mitochondrial function from the literature, namely: 1) ~10% of LRRK2 is localized to the outer mitochondrial membrane (OMM) (West et al., 2005); 2) a reported interaction between LRRK2 and Parkin (Smith et al., 2005), 3) structural similarity between LRRK2 and protein domains required for mitochondrial membrane dynamics (discussed in (Li and Beal, 2005)); and, 4) results that stemmed from a preliminary biased screen between LRRK2 and: Pink1, Parkin and DJ-1 indicated a genetic interaction (reported in Chapter 2 – (Venderova et al., 2009)), our goal was to test the effects of an environmental neurotoxin that targeted mitochondrial function (MPTP) and its ability to sensitize murine models of *LRRK2*-linked PD to cell death.

1.8.1. Main Objectives:

1. Create a system to investigate unbiased LRRK2 interactions, *in vivo* – Chapter 2; and
2. Investigate the LRRK2 gene-environmental interaction in genetic murine models of *LRRK2*-linked PD using the environmental neurotoxic MPTP model – Chapter 3.

1.8.2. Research Hypotheses:

Chapter 2:

A LRRK2-linked drosophila model of PD will replicate disease-relevant phenotypes and provide a genetic high-throughput screening tool for identifying interactors of LRRK2.

Chapter 3:

We hypothesize that mutant LRRK2 sensitizes neurons to cell death following exposure to MPTP/MPP^+ treatment.

CHAPTER 2:
Investigating LRRK2 Interactions in a Drosophila Model of PD

PART 2.1:

***Leucine-rich repeat kinase 2* interacts with *Parkin*, *DJ-1* and *PINK-1* in a *Drosophila melanogaster* model of Parkinson's disease**

This is a pre-copyedited, author-produced PDF of an article accepted for publication in Human Molecular Genetics following peer review. The version of record
Katerina Venderova *et al*. Leucine-rich repeat kinase 2 interacts with Parkin, DJ-1 and PINK-1 in a Drosophila melanogaster model of Parkinson's disease. *Human Molecular Genetics (2009) 18(22): 4390-4404* is available online at: http://hmg.oxfordjournals.org/content/18/22/4390.long .

PART 2.2:

Screening for Genetic Interactors of LRRK2 Using a *Drosophila melanogaster* model of *LRRK2*-linked PD

PART 2.1: *Leucine-rich repeat kinase 2* interacts with *Parkin*, *DJ-1* and *PINK-1* in a *Drosophila melanogaster* model of Parkinson's disease

Statement of Authors Contribution

The co-first authors (*) contributed equally to this project. K. Venderova* designed the experimental plan; composed the manuscript; prepared all of the final figures; performed all of the statistical analysis, and was lead on: survival, dopaminergic and locomotor data sets – Fig. 3, 4, 6, 7, 8, Table 1 and 2 and analyzed images for Fig. 11, 12 and 13. G. Kabbach* supported in experimental design; mapped and balanced the stock UAS-transgene inserts; created stable double-transgenic lines and performed experiments for: Fig. 1, 2, 8, 9, 10, 11, 12, 13 and Tables 3 and 4.

E. Abdel-Messih contributed to the performance of almost every experiment via: the majority of all food and media preparations, the majority of stock line maintenance, the collection of virgins and males for use in performing genetic crosses, performing many of the genetic crosses, collecting and maintaining F1 experimental progeny for the purpose of collecting experimental data for a variety of assessments (locomotor, behaviour, fertility (Fig. 6), analysis of neuronal loss, supported for samples for western blot analysis and interaction studies); Stained sets of samples for Fig. 2; Performed, recorded and collected data for the life-span experiments under temperature-controlled conditions (Fig 6) and for the control set of the rotenone life-span experiment (Fig. 7).

Y. Zhang performed cortical survival experiments (Fig. 4) using the custom-designed viral constructs (R.J. Parks). E. M. Haque designed, prepared the pUAST-*hLRRK2* plasmid for generation of transgenic fly lines, supported in mapping, balancing and designing experimental plan.

Leucine-rich repeat kinase 2 interacts with *Parkin, DJ-1* and *PINK-1* in a *Drosophila melanogaster* model of Parkinson's disease

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Abstract

Mutations in the *LRRK2* gene are the most common genetic cause of familial Parkinson's disease (PD). However, its physiological and pathological functions are unknown. Therefore, we generated several independent *Drosophila* lines carrying WT or mutant human *LRRK2* (mutations in kinase, COR, or LRR domains, resp.). Ectopic expression of WT or mutant *LRRK2* in dopaminergic neurons caused their significant loss accompanied by complex age-dependent changes in locomotor activity. Overall, the ubiquitous expression of *LRRK2* increased lifespan and fertility of the flies. However, these flies were more sensitive to rotenone. *LRRK2* expression in the eye exacerbated retinal degeneration. Importantly, in double transgenic flies, various indices of the eye and dopaminergic survival were modified in a complex fashion by a concomitant expression of *PINK1, DJ-1* or *Parkin*. This evidence suggests a genetic interaction between these PD-relevant genes.

Introduction:

Parkinson's disease (PD) is the most common neurodegenerative movement disorder. Its pathophysiology involves, although is not limited to, progressive loss of nigrostriatal dopaminergic neurons. PD is considered idiopathic in most patients. However, ~10% of patients have a family history of PD and some cases have a clear genetic component. Several genes, including *LRRK2* (reviewed in (Belin and Westerlund, 2008)), have been linked to familial forms of PD.

LRRK2 is a large multidomain protein with kinase and GTP-ase activities (reviewed in (Mata et al., 2006)). *LRRK2* mutations are the most common cause of familial PD, accounting for up to 39% of all cases in certain populations. The manner by which mutations in *LRRK2* induce PD is unclear. Overexpression of wild-type (WT) or mutant *LRRK2* causes cell death *in vitro* (MacLeod et al., 2006; Smith et al., 2005; West et al., 2007). There is also some evidence it may be involved in sorting (Sakaguchi-Nakashima et al., 2007) and endocytosis of synaptic vesicles (Shin et al., 2008), and in regulation of neurite length and branching (MacLeod et al., 2006). However, the physiological or pathological role of this protein remains largely unknown. Importantly, the role of full-length mutant *LRRK2 in vivo* remains largely unreported.

In order to better understand the mechanism of *LRRK2* induced pathology, several groups have recently generated *Drosophila* lines expressing either fly *LRRK* (*dLRRK*) (Imai et al., 2008; Lee et al., 2007) or human *LRRK2* (*hLRRK2*) (Liu et al., 2008). However, the reported neurochemical and behavioural phenotypes of these flies differed considerably. For example, one study shows no loss of dopaminergic neurons

or deficits in climbing ability (Lee et al., 2007), while other show loss of dopamine and dopaminergic neurons accompanied by behavioral deficits (Imai et al., 2008; Liu et al., 2008). Given these disparate observations, we generated human WT *LRRK2* and several other independent mutants including the LRR domain *hLRRK2(I1122V);* COR domain *hLRRK2(Y1699C);* and kinase domain *hLRRK2(I2020T*) mutants. All of these mutations have been identified in PD patients.

Our transgenic flies expressing *hLRRK2* consistently display loss of dopaminergic neurons. Importantly, *hLRRK2* expression also sensitizes flies to environmental toxins, such as rotenone. However, its effects on other important indices, such as behavior and natural lifespan, are much more complex. Interestingly, our results also reveal a complex genetic interaction between *LRRK2* and other genes relevant to PD.

Results:

Generation of LRRK2 transgenic lines

We first generated WT and mutant *LRRK2* transgenic flies by microinjecting a *UAS-hLRRK2*-containing vector into w1118 embryos and selecting the appropriate flies. To ectopically express the transgenes, we used a *UAS/Gal4* bipartite system.

To confirm that we indeed expressed *hLRRK2* in the fly, we first performed RT- PCR. All transgenic flies were positive for transgene expression (Fig. 2.1.1A). To confirm this, we also assessed the expression of hLRRK2 protein by western blot. We observed a strong band at more than 250 kDa that was not present in the control *GMR/+* fly (Fig. 2.1.1B). We thus concluded that all of our lines express the *LRRK2* transgenes.

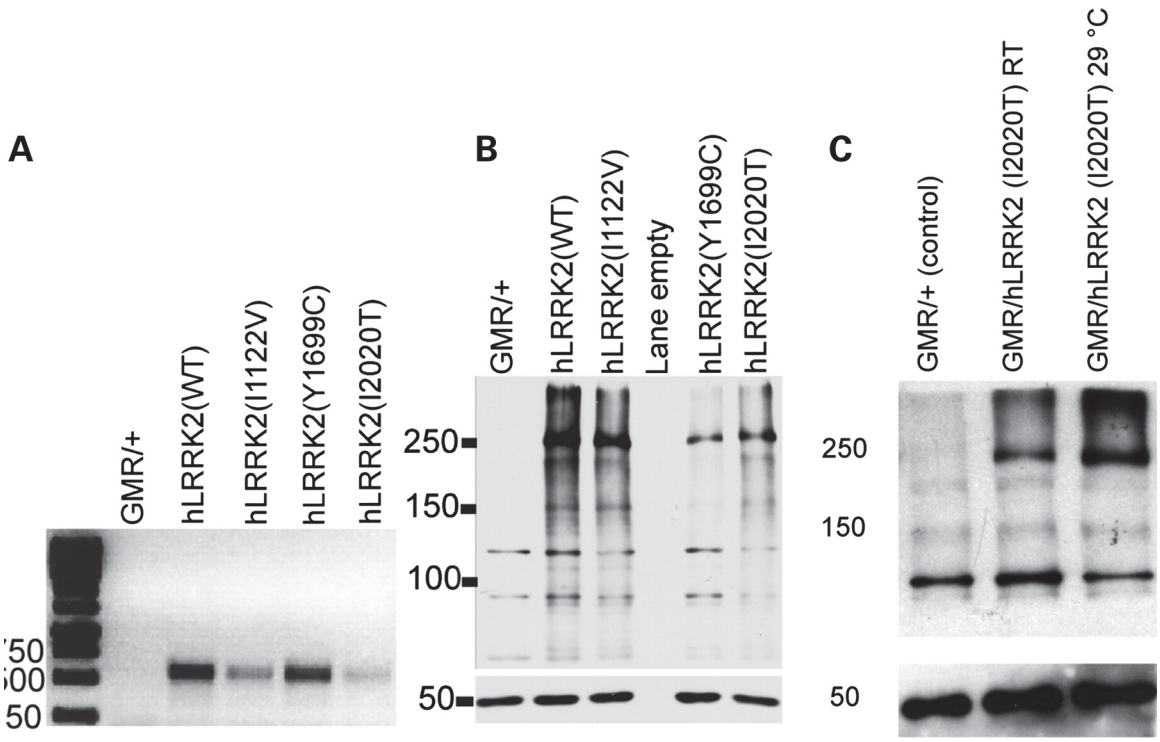

Fig. 2.1.1. Expression of *hLRRK2* in *Drosophila.*

(**A**) RT-PCR of all lines expressing *hLRRK2* under *Da* promoter; (**B**) western blot from heads expressing *hLRRK2* in *GMR* fashion. The band above the 250 kDa marker corresponds to *hLRRK2*. β-tubulin is a loading control. (**C**) Effect of temperature on *LRRK2* expression levels under the *UAS-Gal4* expression system. Western blot from heads expressing *hLRRK2* in *GMR* fashion. β-tubulin is a loading control.

Previous reports suggest that axon outgrowth may be affected by *LRRK2* expression (MacLeod et al., 2006). If this were the case, we might expect to see an abnormal development of the nervous system. To assess this, we expressed the transgenes in the nervous system using the *Elav* driver and examined the axonal growth in embryos by looking at abnormalities including breaks, thinning or fusions in longitudinal connectives and commissures. There were no significant differences in these parameters in *hLRRK2(I2020T)* expressing embryos, compared to controls (Fig. 2.1.2A and B). Accordingly, gross development appeared normal with flies expressing *hLRRK2(I2020T)*.

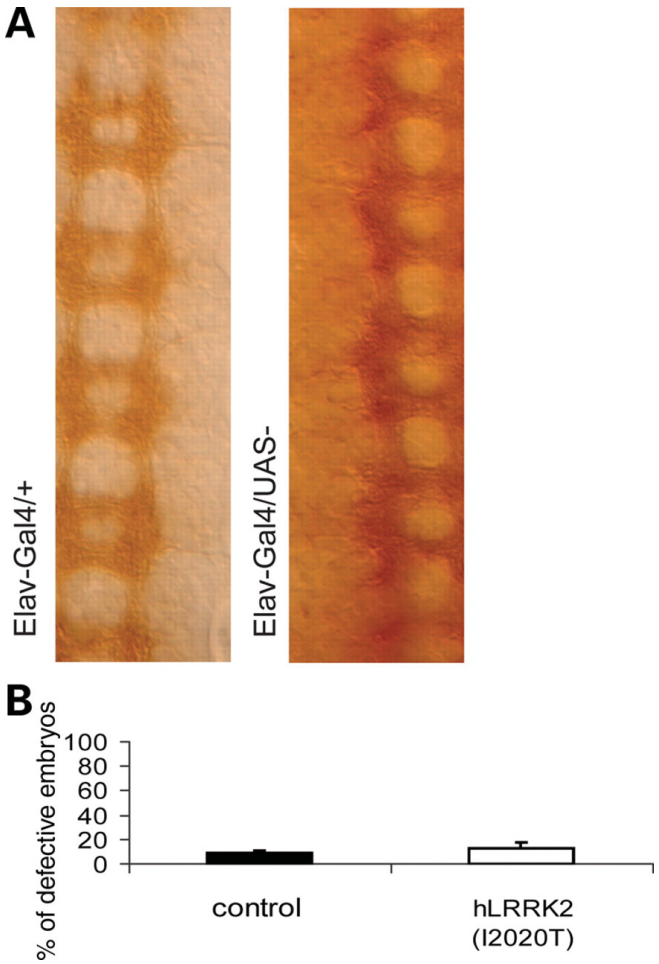

Fig. 2.1.2. *hLRRK2* expression has no effect on axonal growth in embryonic development.

(**A**) *hLRRK2 (I2020T)* expression does not affect axonal growth in embryonic development. Representative images of control (*Elav/+*) and *hLRRK2 (I2020T)*-expressing embryos, respectively. (**B**) Graph summarizing the above data. 304-455 embryos were analyzed per genotype. Unpaired two-tail Student's *t*-test.

Loss of dopaminergic neurons

Loss of dopaminergic neurons is a pathological hallmark of PD. We therefore investigated whether expression of WT or mutant *hLRRK2* results in degeneration of these neurons. We analyzed all four posterior paired dopaminergic clusters in the fly brain: dorsolateral posterior protocerebral (PPL1), lateral posterior protocerebral (PPL2), and two dorsomedial posterior protocerebral clusters (PPM1/2 and PPM3) (Fig. 2.1.3E).

We expressed the transgenes in dopaminergic neurons under control of the *tyrosine hydroxylase (TH)* gene promoter. In control flies, the TH positive neurons in the four clusters did not change significantly in number or morphology during aging (Fig. 2.1.3B and C), data which are in agreement with previous reports (Chen and Feany, 2005). All WT and mutant *LRRK2* flies that were aged for 50 days at room temperature show some degree of neuronal loss in PPM1/2 and/or PPL1 cluster (Fig 2.1.3A and B). The effect in PPM3 and PPL2 clusters did not reach statistical significance, despite a trend towards loss of neurons. Overall, the most prominent effect was observed in the *hLRRK2(I2020T)* mutant (loss of 47.1 ± 6.6% in PPM1/2 cluster and 63.1 ± 5.2% in the PPL1 cluster). The loss of neurons was already apparent at 10 days post-eclosion (Fig. 2.1.3C).

Gal4 enhancer traps generally produce stronger effects at higher temperatures due to higher expression levels of the transgene. For example, increasing temperature to 29°C increased the expression of GMR-driven *hLRRK2* in the eye (Fig. 2.1.1C). However, we saw a similar degree of loss of TH neurons 20 days post-eclosion at 29°C, compared to room temperature, with the greatest loss again in *hLRRK2(I2020T)* (Fig. 2.1.3D).

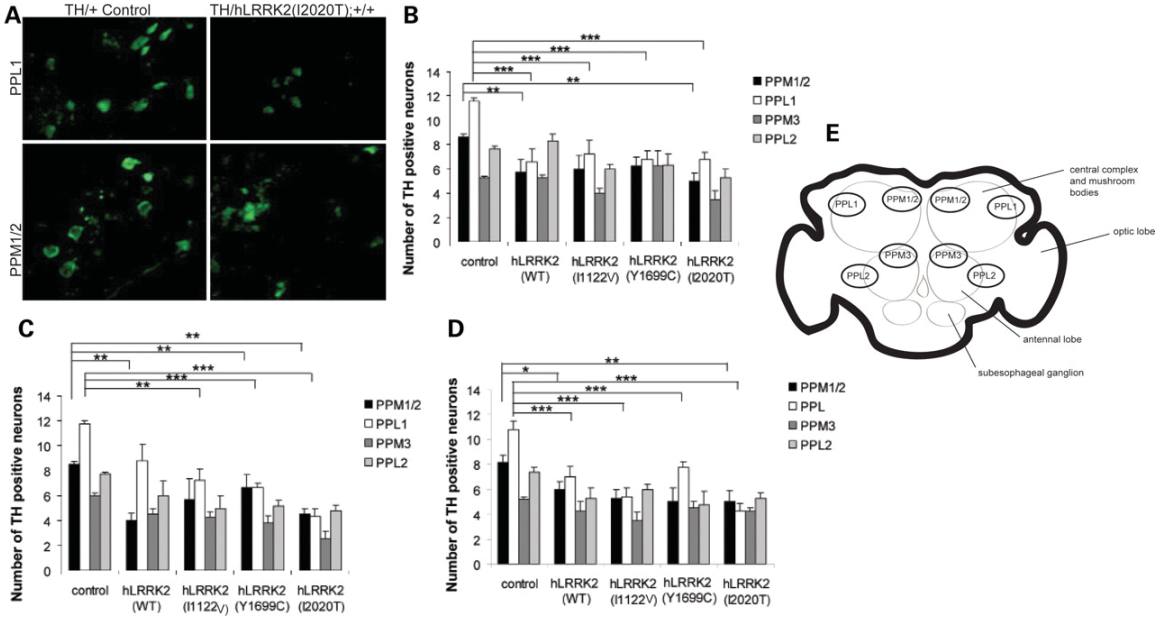

Fig. 2.1.3. *hLRRK2* expression causes loss of dopaminergic neurons.

(**A**) Representative images of TH staining in PPL1 and PPM1/2 DA clusters in 50-day-old control and *TH-Gal4/UAS-hLRRK2(I2020T)* flies maintained at room temperature. (**B**) Graph summarizing the findings above. (**C**) The effect of *hLRRK2* expression on the number of dopaminergic neurons in 10-day-old flies at room temperature; (**D**) 20-day-old flies at 29˚C. (**E**) Schematic picture depicting dopaminergic clusters in Drosophila brain.

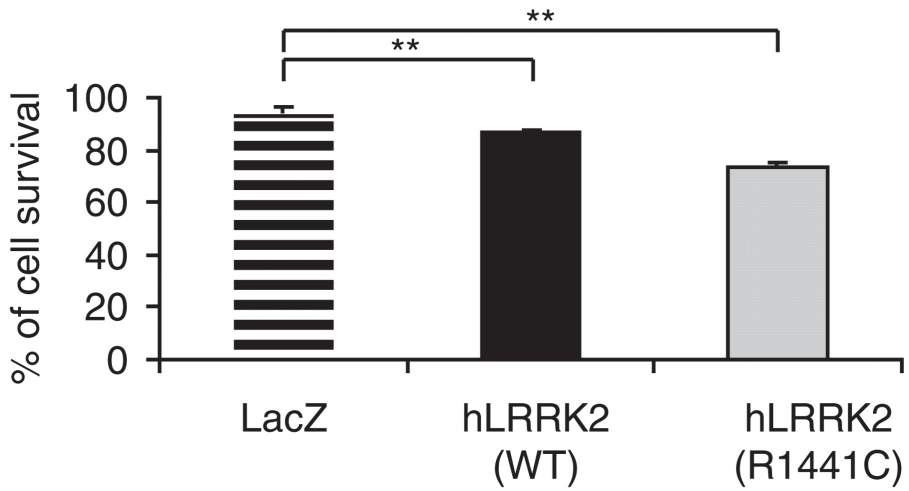

Fig. 2.1.4. Expression of WT or mutant *hLRRK2* kills primary cortical neurons.

Expression of WT or mutant *hLRRK2* kills primary cortical neurons. *n = 3*, the experiments were performed in triplicates. One-way ANOVA followed by Dunnett's post-test.

To verify that expression of *hLRRK2* causes cell death and to extend our findings to the mammalian system, we also expressed full-length *hLRRK2(WT)* or *hLRRK2(R1441C)* mutant in primary cortical neurons via adenoviral delivery. In both cases, we observed a statistically significant neuronal loss (13.4 ± 0.9% in *hLRRK2(WT)* and 26.2 ± 1.2% in *hLRRK2(R1441C)*; compared to 4.90 ± 1.17% in *lacZ* control) (Fig. 2.1.4).

Locomotor activity

PD is a movement disorder. Therefore we proceeded to investigate how *LRRK2* overexpression affects locomotion in the transgenic flies. We performed a climbing assay that has previously been used in transgenic fly models of PD. These deficits were reversed by treatment with levodopa (Pendleton et al., 2002). In this assay, flies are placed in a transparent vial, tapped down and allowed 10 or 20 s to climb up to a horizontal line.

We analyzed the *LRRK2* transgenic fly driven with *TH*, as described above. The climbing ability of both control and transgenic flies progressively deteriorated with age – only 0.6-15.6% of our control flies maintained at 29˚C crossed the line within 10 s at 30 days of age, compared to 62.0-88.0% at 10 days of age (Fig.2.1.5A). The remaining flies stayed at the bottom, attempted to climb up but fell back or very slowly climbed up.

The effects of *hLRRK2* expression on behavior were complex and dependent upon the age of the flies. The climbing ability of all transgenic *LRRK2* lines was significantly impaired at 10 days of age with the most sizable locomotor deficit (27.6 ± 4.2% compared with control) being observed in *hLRRK2(I2020T)* mutants. Intriguingly, however, the *LRRK2* transgenic flies performed slightly better at the later 20 day time point compared

with control (Fig. 2.1.5A). The longevity of flies maintained at 29˚C is known to be significantly shorter than at room temperature (approximately 30 days). Indeed, most of the 30-day-old flies, except for *hLRRK2(I1122V)* mutants, were unable to climb up to the line within 10 s. We therefore chose to extend the observation period and record them for 20 s. Surprisingly, the climbing ability of all of the older *hLRRK2* WT or mutant flies was significantly better (by 262.5-537.5%) than that of the control flies (Fig. 2.1.5B). In comparison, the 10-day-old *hLRRK2* flies were less able to climb up the vial within 10 s compared with controls (Fig. 2.1.5A); this difference was smaller when observing the same flies for 20 s (Fig 2.1.5B). This may suggest that the flies have difficulty initiating the movement. However, once the movement is initiated, the flies appear to move quite efficiently. In addition, any deficits observed in the *LRRK2* transgenic flies are, compared with control, clearly not sustained with aging (Fig. 2.1.5A and C). A very similar pattern of initial depression of movement 30 and 50 days post-eclosion followed by its significant improvement compared with control was also observed at room temperature in *hLRRK2(I1122V)* and *hLRRK2(I2020T)* flies, respectively (Fig. 2.1.5C). The implications of this finding, particularly in relation to loss of dopaminergic neurons, are discussed further below.

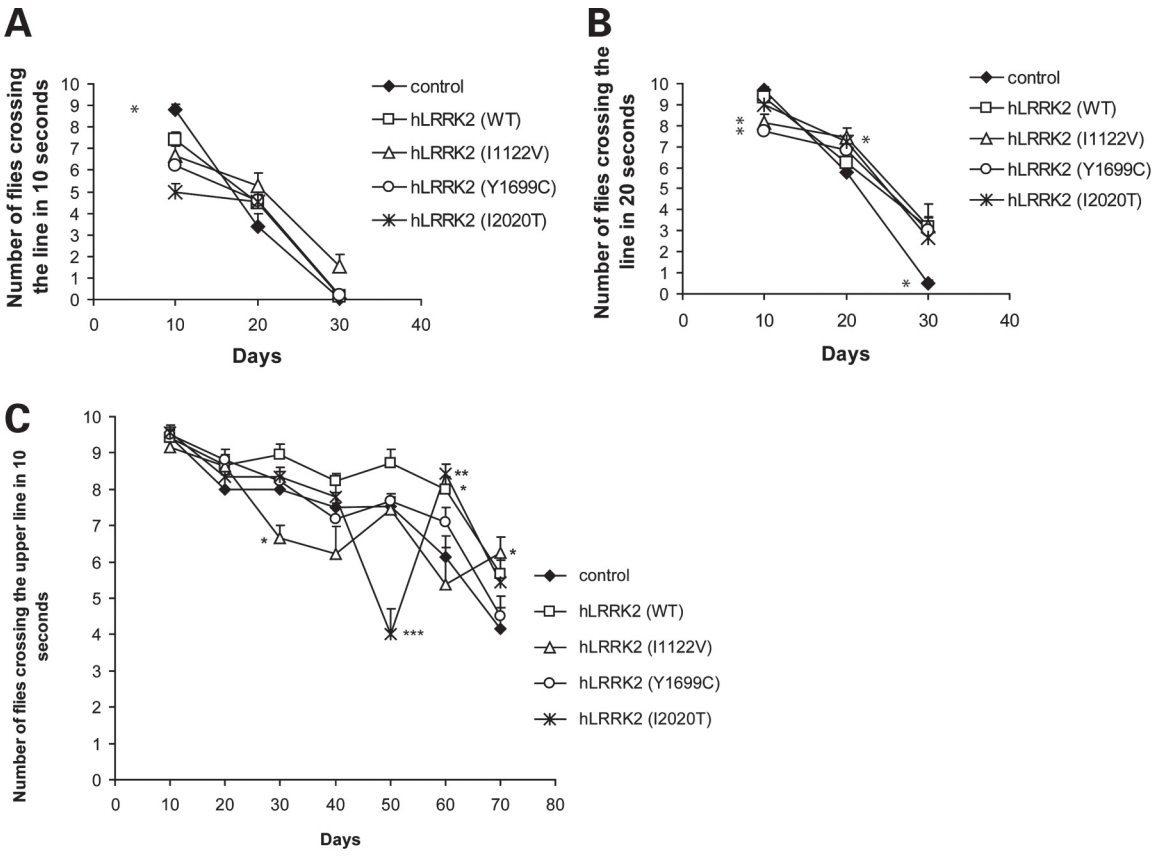

Fig. 2.1.5. *hLRRK2* expression causes alterations in locomotor activity.

Climbing behavior of *TH-Gal4/UAS-hLRRK2(WT), TH-Gal4/UAS-hLRRK2(I1122V), TH-Gal4/+;UAS-hLRRK2(Y1699C)/+ and TH-Gal4/UAS-hLRRK2(I2020T)* flies raised and maintained at 29˚C and recorded for 10 s (**A**) r 20 s (**B**). n = 4-7 sets of 10 for each time point per genotype. (**C**) Climbing behavior of transgenic flies kept at room temperature and recorded for 10 s. Each cohort was recorded three times. All data were analyzed by one-way ANOVA, Bonferoni's post-test. Asterisks next to control signify that all genotypes were significantly different from control.

Lifespan and sensitivity to oxidative stress

Next, we investigated the effect of *hLRRK2* expression on lifespan of the flies and their response to oxidative stress. These experiments were performed at room temperature. Unexpectedly, ubiquitous expression of either *hLRRK2(WT), hLRRK2(Y1699C)* or *hLRRK2(I2020T)* significantly extended the basal lifespan of these flies when compared with controls (Fig. 2.1.6A; Table 2.1.1). The *hLRRK2(I1122V)* mutant was not significantly different from control.

We repeated the experiments with a pan-neuronal *Elav* driver to test whether neuronal expression of *LRRK2* is responsible for this effect. Similar to ubiquitous expression, the pan-neuronal *hLRRK2(WT)* expression significantly increased lifespan at room temperature (Fig. 2.1.6B; Table 2.1.1). However, in this case, none of the *LRRK2* mutants had a significantly different lifespan at room temperature compared with control. In contrast, at 29˚C, there was no significant difference in lifespan between control and *Elav/hLRRK2* flies (Fig. 2.1.6C). Taken together, our data indicate that expression of WT or mutant *LRRK2* has the surprising potential to increase lifespan, depending upon the conditions.

Female fecundity (egg laying) and number of progeny is known to negatively correlate with lifespan. Thus, to determine whether the extended lifespan seen in our lines at room temperature is accompanied by lower numbers of progeny, we analyzed the fertility of flies ubiquitously expressing *hLRRK2*. Surprisingly, all WT and mutant *hLRRK2* lines, except for *hLRRK2(Y1699C)*, had a significantly greater number of progeny compared with control flies, by 39.4-59.32% (Fig. 2.1.6D). This finding is consistent with observations in *dLRRK* loss-of-function mutants where fertility and

fecundity is decreased (Lee et al., 2007). Therefore, the lifespan extension seen in flies expressing *hLRRK2* cannot be attributed to a decrease in fertility. Furthermore, there was no significant effect on male-to-female ratio or on the genotype probability of the progeny, compared to control (data not shown). The experiment was carefully standardized and controlled, as crowding may have a profound effect in this type of experiment.

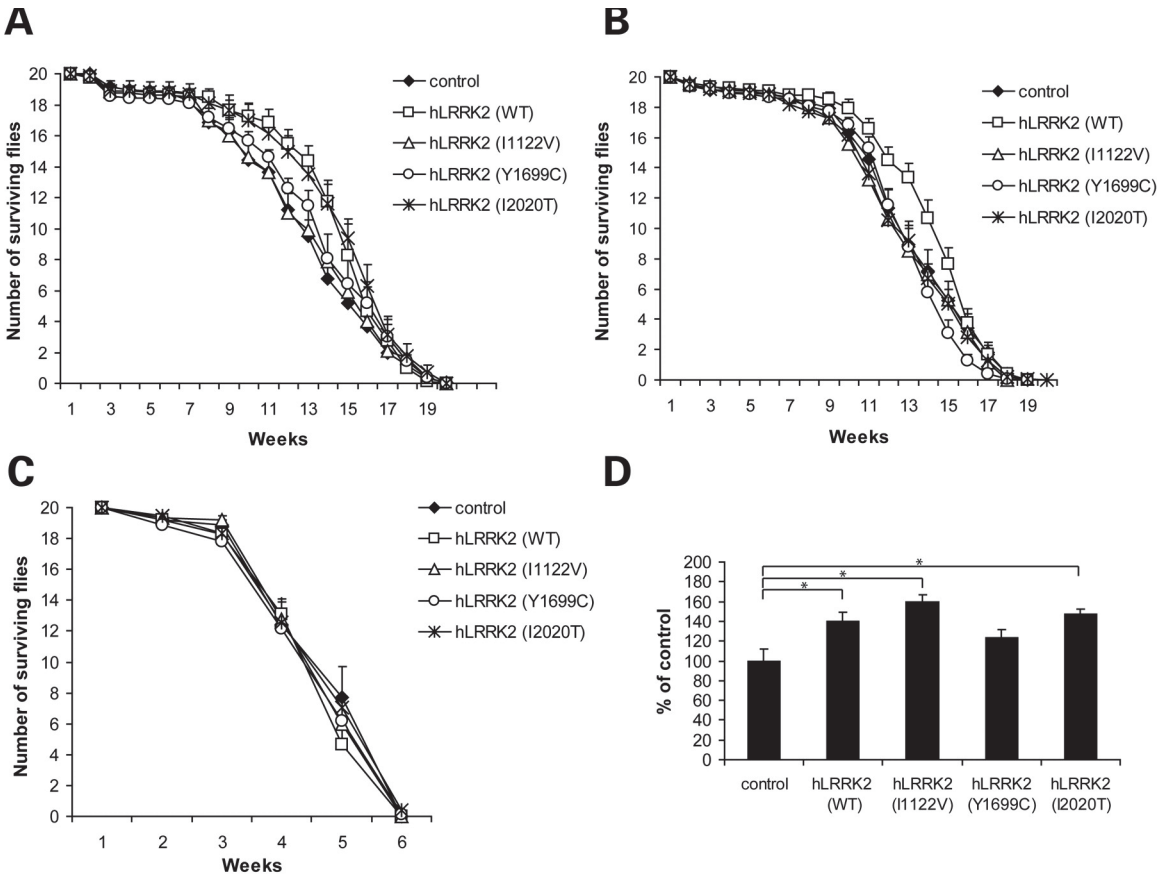

Fig. 2.1.6. *hLRRK2* expression affects the lifespan and the number of progeny.

(**A**) Effect of ubiquitously expressed *hLRRK2* on the lifespan at room temperature. n = 10-17 sets of 20 per genotype. (**B**) Effect of pan-neuronally expressed *hLRRK2* on lifespan at room temperature (n = 20-32 sets of 20 per genotype). (**C**) Effect of pan-neuronally expressed *hLRRK2* on lifespan at 29˚C (n = 10 sets of 20 per genotype). (**D**) *hLRRK2* expression increases the number of progeny. n = 5-8 sets of parents ubiquitously expressing *hLRRK2*. One-way ANOVA, Bonferoni's post-test.

Table 2.1.1. *hLRRK2* expression increases basal fly lifespan at RT. Summary of the effects of *hLRRK2* expression on basal fly lifespan at room temperature. The data were analyzed by nonlinear regression analysis.

genotype	50% survival (weeks)	95% CI	P
Da-Gal4/+	13.0	12.3 - 13.6	N/A
Da-Gal4/UAS-hLRRK2(WT)	14.8	14.2 - 15.3	0.0003
Da-Gal4/UAS-hLRRK2(I1122V)	13.3	12.2 - 14.3	0.6 (n.s.)
Da-Gal4/+;UAS-hLRRK2(Y1699C)/+	14.4	13.4 - 15.4	0.012
Da-Gal4/UAS-hLRRK2(I2020T)	15.2	14.3 - 16.1	0.0002
Elav-Gal4/+	13.0	12.4 - 13.6	N/A
Elav-Gal4/UAS-hLRRK2(WT)	14.8	14.1 - 15.5	0.0006
Elav-Gal4/UAS-hLRRK2(I1122V)	13.0	12.5 - 13.4	0.7044 (n.s.)
Elav-Gal4/UAS-hLRRK2(Y1699C)	12.8	12.6 - 13.1	0.3481 (n.s.)
Elav-Gal4/UAS-hLRRK2(I2020T)	13.0	12.6 - 13.4	0.7223 (n.s.)

Many of the identified PD genes modulate sensitivity to reactive oxygen species. In this regard, *LRRK2* has been shown to increase sensitivity to H_2O_2 in primary cortical neurons (West et al., 2007). Accordingly, we examined the sensitivity of our transgenic lines to rotenone, a pesticide which leads to oxidative stress. Importantly, chronic exposure to low concentrations of rotenone (100 μM) at room temperature rendered all flies ubiquitously expressing *hLRRK2* significantly more susceptible to this toxin compared with control (Fig. 2.1.7A; Table 2.1.2). More importantly, chronic exposure of flies expressing *hLRRK2* in dopaminergic neurons to low doses of rotenone also significantly increased dopaminergic neuron death in these flies, compared with both non-treated *hLRRK2* expressing flies, or control rotenone-treated flies (Fig. 2.1.7B).

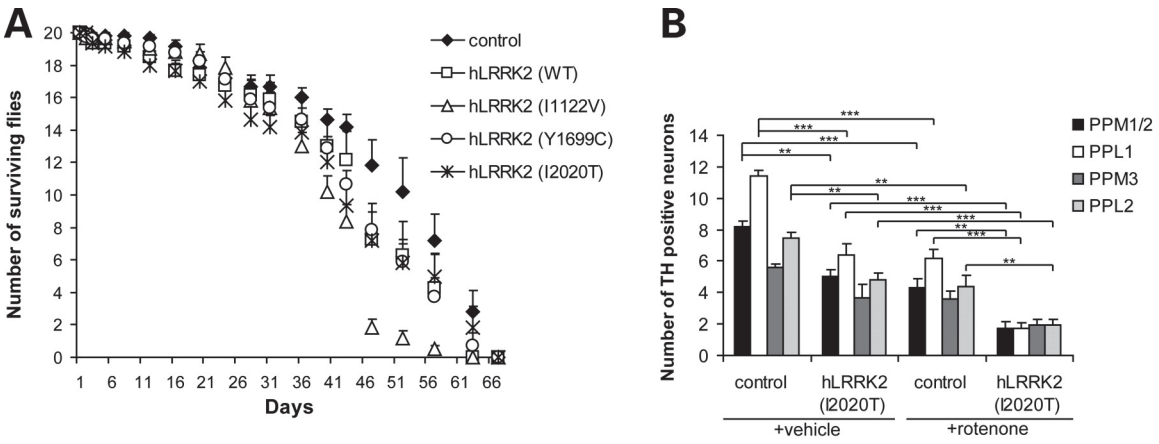

Fig. 2.1.7. *hLRRK2* expression increases sensitivity to oxidative stress.

(**A**) Effect of rotenone at room temperature on lifespan of flies ubiquitously expressing *hLRRK2*. n = 6-8 sets of 20 per genotype. (**B**) Effect of rotenone on survival of dopaminergic neurons in flies expressing *hLRRK2* specifically in TH-positive neurons. One-way ANOVA, Bonferonni's post-test.

Table 2.1.2: *hLRRK2* expression increases sensitivity of the flies to rotenone

Summary of the effects of *hLRRK2* expression on sensitivity to rotenone at room temperature. The data were analyzed by nonlinear regression analysis

genotype	50% survival (days)	95% CI	P
Da-Gal4/+	47.9	47.5 - 48.3	N/A
Da-Gal4/UAS-hLRRK2(WT)	44.1	43.6 - 44.5	< 0.0001
Da-Gal4/UAS-hLRRK2(I1122V)	41.0	40.7 - 41.2	0.0003
Da-Gal4/+;UAS-hLRRK2(Y1699C)/+	43.9	43.6 - 44.2	< 0.0001
Da-Gal4/UAS-hLRRK2(I2020T)	41.0	40.7 - 41.5	0.0003

Eye defects

We examined multiple eye parameters in our transgenic lines crossed with the eye-specific *GMR* driver. We first analyzed for the effects of *LRRK2* expression at room temperature. No effect was observed in any *hLRRK2* fly lines. Accordingly, we next looked for the presence of any abnormalities at a higher temperature, 29˚C. First, we examined overall appearance of the eyes under optical microscope. While *GMR* control flies appeared normal under an optical microscope, eyes of males of all of the transgenic lines were defective (Fig. 2.1.8). To be able to quantify the defects, we examined two parameters of the whole eye. First, we examined for the loss of pigment, defined as spotty lighter areas. Here, any loss of pigment over *GMR* controls was scored as defective. There appeared to be a 99.6-100% penetrance of this eye defect in our *LRRK2* transgenic lines (Table 2.1.3). Similar phenotype with pigmentation loss has been attributed to decreased lens and pigment deposition due to oxidative stress-induced loss of lens-secreting cone and pigment cells (Yarosh et al., 2008). Secondly, we evaluated the presence of black lesions previously reported in several other transgenic lines, including *PINK1-RNAi* flies (Wang et al., 2006). Hereto, less than 1% of *GMR* controls displayed the black lesions while 15.6% (±7.4) to 53.6% (± 23.3) of all the WT or mutant *LRRK2* lines, except for *hLRRK2(I1122V)*, showed significantly more black lesions compared with control, with the highest prevalence in *hLRRK2(WT)* flies (Figs. 2.1.8 and 2.1.9A).

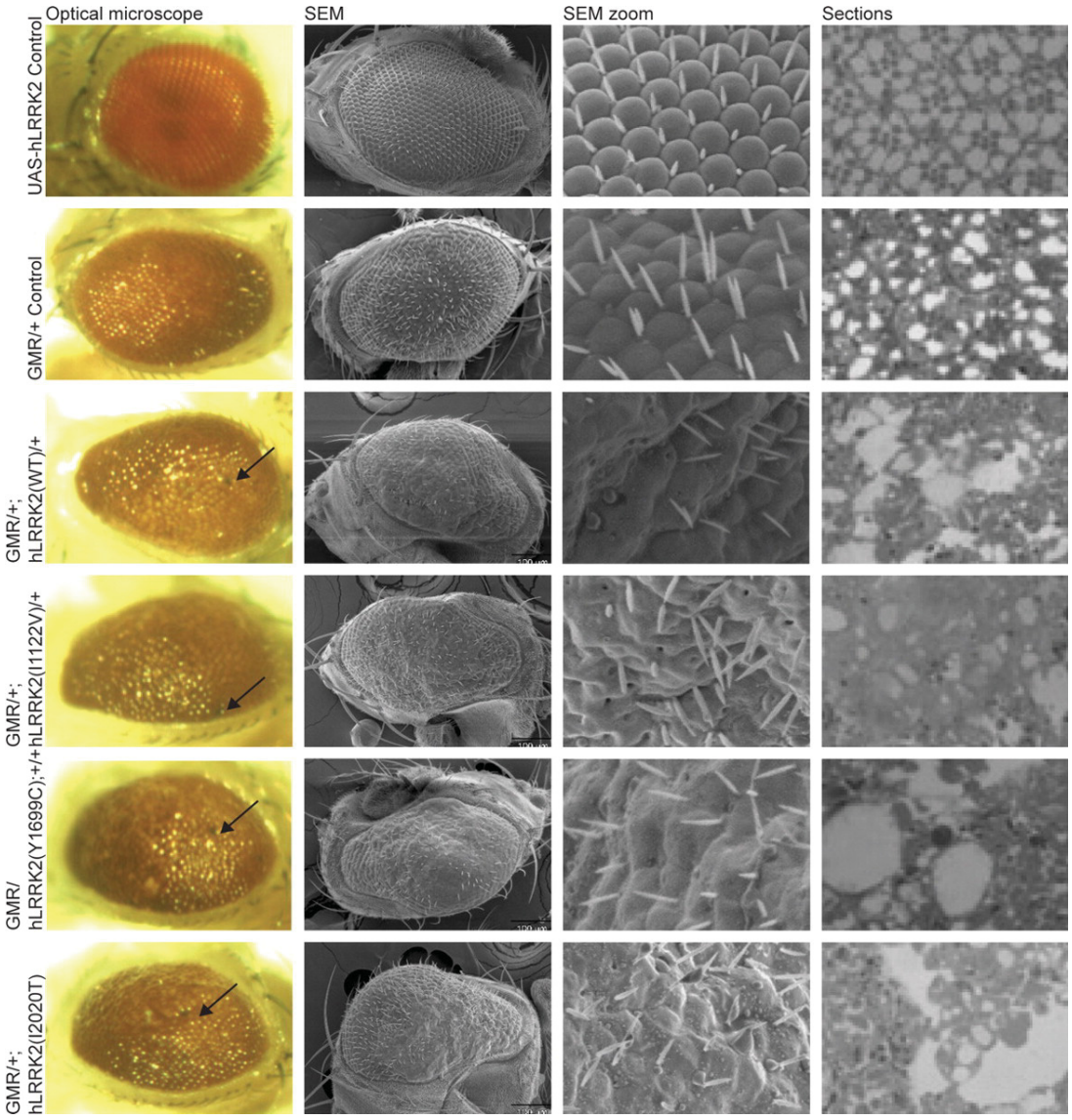

Fig. 2.1.8: *hLRRK2* expression causes structural and pigmentation abnormalities at 29˚C.

Representative images from optical microscope, SEM and tangential eye sections. The arrows point to a black lesion.

Next, we analyzed the eyes using scanning electron microscopy (SEM). It is important to note that *GMR* can cause known defects at 29˚C and the eyes of these control animals were not absolutely structurally normal compared to non-GMR controls (Fig. 2 . 1 . 8). However, overexpression of WT or mutant *hLRRK2* again caused a larger defect, including glossy and rough, sometimes collapsing, surface of the eye, disorganization of mechanosensory interommatidial bristles and irregular lens shape (Fig. 2.1.8). The rough phenotype may be reflective of mispatterning of lattice cells which may be due to a failure in apoptosis regulation (Yarosh et al., 2008). Some facets, preferentially but not exclusively localized in one area close to the edge of the eye, had holes. These holes are likely caused by a complete absence of corneal lens. The *hLRRK2*-expressing flies had significantly more holes compared with control. In addition, the lens material of the adjacent ommatidia was often clearly fused together and the interommatidial bristles displayed profound disorganization and were occasionally shorter. No significant loss of bristles was apparent in any of the *LRRK2* transgenic lies, except for *hLRRK2(Y1699C)*.

Finally, we examined the ommatidial structure on sections. Here again, *GMR* controls did show substantial defects in the ommatidial organization. Importantly, however, this structural defect was greatly exacerbated by WT or mutant *hLRRK2* expression (Fig. 2.1.8). The regular trapezoidal arrangement of the photoreceptor cells was very severely disrupted. The cell lattice between photoreceptor cell arrays of different ommatida was completely absent and the ommatidia were sometimes fused together. Importantly, the sections from these flies repeatedly displayed large holes that significantly altered the architecture of the ommatidial array. Similar holes were observed

in other fly models of neurodegenerative disorders (Marsh et al., 2000).

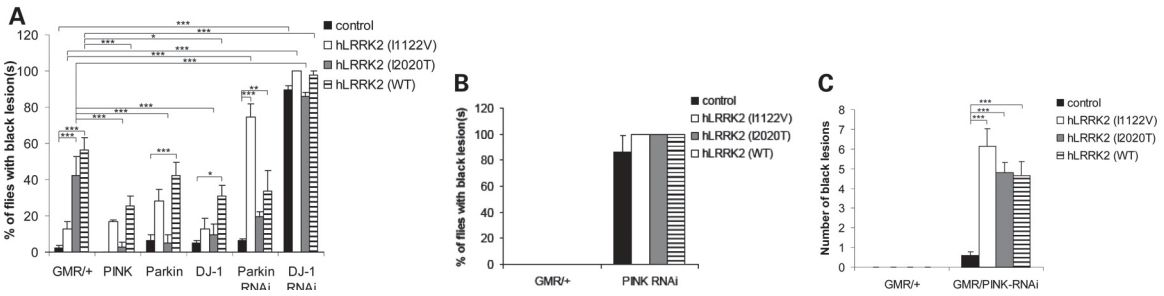

Fig. 2.1.9: *hLRRK2* interacts with *hParkin*, *hPINK1* and *hDJ-1* in the eye.

Summary of *hLRRK2* effects and of interaction with *PINK1*, *DJ-1* and *Parkin* on the formation of black lesions (88-412 flies per genotype). (**A**) The effects at room temperature; (**B** and **C**) the effect of *LRRK2* overexpression in *PINK1* deficient flies at 29°C. One-way ANOVA followed by Bonferonni's *post hoc* test.

Interactions of LRRK2 with other PD causing genes

The presence of eye defects allowed us to screen for genetic interactions with other known Parkinson's disease genes. Accordingly, we next investigated possible genetic interactions between *LRRK2*, *PINK1, DJ-1* and *Parkin*. First, we examined the genetic interaction between *hPINK1, hLRRK2(WT)* and two *hLRRK2* mutant lines with the strongest eye phenotype, *hLRRK2(I1122V)* and *hLRRK2(I2020T)*. The flies were, again, maintained at 29˚C.

hPINK1 overexpression alone did not cause a significant formation of black lesions (Fig. 2.1.9A), holes or loss of pigmentation (Table 2.1.3), but the eyes had some loss and disorganization of interommatidial bristles (Fig. 2.1.10). This is consistent with recently published data (Poole et al., 2008). The overexpression of *hPINK1* in the eye did not significantly ameliorate the loss of pigmentation (Table 2.1.3), the roughness of the eye surface (Fig. 2.1.10) or the formation of holes (Fig. 2.1.10) observed in both mutant or WT LRRK2 lines by optical or SEM. In fact, expression of *hLRRK2(WT) or hLRRK2(I1122V)*, and to some extent *hLRRK2(I2020T),* significantly potentiated the *hPINK1*-induced loss of ommatidial bristles (Fig. 2.1.10). This effect was not additive because *hLRRK2* expression on its own did not cause any bristle loss. In contrast, *hPINK1* expression rescued the *hLRRK2(WT)*- and *hLRRK2(I2020T)*–induced formation of black lesions on the eye surface (Fig. 2.1.9A). Therefore, *hPINK1* expression appears to alleviate some (black lesions) but not all indices of *hLRRK2*-induced eye defects (pigmentation loss), while *hLRRK2* overexpression potentiates the bristle loss phenotype of *hPINK1*. In order to better analyze these interactions, we further performed loss-of-function experiments. However, because the double transgenic *GMR/PINK1-RNAi;*

hLRRK2/+ flies were not viable at 29°C, the experiments were performed at room temperature. As expected, *PINK1-RNAi* flies exhibited an eye phenotype characterized by pigmentation deficits and bristle loss (Fig. 2.1.11 and 2.1.12; Table 4). In addition, nearly all *PINK1-RNAi* flies displayed black lesions (Fig. 2.1.9B and C and 2.1.12). Co-expression of *hLRRK2* did not significantly elevate the number of animals with black lesions (because *PINK1-RNAi* alone caused a dramatic effect) (Figure 2.1.9B). However, the number of black lesions per animal was dramatically elevated with *hLRRK2* (WT and mutants) expression (Fig. 2.1.9C and 2.1.12) moreover, these flies show a mild albeit significant increase in the number of holes. Altogether, these findings strongly suggest an interaction between *PINK1* and *LRRK2*.

Table 2.1.3: Summary of the effects of *hLRRK2* expression on eye pigmentation at 29°C. Interaction with *hPINK1*, *hParkin* and *hDJ-1*.

hLRRK2 expression causes loss of pigmentation in the eye at 29°C. Interactions with *PINK1, DJ-1* and *Parkin*. 118-570 flies per genotype. One-Way ANOVA, Bonferonni's *post-hoc* test.

Genotype	% of flies with loss of pigmentation (+SEM)
GMR/+	0.07 (± 0.07)
GMR/hLRRK2 (WT)	99.61 (± 0.28)
GMR/hLRRK2 (I1122Y)	99.91 (± 0.09)
GMR/hLRRK2 (Y1699C)	100.00 (± 0)
GMR/hLRRK2 (I2020T)	99.70 (± 0.30)
GMR/hPINK-1	0 (± 0)
GMR/hPINK-1; hLRRK2 (I1122V)/+	94.95 (±3.94)
GMR/hPINK-1; hLRRK2 (I2020T)/+	100.00 (± 0)
GMR/hPINK1; hLRRK2 (WT)/+	97.61 (±2.38)
GMR/hParkin	63.39 (±6.92)
GMR/hParkin; hLRRK2 (I1122V)/+	99.59 (± 0.41)
GMR/hParkin; hLRRK2 (I2020T)/+	99.21 (± 0.79)
GMR/hParkin; hLRRK2 (WT)/+	99.56 (± 0.44)
GMR/hDJ-1	100.00 (± 0)
GMR/hDJ-1; hLRRK2 (I1122V)/+	100.00 (± 0)
GMR/hDJ-1; hLRRK2 (I2020T)/+	100.00 (± 0)
GMR/hDJ-1; hLRRK2 (WT)/+	100.00 (± 0)
GMR/Parkin-RNAi	100 (± 0)
GMR/Parkin-RNAi; hLRRK2 (I1122V)	100 (± 0)
GMR/Parkin-RNAi; hLRRK2 (I2020T)	100 (± 0)
GMR/Parkin-RNAi; hLRRK2 (WT)	100 (± 0)
GMR/DJ-1-RNAi	100 (± 0)
GMR/DJ1-RNAi; hLRRK2 (I1122V)	100 (± 0)
GMR/DJ1-RNAi; hLRRK2 (I2020T)	100 (± 0)
GMR/DJ1-RNAi; hLRRK2 (WT)	100 (± 0)

Next, we examined the effects of *hParkin* expression. At 29˚C, *hParkin*expression by itself showed a dramatic phenotype by both SEM (bristle loss, holes and rough surface) (Fig. 2.1.10), as well as counts of pigment loss (Table 2.1.3). This phenotype was not rescued by expression of WT or mutant *hLRRK2*. (Fig. 2.1.10). However, *hParkin* expression, similar to *hPink1* expression, diminished the formation of black lesions in *hLRRK2(I2020T)* lines (Fig. 2.1.9A). Again, *Parkin-RNAi* mutants exhibited a strong eye phenotype. Importantly, expression of one of the *hLRRK2* mutants, *I1122V*, dramatically exacerbated the formation of black lesions in *Parkin-RNAi* flies (Fig. 2.1.9A). This is also consistent with the observation with *PINK1-RNAi* flies discussed above. *Parkin-RNAi* alone induced an effect on bristles and holes that was larger than that observed for *hLRRK2* expression alone in the eye. The *Parkin-RNAi*-mediated effect was surprisingly blunted by *hLRRK2* expression (Fig. 2.1.13). This again indicates a complex interaction between *Parkin* and *LRRK2*.

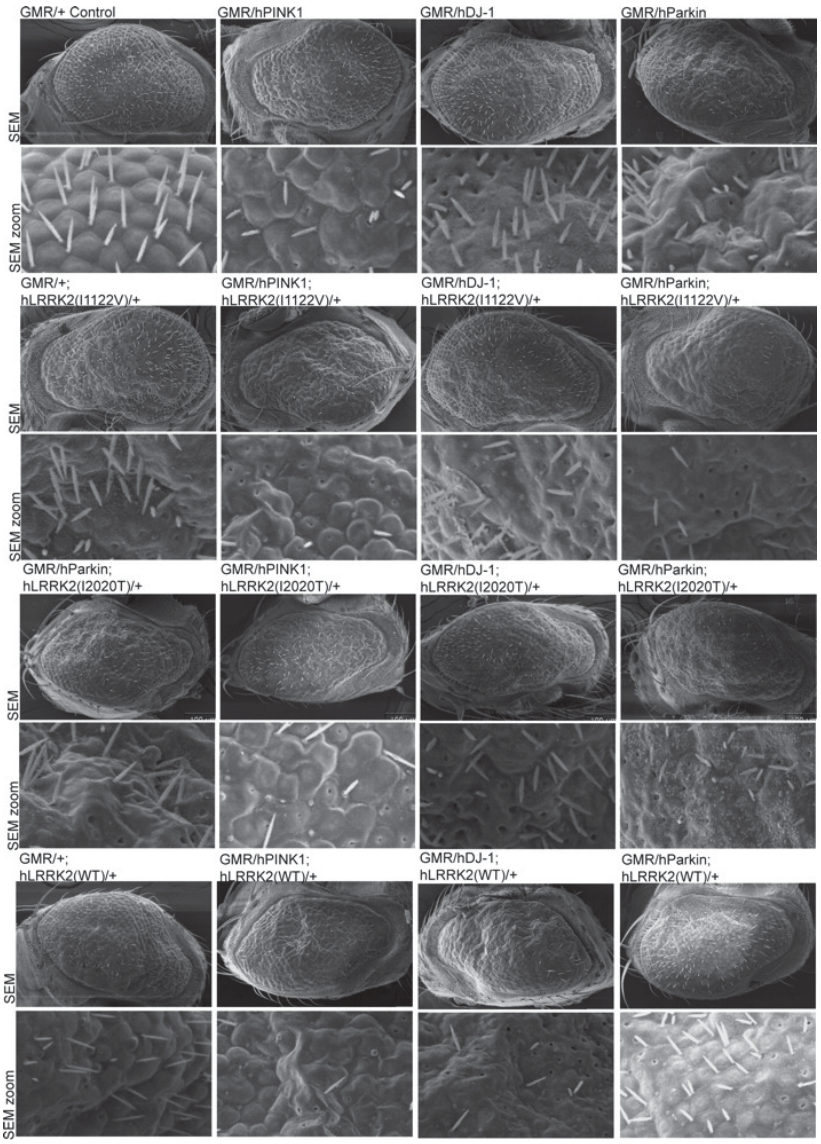

Fig. 2.1.10: *hLRRK2* interacts with *hParkin, hPINK1* and *hDJ-1* in the eye at 29°C.

Representative images from SEM.

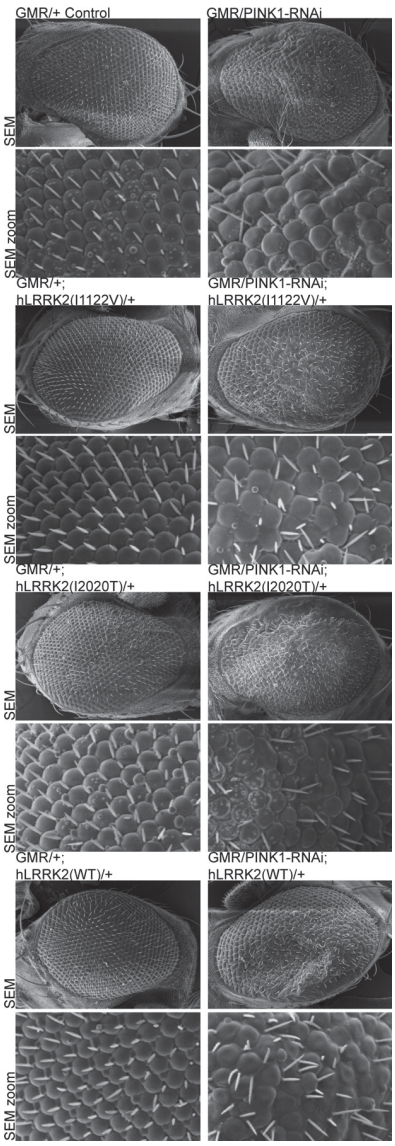

Fig. 2.1.11: Interactions of *hLRRK2* with *PINK1-RNAi* in the eye at room temperature.

Representative images from SEM.

Similar to *Parkin*, expression of *hDJ-1* by itself, at 29˚C, caused a rough eye phenotype with holes (Fig. 2.1.10) and pigmentation loss (Table 2.1.3). Expression of either of the two *hLRRK2* mutants did not rescue this *hDJ-1* phenotype. Indeed, *hLRRK2(I1122V)*, *hLRRK2(WT)*, and to a lesser extent, *hLRRK2(I2020T)* caused a significant exacerbation of the *hDJ-1* phenotype - especially a pronounced loss of interommatidial bristles, as evidenced by SEM analysis (Fig. 2.1.10). In common with *hPINK1* and *hParkin, hDJ-1* expression significantly ameliorated black lesions formation in *hLRRK2(I2020T)* or *hLRRK2(WT)* (Fig. 2.1.9A). Under SEM, loss of *DJ-1* led to a phenotype that was qualitatively and quantitatively similar to *hDJ-1* overexpression (Fig. 2.1.13). Moreover, this phenotype was potentiated by *hLRRK2* expression in a similar fashion (Fig. 2.1.13). Unlike with *DJ-1* expression, however, *DJ-1* loss led to the appearance of black lesions on the eye surface and this effect was not altered by *LRRK2* expression (Fig. 2.1.9A).

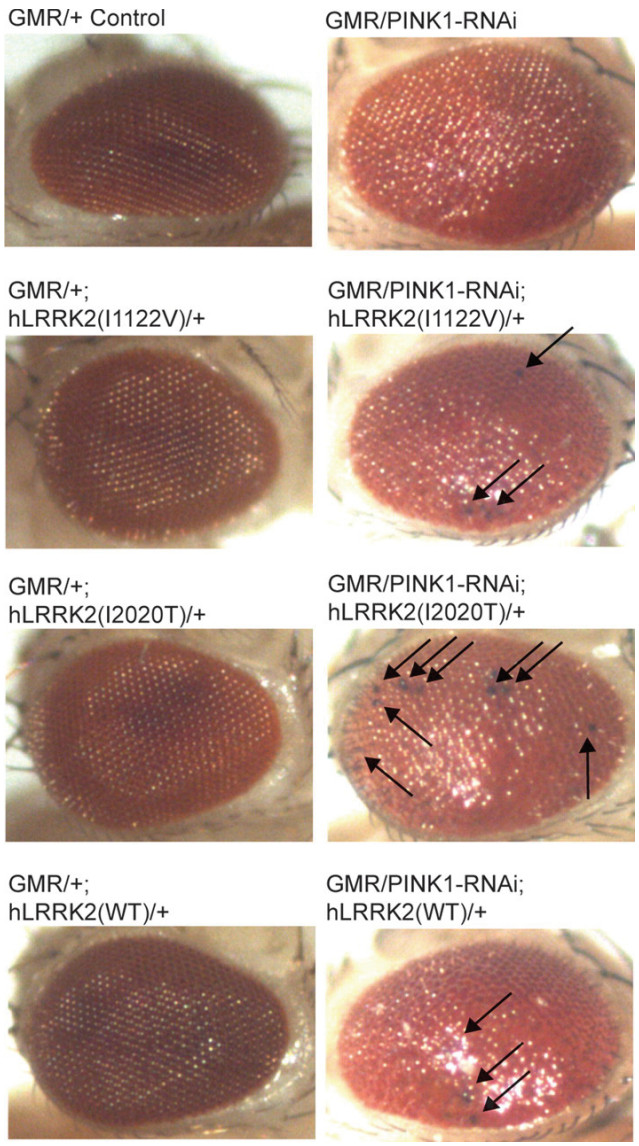

Fig. 2.1.12: Interactions of *hLRRK2* with *PINK1-RNAi* in the eye at room temperature.

Representative images from optical microscope. The arrows point to a black lesion.

Table 2.1.4: Summary of the effects of *hLRRK2* expression on eye pigmentation at RT. Interaction with *PINK1*.

Loss of pigmentation in the eye and formation of black lesions, as seen under optical microscope. Interactions of *hLRRK2* with *PINK1-RNAi* at room temperature.

Genotype	% of flies with loss of pigmentation (±SEM)
GMR/+	0 (± 0)
GMR/hLRRK2 (WT)	0 (± 0)
GMR/hLRRK2 (I1122V)	0 (± 0)
GMR/hLRRK2 (Y1699C)	0 (± 0)
GMR/hLRRK2 (I2020T)	0 (± 0)
GMR/PINK1-RNAi	42.93 (± 10.42)
GMR/PINK1-RNAi; hLRRK2 (I1122V)	100 (± 0)
GMR/PINK1-RNAi; hLRRK2 (I2020T)	100 (± 0)
GMR/PINK1-RNAi; hLRRK2 (WT)	100 (± 0)

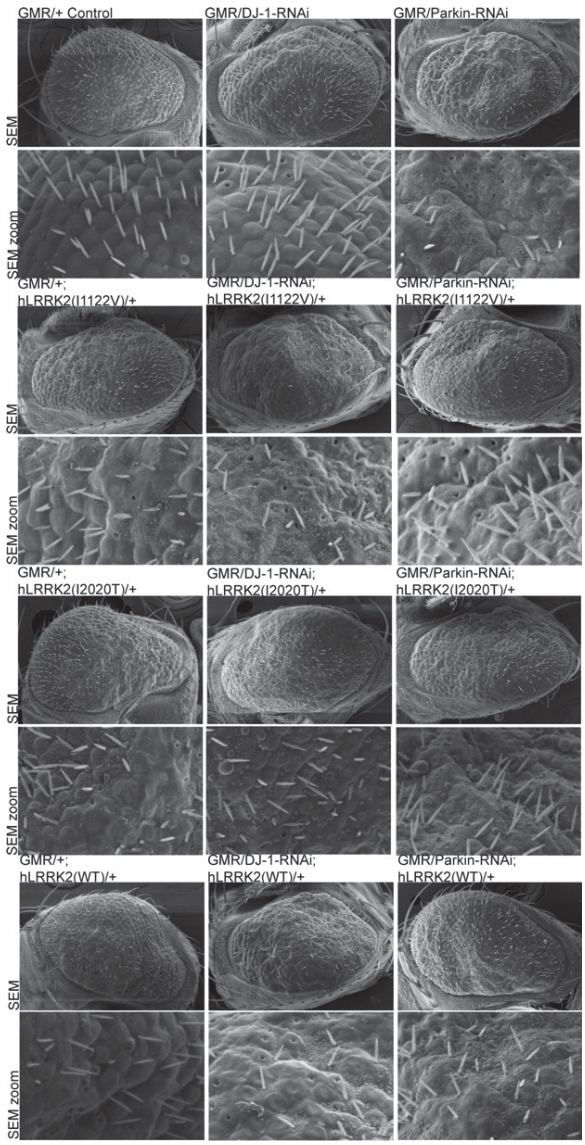

Fig. 2.1.13: Interactions of *hLRRK2* with DJ-RNAi and Parkin-RNAi in the eye at 29°C.

Representative images from SEM.

In conclusion, expression of all three recessive PD genes inhibits black lesion formation suggesting a genetic interaction. This is further supported by the observation that loss of *Parkin* or *PINK1* exacerbates, at least in most cases, black lesion formation. However, clearly all of the defects observed by *LRRK2* are not rescued by these recessive PD genes. Moreover, expression of *hLRRK2(WT)* or *hLRRK2(I1122V)* (or, to a lesser extent *hLRRK2(I2020T)*) substantially potentiated bristle loss seen in *PINK1, DJ-1*, or *DJ-RNAi* flies, respectively.

Discussion:

Our results are of significance because of the following: (i) We have generated *hLRRK2* fly models using several independent *hLRRK2* mutant lines which support a pro-death role of *LRKR2* in dopaminergic neurons. (ii) We also show that *LRRK2* flies have multiple surprising phenotypes not expected of a protein with pro-death function. This includes a complex and nonlinear behavioral phenotype, as well as increased basal lifespan. (iii) We demonstrate that these transgenic flies show increased sensitivity to rotenone both in terms of lifespan and dopaminergic loss, suggesting a potentially important relationship between environment and genetics. (iv) Finally, we show a complex interesting genetic interaction between *LRRK2* and the recessive PD genes.

Recently, two papers have described the effects of expression of the fly orthologue of *hLRRK2, dLRRK*, with conflicting results. Lee *et al.* shows no loss of dopaminergic neurons or deficits in climbing ability (Lee et al., 2007). In contrast, Imai *et al*. shows loss of dopamine and of dopaminergic neurons accompanied by

behavioral deficits (Imai et al., 2008). Because *hLRRK2* and *dLRRK* exhibit only 38-44% similarity in their domains, and because questions have been raised as to whether *dLRRK* is a true orthologue of *hLRRK2* (Poole et al., 2008), it is important to assess the effect of *hLRRK2* expression. Accordingly, we have developed and characterized independent lines of WT and mutant *hLRRK2*-expressing *Drosophila*. First and foremost, these flies display no overt developmental defects, notably a lack of nervous system pathology. This is perhaps unexpected given the association of *LRRK2* with axonal development and outgrowth (MacLeod et al., 2006). Thus, subtle effects on nervous system integrity cannot be ruled out at this point. Clearly, however, our results indicate that expression of any of the human or other *LRRK2* mutants result in loss of dopaminergic neurons. These results are consistent with the notion that *LRRK2* expression results in selective dopaminergic loss in *Drosophila* without overt effects on other neuronal subpopulations. Recently, Liu *et al* showed a similar degree of loss of dopaminergic neurons in all clusters for both WT and kinase domain mutant of *hLRRK2* (Liu et al., 2008). Taken together with our current evaluation of WT and three independent *LRRK2* mutants, these data strongly support a pro-death role for ectopic *LRRK2* expression, at least in *Drosophila.*

The effects of *LRRK2* expression on locomotor behavior are complex. After an expected initial deterioration in performance compared with control (that correlates with loss of DA neurons), all the transgenic lines outperformed the control flies at later time points. While the earlier diminution of activity is consistent with that reported by others with *dLRRK* expression or expression of the human G2019S mutant, our results suggest that the consequences of dopaminergic loss may be quite complex at later time

points. Clearly, at these points, behavior does not correlate with dopaminergic loss. However, we speculate that this effect reflects a dopaminergic or non-dopaminergic compensatory mechanism resulting from loss of dopaminergic neurons. Consistent with this, it is known that in mice treated with the dopaminergic toxin MPTP exhibit an increase in dopamine turnover. This may reflect a mechanism by which the surviving dopaminergic terminals compensate for a decrease in the neuronal population. In fact, numerous reports have suggested that under certain conditions, mice may display increased locomotor activity upon MPTP treatment (Sedelis et al., 2001). Similarly, we propose that the later increase in locomotor activity observed in flies may be a compensatory response to loss of a subset of dopaminergic terminals.

Our results also show some surprising results when it comes to basal lifespan of *hLRRK2* transgenic flies. For example, under room temperature conditions with ubiquitous expression, most transgenic *hLRRK2* lines showed increased lifespan compared with controls. Interestingly, in support of our data, *dLRRK* loss-of-function mutants have a slightly shorter lifespan (Wang et al., 2008). This suggests that *LRKK2*, in addition to its pro-death function as it relates to dopaminergic neurons, may possess properties which are protective. It is important to note that these results contrast with a recently published paper (Liu et al., 2008) which shows a shortened lifespan of flies expressing WT or kinase mutant of *hLRRK2*. The reason for this discrepancy is unclear. However, we noted that our flies were grown under less crowded conditions than previously reported and that the control flies in the aforementioned report showed significantly shorter lifespan than our own controls (Liu et al., 2008). Finally, it is important to note that specific neuronal expression of mutant *LRRK2* (in contrast to

ubiquitous expression of *LRRK2* or expression of WT *LRRK2* in neurons) did not promote differences in lifespan at any temperature. However, *LRRK2* mutant expression in TH positive neurons still affected climbing behavior in a complex pattern. It is therefore, unlikely that the observed behavioral differences are due to alterations in relative lifespan.

Due to the relatively low lifetime penetrance of *LRRK2* mutations, it is likely that environmental factors play an important role in the etiology of familial PD. Rotenone is a commonly used pesticide and a complex I inhibitor that increases a production of reactive oxygen species. It has been used to model PD in rodents (Sherer et al., 2003) and in *Drosophila* (Coulom and Birman, 2004). We utilized a chronic paradigm with lower doses of rotenone that would more realistically mimic a possible exposure to environmental toxins. Hence, the maximum survival of our control flies in this experiment was relatively long, over 2 months. All *hLRRK2*-expressing lines were significantly more sensitive to rotenone than controls. These results are consistent with the notion that mutations in other PD genes, such as *DJ-1* and *Parkin,* also render cells more sensitive to a variety of external stressors. Moreover, rotenone-treated *hLRRK2* flies exhibited the greatest degree of dopaminergic loss, compared with both rotenone-treated controls, or vehicle-treated *hLRRK2* expressing flies. Taken together, these results point to a potentially important interaction between environmental factors, such as rotenone, and genetic makeup in the control of loss of dopaminergic neurons.

The reasons why *LRRK2* expression increases basal lifespan while increasing susceptibility to exogenous environmental stress are unclear. *LRKK2* has recently been

shown to regulate responses to oxidative stress through phosphorylating 4E-BP (Imai et al., 2008). 4E- BP, in its un-phosphorylated state, acts as a brake on a cap-dependent translation mediated by eIF4E. Clearly, the regulation of this pathway (and protein translation) has a large number of consequences depending upon the circumstances. Overexpression of *dLRRK* has been linked to oxidative stress via this pathway (Imai et al., 2008). Interestingly, some authors noted that low levels of oxidative stress result in increased longevity (Schulz et al., 2007). It is possible, for example, that overexpression of *hLRRK2* may result in such an increase under low stress (basal) conditions, but reduce longevity when confronted with higher levels of environmental stressors. Further studies are required to explore these possibilities.

The transgenic flies showed a complex eye phenotype, including glossy and rough surface with necrotic lesions, pigmentation loss, holes, disorganization and/or loss of interommatidial bristles and disorganization of the ommatidial array. This phenotype allowed us to analyze the interaction of *LRRK2* with other known PD genes. We have presented strong evidence that the three recessive PD genes interact with *LRRK2*. However, the genetic interactions are not straightforward. The fact that they don't always follow what one would expect (e.g. that overexpression of *PINK1* is protective) highlights the complexity of the matter. Just as one example, *PINK1* (as well as *Parkin* or *DJ-1*) clearly present a relatively straightforward interaction with *LRRK2* when it comes to the formation of black lesions. In most cases, expression of *PINK1* leads to a reduction in black lesions while loss of *PINK1* exacerbates these black lesions. This would strongly implicate a protective role of *PINK1* in black lesions formation with respect to *LRRK2*. However, when one looks at other parameters, such as bristle loss,

PINK1 expression in fact exacerbates the *LRRK2* phenotype. It seems that the right dose of (or balance between) *LRRK2, PINK1, DJ-1* and *Parkin* is crucial for cell survival. In the case of *PINK1*, this might make sense considering growing evidence of the importance of *PINK1* in mitochondrial dynamics and quality control (Yang et al., 2008). In this case, too much *PINK1* activity might have a deleterious effect, similar perhaps to loss of function. This observation also adds a level of complexity to the understanding of the protective role of *PINK1* reported by several groups, including our own (Gautier et al., 2008; Haque et al., 2008). We propose that the direction of the interaction (suppression vs. enhancement of the phenotype) depends on several other factors, especially the parameter/cell type studied.

LRRK2 impacts a subset of signaling pathways common to these PD genes, although the biochemical underpinnings of the interaction between *LRRK2* and the other Parkinson's genes are unknown. For example, *DJ-1* has been shown to modulate the PI3 kinase/AKT pathway in flies (Yang et al., 2005), an upstream branch of mTOR pathway which regulates 4E-BP. In addition, *Parkin* has been shown to interact with *LRRK2* in mammalian cells *in vitro* (Smith et al., 2005). It is important to emphasize that only certain *hLRRK2* mutations affect the different parameters analyzed and/or genetically interact with *hPINK1*, *hParkin* or *hDJ-1*. The reason for this is unclear but may relate to potentially different signaling pathways affected by different mutants.

In conclusion, we have generated a *hLRRK2* fly model of PD and identified *PINK1, Parkin* and *DJ-1* as *LRRK2* interactors. This demonstrates that this model is suitable for a suppressor/enhancer screening.

Materials and Methods

Drosophila genetics:

The flies were maintained on a standard cornmeal/agar medium at RT or at 29˚C. The cDNA encoding human WT or mutant *LRRK2* were obtained from *pcDNA3.1 (+)* with *BamH1/Xho1* double digests and cloned to *pUAST* vector at Bglll/Xhol site. The plasmids were microinjected to w1118 fly embryo (Genetic Services, Cambridge, MA). The other fly stocks were described earlier. *UAS-hParkin* (Yang et al., 2003), *UAS-hPINK1* (Yang et al., 2008), *UAS-PINK-RNAi* (Wang et al., 2006) and *UAS-hDJ-1* (Yang et al., 2005). *TH-Gal4, Elav-Gal4* and *Da- Gal4* flies were obtained from Bloomington Drosophila Stock Centre, *UAS-DJ-1-RNAi*, *GMR-Gal4/BC* and w1118 flies were a gift from Dr. Bingwei Lu, Dr. Yong Rao (Cafferty et al., 2006) and Dr. Margaret Sonnenfeld (Sun et al., 2006), resp. *UAS-Parkin-RNAi* flies were obtained from the Vienna Drosophila Research Centre.

RT-PCR and Western blot:

Samples were reverse-transcribed and RT-PCR was performed with the following primers: 5'-CGATCCATGGCTAGTGGCAGCTGT-3' (forward) and 5'-CCTCTGAGACTCTCTCAAACAGC-3' (reverse). For the Western blot, we used an anti-LRRK2 rabbit polyclonal antibody (Novus Biologicals), and E7 mouse monoclonal anti-β-tubulin antibody (Developmental Studies Hybridoma Bank) for loading control.

Quantification of dopaminergic neurons:

Male flies expressing *LRRK2* under the control of the *TH* promoter (and *TH-Gal4/+*

controls) were aged at RT for 10 and 50 days (or for 20 days at 29˚C, as indicated). Dissected brains were fixed and TH positive neurons of the posterior clusters were visualized by staining with polyclonal rabbit anti- TH primary Ab (Novus Biologicals) and a fluorescent Ab (Alexa 488). Each whole brain was scanned using optical sections and the collected Z-series images were projected into a 3-D animation to quantify numbers of TH-positive neurons.

Locomotor behavior:

Males were aged for 10-70 days and divided into sets of 10 the day before the experiment. Next day, the flies were transferred into transparent tubes with a horizontal line 8 cm above the bottom. After 10 min at room temperature, the flies were tapped down and filmed. The number of flies that crossed the line in 10 and 20s was recorded, as indicated. All behavioral experiments were carried out at room temperature under standard light conditions.

Lifespan:

hLRRK2 flies (or w1118 control) were crossed with *Da*, or *Elav* driver flies. The crosses were performed and flies were maintained at RT or at 29 ˚C, as indicated. The conditions of the cross, including the number of parent males and females, were kept the same for all genotypes. The flies from each genotype were collected within 48h *post*-eclosion, divided into sets of 20 and aged. The vials were changed every 3-7 days.

Rotenone sensitivity:

Individual stocks of rotenone (Sigma) that were dissolved in dimethyl sulfoxide, kept frozen and protected from light, were mixed with water used to rehydrate the instant fly food media (Carolina Biologicals) (final concentration in the food: 100 μM). The food containing rotenone was made fresh and changed every 2-4 days. *LRRK2* or w1118 control flies were crossed with *Da* driver flies at RT. The conditions of the cross, including the number of parent males and females, were kept the same for all genotypes. The flies from each genotype were collected within 24h post-eclosion, divided into sets of 20, placed in the rotenone-containing vials and aged at RT protected from light. For rotenone sensitivity of the DA neurons, flies were treated for 1 month.

Eye phenotype:

Flies were crossed and maintained at 29˚C. For SEM, heads of males expressing *hLRRK2* under the eye-specific *GMR* promoter and control were fixed and dehydrated. To study the ommatidial organization, tangential sections of the heads in Durcupan resin were cut at 2μm, mounted and stained with toluidine blue. The SEM and sectioning was done by the Advanced Bioimaging Center, Mount Sinai Hospital, Toronto. All flies were analyzed 10 days post-eclosion.

Progeny quantification:

Both male and female parents came from standardized fly cultures (same number of male and female parents in all crosses). Eight 0-1 days old ubiquitously expressing *hLRRK2,* or control *Da/+*, males were crossed with ten 0-1 days old unmated females of

the same genotype. After 5 days of laying eggs, these parents were placed in a fresh vial and allowed to lay eggs for 5 more days. Newly eclosed flies were periodically removed from the vials; they were allowed 23 days to eclose.

Cell culture and recombinant adenovirus infection:

The primary culture of mouse cortical neurons was carried out as described previously (Zhang et al., 2006). The adenoviruses expressing *lacz*, wild-type (WT) or R1441C mutant forms of *LRRK2* were engineered. The experiments were performed at a multiplicity of infection of 100 plaque-forming units per cell. Adenoviral vectors were added to cell suspension immediately before plating. Two days after plating, cells were fixed and stained with Hoechst 33258 and neuronal survival was evaluated by assessing nuclear integrity of GFP-positive or lacz-positive neurons as previously described (Aleyasin et al., 2004).

Embryo staining:

Elav/hLRRK2 embryos were aged for 10-11 hours on standard agar-apple juice plates at 4˚C, fixed and incubated with the primary mouse anti-CNS axons BP-102 antibody (Developmental Studies Hybridoma Bank) followed by the goat anti-mouse HRP-conjugated secondary antibody (Promega, Madison). HRP activity was detected by precipitation of 3-3' diaminobenzidine (DAB) in the presence of H_2O_2. The embryos were scored as defective if there was one or more breaks in longitudinal connectives or commissures.

Statistical analysis:

The data were analyzed as specified, expressed as means ± standard error of means, and denoted * if $P \leq 0.05$, ** if $P \leq 0.01$ and *** if $P \leq 0.001$.

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Conflict of Interest statement. None Declared.

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Part 2.2: Screening for Genetic Interactors of LRRK2 Using a *Drosophila melanogaster* model of *LRRK2*-linked PD

Introduction

Following the successful characterization of our *D. melanogaster* model of LRRK2-linked PD (Venderova et al., 2009), we proceeded to use this system to better delineate the biological function(s) of the LRRK2 protein. When we began the screening, LRRK2's gross anatomical location (Higashi et al., 2007b; Higashi et al., 2007a; Melrose et al., 2007), subcellular distribution (Berger et al., 2010; Biskup et al., 2006) and "toxic" nature (Iaccarino et al., 2007; Smith et al., 2006; West et al., 2007), was generally demonstrated and accepted. However, very little was reported or confirmed regarding its protein-protein interactions. Therefore, we utilized our *D. melanogaster* model of LRRK2-linked PD to perform an unbiased, genome-wide, high-throughput screen (as reviewed in (St Johnston, 2002)). This *in vivo,* genetic screening method has been used successfully (Collins and Cohen, 2005; Fernandes and Rao, 2011; Kim et al., 2005b; Lee et al., 2001; McElwain et al., 2011).

The purpose of this screen was to screen for genetic interactors across all of the major chromosomes of the *D. melanogaster* system. This comprises the second and third chromosome, which represent the majority (~80%) of the melanogaster genome (dos Santos et al., 2015; Fernandes and Rao, 2011). The first and fourth chromosomes are not routinely tested, as they represent the sex chromosome and a negligently sized chromosome that lacks key genes, respectively (dos Santos et al., 2015). As such, we could perform an almost complete genome-saturated, unbiased screen to identify individual gene(s) and pathways whose roles could provide insight into potential LRRK2-associated cellular functions.

Methods

Using the GAL4/UAS system, we generated fly lines that stably and ectopically over-expressed various forms of hLRRK2 (WT and mutants: I1122V, Y1669C, R1441C, and I2020T) under the control of the tissue-specific glass multiple reporter (GMR) promoter element. This GMR system selectively drives the expression of the hLRRK2 transgenes in the developing drosophila compound eye (Freeman, 1996; Song et al., 2000). This lead to an eye phenotype consisting of: reduced pigmentation, increased holes and roughness and the appearance of black spots (Venderova et al., 2009). By crossing these flies with lines that lacked genomic sequences at 29°C, I screened for genetic interactions that either exacerbated (genetic enhancers) or attenuated (genetic suppressors) the established hLRRK2 eye phenotype.

The stably expressing hLRRK2 flies (GMR/CyO ; UAS-hLRRK2/TM6B) were crossed with Bloomington Deficiency Kit drosophila lines, made commercially available by the Bloomington Drosophila Stock Centre (BDSC) at Indiana University (http://flystocks.bio.indiana.edu/bloomhome.htm). The Bloomington Deficiency Kit contains a library of *D. melanogaster* models that have varying sized regions of their genome deleted in a manner that is tracked using balancer chromosomes (BC). The resulting F1 progeny had eyes that over-expressed hLRRK2 in the background of a haplo-insufficient, partial chromosomal deletion. The proper progeny were identified by the absence of any balancer chromosome markers. Please refer to Figure 2.2.1. for screening strategy and processing. The F1 progeny eyes were visually assessed under a light microscope. The eye phenotype was scored against various parameters of overall morphology including: roughness, deformation, holes, loss of pigmentation and the presence, number and size of black spots. Flies were simultaneously compared to controls, where hLRRK2 or the deficiency-line alone was present with the GMR-GAL4 driver. A suspected enhancement or suppression of the basal LRRK2-induced phenotype was repeated with a second independent cross and assessed by two separate investigators within the lab. Once

confirmed, the stock line was identified as a candidate interaction and moved forward along the experimental pipeline.

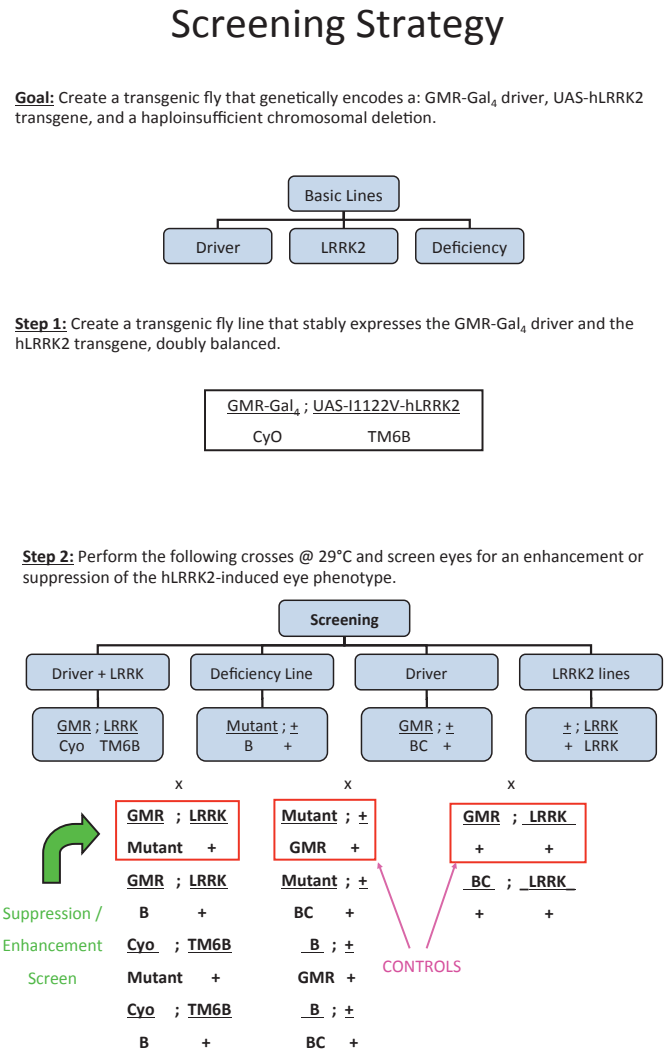

Figure 2.2.1. Suppressor/enhancer genetic screening strategy.

D. melanogaster models stably expressing a UAS-hLRRK2 transgene product in the compound eye of the fruit fly, under the control of the GMR-Gal4 driver system, were generated and crossed with a library of flies carrying chromosomal deletions commercially available through the Bloomington Deficiency Kit. The resulting target progeny's eye phenotype was scored and simultaneously compared to their appropriate dual controls. GMR: Glass Multiple Reporter; B: Balancer; BC: Balancer Chromosome; CyO = Curly wing balancer; TM6B = Tubby humeral balancer.

Results

The screen originated with the GMR/CyO ; I1122V-hLRRK2/TM2 fly line and the Primary Bloomington Deficiency Kit corresponding to the right arm of the second (2R) chromosome (http://flystocks.bio.indiana.edu/Browse/df/dfkit2r.php). I screened 64% of the original 2R chromosome stocks ordered (summarized in Table 2.2.1.). This primary 2R deficiency screening produced: 26 non-interacting deficiency kit stock lines; 2 lethal lines which were not viable at 29°C temperatures; 4 unclear results in need of reassessment; 5 stocks whose balancer identification needed confirmation; and excitingly 1 validated interacting stock line – #7876, confirmed twice by two independent investigators, myself and another screening colleague (G. Kabbach), who remained blinded.

The 7876 fly candidate (genotype denoted as GMR/7876 ; I1122V/+) exhibited an exacerbated or "enhanced" eye phenotype. Specifically, it presented with an increase in the number and size of black spots present (Figure 2.2.2.). This primary stock represents a deficiency denoted as "*Df(2R)Exel7131,*" based on its generator Exelixis, Inc. (Parks et al., 2004). The enhancement suggested that the summation of the single-gene deficient interactions that spanned this region resulted in more damage and could be interpreted to mean that the overall presence of that region would naturally have played a protective role against the toxicity of over-expressed hLRRK2.

Table 2.2.1. Right arm of second chromosome screening summary.

Summary of the screening work personally conducted on the right arm of the second chromosome (2R) of the *D. melanogaster* genome.

Set #	Stock #	Observations	Remaining	Re-Ordered
1	739	No Interaction Phenotype Noted	1702	**6779**
	6647	No Interaction Phenotype Noted	1547	**6609**
	7145	No Interaction Phenotype Noted	3518	**3520**
	7896	No Interaction Phenotype Noted	1682	**6780**
	9596	No Interaction Phenotype Noted	2471	**3467**
2	**7876**	**ENHANCER**		**749**
	5574	Unclear - Repeat		**7273**
	5680	Unclear - Repeat		**9069**
	282	No Interaction Phenotype Noted		**7445**
	201	No Interaction Phenotype Noted		**9410**
3	1888	No Interaction Phenotype Noted		**5879**
	3368	No Interaction Phenotype Noted		**4966**
	3909	No Interaction Phenotype Noted		**1007**
	442	No Interaction Phenotype Noted		**6455**
	6516	No Interaction Phenotype Noted		
	754	No Interaction Phenotype Noted		
4	198	No Interaction Phenotype Noted		
	1743	No Interaction Phenotype Noted		
	7414	No Interaction Phenotype Noted		
	2604	No Interaction Phenotype Noted		
	9691	No Interaction Phenotype Noted		
5	11501	Repeat after re-balancing		
	7598	Repeat after re-balancing		
	8074	Repeat after re-balancing		
	6965	Repeat after re-balancing		
6	7146	No Interaction Phenotype Noted		
	6866	No Interaction Phenotype Noted		
	190	No Interaction Phenotype Noted		
	4961	Unclear - Repeat		
	757	No Interaction Phenotype Noted		
7	7875	No Interaction Phenotype Noted		
	1145	Unclear - Repeat		
	9496	Un-identifiable Balancer		
	3591	No Interaction Phenotype Noted		
	5246	No Interaction Phenotype Noted		
8	7441	No Interaction Phenotype Noted		
	6917	No Progeny - Repeat at Low Temp.		
	4960	No Progeny - Repeat at Low Temp.		
TOTAL	**38**		**5**	**14**

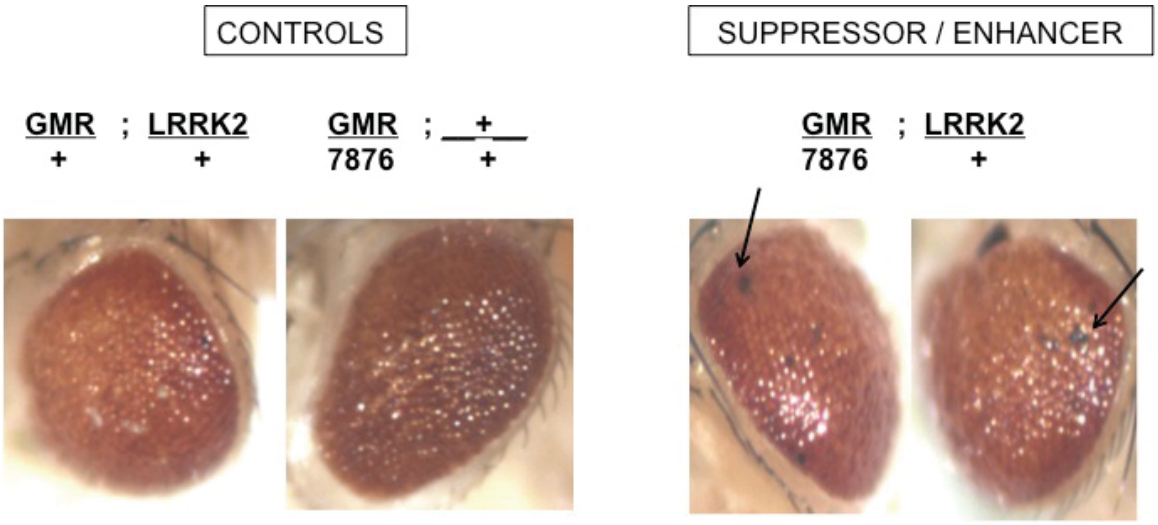

Figure 2.2.2. Primary deficiency kit LRRK2 genetic interacting stock line 7876. Zone Representative images of the proper controls (GMR/+ ; LRRK2/+ and GMR/7876 ; +/+) and the enhanced F1 interactor progeny (GMR/7876 ; LRRK2/+) identified while screening the right arm of the second (2R) chromosome. Stock #7876 corresponds to deficiency line *Df(2R)Exel7131*. Notice the increased number and size of the black spots, as indicated by the black arrows. Images captured by 10X objective optical microscope.

In this case, the *Df(2R)Exel7131* chromosomal deletion corresponded to a region with 16 fully deleted genes and another 2 disrupted genes (Appendix II - Table S.1). Only those genes with corresponding human homologs were pursued. Subsequent screening of the *Df(2R)Exel7131* (7876) interacting region was conducted, by Ghassan Kabbach (M.Sc.), Paul Marcogliese (Ph.D. Candidate) and Sarah Seang (B.Sc. Honour's). Using stock lines that corresponded to single-gene manipulations, either down-regulation (RNAi) or over-expression (enhanced promoter) lines, they successfully identified a total of 3 candidate genes: Mdr50 (p-glycoprotein), convoluted (insulin-like growth factor binding protein, acid labile subunit), and Hsc-70-5 (heat shock 70kDa protein 9B) as positive, genetic interactors of LRRK2 (summarized in Table 2.2.2). The nature of the interaction suggests that the genes in question possess a protective role against LRRK2, as they revealed their interactions in the form of enhancements.

Table 2.2.2. Follow-up individual genetic interaction screening results from stock line 7876. Summary of the follow-up data produced by team members: G. Kabbach, P. Marcogliese, and S. Seang, who completed the subsequent screening stages following my discovery of the first positive candidate primary region stock line, #7876. S: Suppression, E: Enhancement, ND: No Difference and NC: Not completed, N/A: Not Applicable.

Candidate Hit #1:					
***D. melanogaster* Gene: Heat Shock Protein cognate 5 (Hsc70-5)** **Type of Disruption: dsRNA** ***H. sapiens* ortholog: Heat Shock Protein cognate (Hsc) 70KDa Protein 9 Precursor (mortalin)**					
Parameter / hLRRK2 Transgene	**WT**	**I1122V**	**Y1669C**	**R1441C**	**I2020T**
GMR-GAL4 Eye Screen (29°C)	S	S	S	S	S
GMR-GAL4 Eye Screen (25°C)	N/A	N/A	N/A	N/A	N/A
TH-GAL4 TH Neuron System Screen					ND
Candidate Hit #2:					
***D. melanogaster* Gene: Multi Drug Resistance 50 (Mdr50)** **Type of Disruption: dsRNA** ***H. sapiens* ortholog: p-glycoprotein**					
Parameter / hLRRK2 Transgene	**WT**	**I1122V**	**Y1669C**	**R1441C**	**I2020T**
GMR-GAL4 Eye Screen (29°C)	N/A	N/A	N/A	N/A	E
GMR-GAL4 Eye Screen (25°C)	E	E	N	E	E
TH-GAL4 TH Neuron System Screen					ND
Candidate Hit #3:					
***D. melanogaster* Gene: convoluted** **Type of Disruption: dsRNA** ***H. sapiens* ortholog: insulin-like growth factor binding protein, acid labile subunit**					
Parameter / hLRRK2 Transgene	**WT**	**I1122V**	**Y1669C**	**R1441C**	**I2020T**
GMR-GAL4 Eye Screen (29°C)	N/A	N/A	N/A	N/A	E
GMR-GAL4 Eye Screen (25°C)	N	N	N	N	E
TH-GAL4 TH Neuron System Screen					S

Outcomes and Future Directions

This project was succeeded from the original screening leads (Elizabeth Abdel-Messih, PhD Candidate, Ghassan Kabbach, M.Sc., Dr. Katerina Verderova, Ph.D.) by Paul Marcogliese (PhD Candidate), Sameera Abuaish, (M.Sc. Graduate), and a team of trainees: Sarah Seang, Cindy Wei, Amanda Perrozo, Gary Li, Alaa Fanous, Yanick Lee, Brent Phillips and Francis Lebrun, who have all contributed and completed the screening. A total of 36 individual gene hits have been identified and confirmed in the eye screen and in the tyrosine hydroxylase (TH) positive clusters of the drosophila CNS. Some have also undergone behavioural analysis and are under investigation in mammalian systems.

1. The manuscript corresponding to this screen is in preparation for submission:

Marcogliese PC, Abuaish S, **Abdel-Messih E**, Kabbach G, Seang S, Li G, Slack R, Haque EM, Venderova K, Park DS. Functional genetic interactors modifying LRRK2-induced eye degeneration and dopaminergic cell loss in Drosophila. *In preparation*.

2. Results from interactors identified using the screen are under investigation and in preparation for publication:

Kim KS, Marcogliese PC, LeBrun FR, Wei C, Yang JW, **Abdel-Messih E**, Kabbach G, Slack RS, Haque EM, Venderova K, Schlossmacher MG, Hayley S, Park DS. LRRK2 regulates phagocytosis via direct phosphorylation of the actin nucleating complex, WAVE2. *In preparation*.

CHAPTER 3
Cellular Responses and Neuronal Survival of R1441-LRRK2 Murine Strains Following MPTP/MPP$^+$ Treatment

Manuscript formatted and in preparation for submission

Statement of Authors Contribution

E. Abdel-Messih designed and performed all of the experiments, with some assistance from authors (described below); maintained and managed all of the murine colonies; performed the majority of the genotyping; prepared all of the figures; performed all of the data analysis; and composed the manuscript in its entirety.

M.W. Rousseaux – performed MPTP injections (chronic paradigm) and analyzed a few brain SNc samples (doubly blinded) to ascertain results (Fig. 3.3).

D-S. Im: Performed MPTP injections for a subset of the metabolite studies (Lrrk2 KI) included in (Fig. 3.7).

S. Abuaish: Assisted with PRC for genotyping of the LRRK2 animal colonies.

P.C. Marcogliese: Quantified a few MPTP injected brains (doubly blinded) to confirm the SNc phenotype being reported (Fig. 3.5).

J. Kulczycki: Performed HPLC for experiments in Fig. 3.6 and 3.7, in the lab of our collaborator, Dr. H. Anisman, Carleton University.

Cellular Responses and Neuronal Survival of R1441-LRRK2 Murine Strains Following MPTP/MPP$^+$ Treatment.

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Abstract

The incomplete penetrance and heterogeneous phenotypes associated with LRRK2-linked PD are likely the result of gene-environment interplay. Accordingly, the ability to replicate LRRK2-linked neuronal death, as seen in PD, in animal models is currently lacking. This could be attributed to the nature of controlled animal husbandry, resulting in the lack of exposure to common environmental factors. To explore gene-environment interactions, Lrrk2 Knock Out (KO), R1441C- Lrrk2 Knock In (KI), and transgenic models expressing WT or R1441G human LRRK2 (hLRRK2) were assessed for their ability to sensitize neurons to death following MPTP/MPP^+ treatment, a model of environmental toxins. We also assessed the mitochondrial length phenotypes in primary cortical neurons following ectopic WT and R1441C-hLRRK2 expression and from germline Lrrk2 KO and WT littermates. In Lrrk2 WT and KO primary neurons we assessed whether mitochondrial length phenotypes were modulated following treatment with MPP^+, *in vitro*. In both cases we found no alteration in the average mitochondrial

length. We did note a difference in the distribution of the percentage of mitochondrial binned into various length ranges. The assessment of survival following treatment with MPP^+ in Lrrk2 KO, R1441C KI and WT or R1441G-hLRRK2 expressing mice did not exhibit enhanced cellular toxicity, *in vitro*. Neither did the Lrrk2 R1441C KI, WT or R1441G-hLRRK2 display any differential response following MPTP treatment *in vivo*. Our data indicates that R1441-LRRK2 pathogenic mutants are not susceptible to MPTP-induced death mechanisms. It also provides evidence that other PD-related environmental stimuli may be more relevant for pathogenesis in *LRRK2*-linked cases.

Introduction

Parkinson's disease (PD) is a neurodegenerative disorder that displays an age-dependent prevalence (Pringsheim et al., 2014). The majority of cases are idiopathic (iPD), with certain factors like ageing and exposure to toxic compounds posing an increased risk of developing the disease (Bennett et al., 1996; Morens et al., 1996). However, ~10% of cases are genetically linked, allowing for the exploration of potentially overlapping pathogenic mechanisms (Trinh and Farrer, 2013). Mutations in the leucine-rich repeat kinase 2 (*LRRK2*) gene (OMIM: 609007) account for ~6% of familial PD cases (Di Fonzo et al., 2005) and are found in ~2% of sporadic occurrences (Gilks et al., 2005). They are inherited in an autosomal dominant manner with an incomplete penetrance that, similar to iPD, increases with age (28% at 59 years up to 74% at age 79) (Healy et al., 2008). *LRRK2*-linked PD generally exhibits a phenotype

clinically indistinguishable to iPD (Wszolek et al., 1995). Hence, it is hypothesized that understanding *LRRK2*-linked PD will positively impact our knowledge of pathogenic mechanisms relating to iPD.

The LRRK2 protein is a complex structure that contains protein-protein interaction domains, such as LRR and WD40, and two catalytic domains: GTPase and Kinase (West et al., 2005). Although numerous roles and interactors of LRRK2 have been reported, few have been confirmed and little is known about its true biological function(s). Using a *D. melanogaster* model of LRRK2-linked PD, we demonstrated that over-expression of WT and mutant human LRRK2 (hLRRK2) caused dopaminergic neuronal death, locomotor deficits, and decreased survival that was exacerbated by environmental rotenone exposure. Using this model we performed a genetic enhancer/suppressor screen and demonstrated that hLRRK2 genetically interacted with the recessive PD-linked genes: Dj-1, Parkin and Pink1 (Venderova et al., 2009). The latter genes are most often associated with management of mitochondrial oxidative stress and quality control (Bouman et al., 2011; Clark et al., 2006; Greene et al., 2012; Haque et al., 2012; Irrcher et al., 2010; Joselin et al., 2012; Narendra et al., 2008; Narendra et al., 2010; Parsanejad et al., 2014b; Poole et al., 2008). Since mitochondrial dysfunction has been reported in the post-mortem PD brain tissue and environmental toxins that increase the risk of developing PD commonly target the mitochondria, we chose to pursue this common element in the context of our *LRRK2* mammalian studies (Beal, 2003; Keeney et al., 2006). A role for LRRK2 and mitochondria is supported by its localization (~10% associates with the mitochondrial outer membrane) and its involvement in mitochondrial dynamics (Niu et al., 2012; Wang et al., 2012b; West et al., 2005). This combined with

LRRK2's reduced penetrance suggested that additional environmental triggers that target the mitochondria may be required to a manifest disease phenotype and reiterated the need to explore the gene-environment interplay believed to be associated with *LRRK2*-linked PD.

To test whether toxin exposure could manifest sensitivity in the histological and behavioural asymptomatic murine models of *LRRK2*-linked PD, we challenged various murine models with the mitochondrial-targeting dopaminergic neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahyrdopyridine (MPTP) or its active metabolite, 1-methyl-4-phenylpyridinium (MPP^+) (reviewed in (Przedborski et al., 2000)). The MPTP/MPP^+ (CID:39484) compounds serve as a model of environmental toxins (NCBI, 2016). Although multiple pathogenic mutations span *LRRK2*, we chose to focus on the pathogenic GTPase mutants, since this domain may be more relevant to mitochondrial dynamics related proteins (described in (Li and Beal, 2005)). The R1441 GTPase mutation site has multiple disease-linked amino acid substitutions, with the pathogenic R1441C/G being investigated (Mata et al., 2005b; Ross et al., 2009; Zimprich et al., 2004). Herein we describe the results of our assessment of mitochondrial length phenotypes and *in vitro* and *in vivo* neuronal survival in toxin challenged murine models of WT and R1441-LRRK2.

Results

Over-expression of R1441C-hLRRK2 and Lrrk2 KO do not alter average mitochondrial length, but alter length distribution phenotype in cortical neurons, *in vitro*.

Reports indicate that LRRK2 affects mitochondrial morphology, interacts with mitochondrial fusion and fission related proteins and phosphorylates dynamin-related protein 1 (Drp1) to modulate fission-related dynamics (Niu et al., 2012; Stafa et al., 2014; Su and Qi, 2013; Wang et al., 2012b). This would be of relevance to our environmental MPTP susceptibility, since MPP^+ affects mitochondrial function and motility, which may also affect mitochondrial dynamics (Kim-Han et al., 2011). Therefore, we assessed the effects of LRRK2 expression on mitochondrial length, as a read-out of mitochondrial dynamics. Infection of control, WT or R1441C hLRRK2 expressing constructs for 72 hours in primary, cortical neurons produced no significant difference in the average mitochondrial length phenotype (Fig. 3.1A,B). The R1441C-hLRRK2 neurons displayed a trend towards a shorter average mitochondrial length that remained non-significant. However, when the percentage of mitochondria quantified was binned into length distribution patterns, the WT and R1441C-hLRRK2 had significantly ($p<0.001$) higher percentages of shorter mitochondria (Fig. 3.1C). This shorter distribution phenotype is much more pronounced in the R1441C-hLRRK2 expressing neurons than that of the WT-hLRRK2, when compared to control.

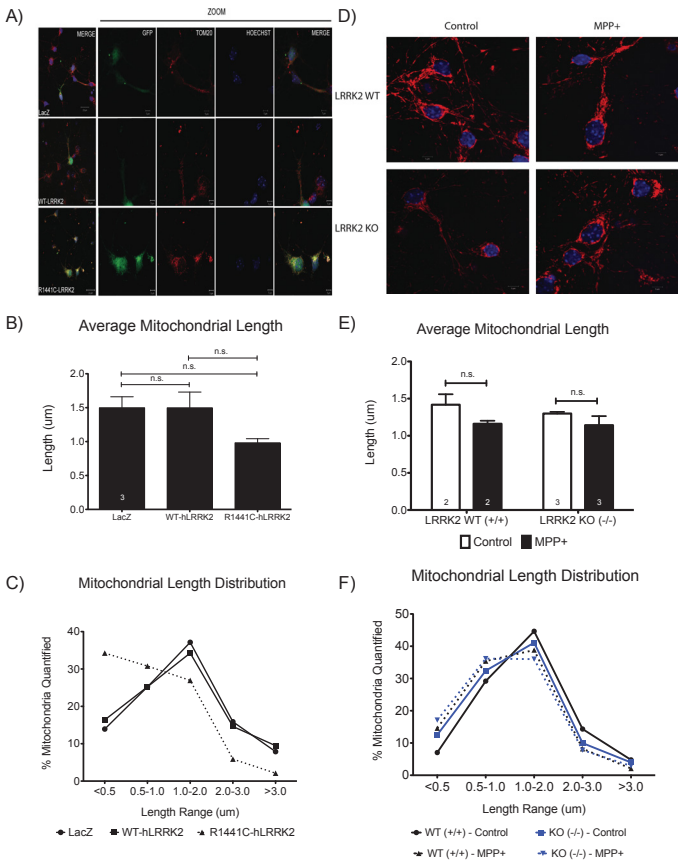

Figure 3.1. Mitochondrial lengths in cortical neurons with varying forms of LRRK2, *in vitro*. (*Left panel*) Assessment of mitochondrial lengths in primary cortical neurons following over-expression of control, WT-hLRRK2 or RG-hLRRK2. (**A**) Representative images of cultures stained with TOM-20 to label mitochondria were quantified and average mitochondrial length (**B**) and mitochondrial length distribution (% of total population quantified) are reported (**C**). Data represents an N=3/vector with ~2500 mitochondria quantified/vector. (*Right panel*) Lrrk2 KO and WT littermate cortical neuron mitochondria following control or 10μM MPP^+ treatment for 6 hrs. (**D**) Representative images quantified to yield: average mitochondrial length (**E**) and length distributions (**F**). Each category represents >5000 mitochondria quantified.

Since the question of both gain and loss of function (GOF/LOF) mutations have been proposed for LRRK2 we tested the opposite scenario; what occurred when Lrrk2 was absent. We assessed the mitochondrial length morphology of cortical neurons from Lrrk2 Knock Out (KO) animals in comparison to their WT littermate controls (Tong et al., 2010). Since the brain morphology of the Lrrk2 KO animals appears normal, and the previous data indicated no overall effect on average mitochondrial length, we chose to assess the mitochondrial morphology of Lrrk2 WT and KO neurons under both basal and 1-methyl-4-phenylpyridinium (MPP^+) treated conditions. We harvested Lrrk2 WT and KO littermate cortical neurons and cultured them for 6 days *in vitro* (DIV). Prior to fixation, cultures were treated with 10μM MPP^+ or control for 6 hours, a time point allowing us to easily detect any shifts in the length distribution curve. There was no significant difference in the average mitochondrial length between the Lrrk2 WT and KO littermate neurons under control or MPP^+ treatment conditions (Fig. 3.1D,E). However, when the percentage of mitochondria was binned into length ranges, a significant difference ($p<0.001$) between the Lrrk2 WT and KO mitochondrial distributions is observed. The Lrrk2 KO neurons display a higher percentage of shorter mitochondria, basally (Fig. 3.1F). Following MPP^+ treatment, both the Lrrk2 WT and KO neurons display a similar shift in their pattern of behaviour – a significant increase in the percentage of shorter mitochondria, with the Lrrk2 KO neurons remaining more fragmented.

Modulating expression of WT or pathogenic R1441-LRRK2 does not alter survival of cortical neuronal cultures exposed to MPP+ treatment, *in vitro*.

The mitochondrial data indicated that no average mitochondrial length changes were noted, but that percentage of length distribution changes can be detected in the mitochondrial length morphology in LRRK2 WT, R1441C and Lrrk2 KO expressing cells. However, whether these changes result in enhanced neuronal sensitivity required further investigation. Therefore, we tested whether LRRK2 played a role in modulating neuronal survival following MPP^+ treatment in transgenic WT, pathogenic (R1441C/G) or KO Lrrk2 murine models. We tested each model using a standard MPP^+ survival time-course paradigm (Haque et al., 2008; Huang et al., 2010; Parsanejad et al., 2014b; Parsanejad et al., 2014a; Qu et al., 2007), wherein primary cortical cultures were exposed to 20 μM of MPP^+ for 12, 24, 36 and 48-hours. We began by assessing the R1441C Lrrk2 KI (Lrrk2 KI) animals, which express the endogenous murine Lrrk2 protein carrying the pathogenic R1441C mutation under the control of the endogenous promoter system (Tong et al., 2009b). When challenged with MPP^+, the homozygous R1441C (KI/KI) mutants responded identically to their WT (+/+) littermate control cultures and did not display any signs of sensitization or alterations in death kinetics (Fig 3.2A). To test whether the loss of Lrrk2 could bring about a phenotype, we compared the survival curves of the Lrrk2 Knock Out (KO) neurons to their WT (+/+) littermate controls (Fig 3.2B). Again we found no difference in the survival percentages.

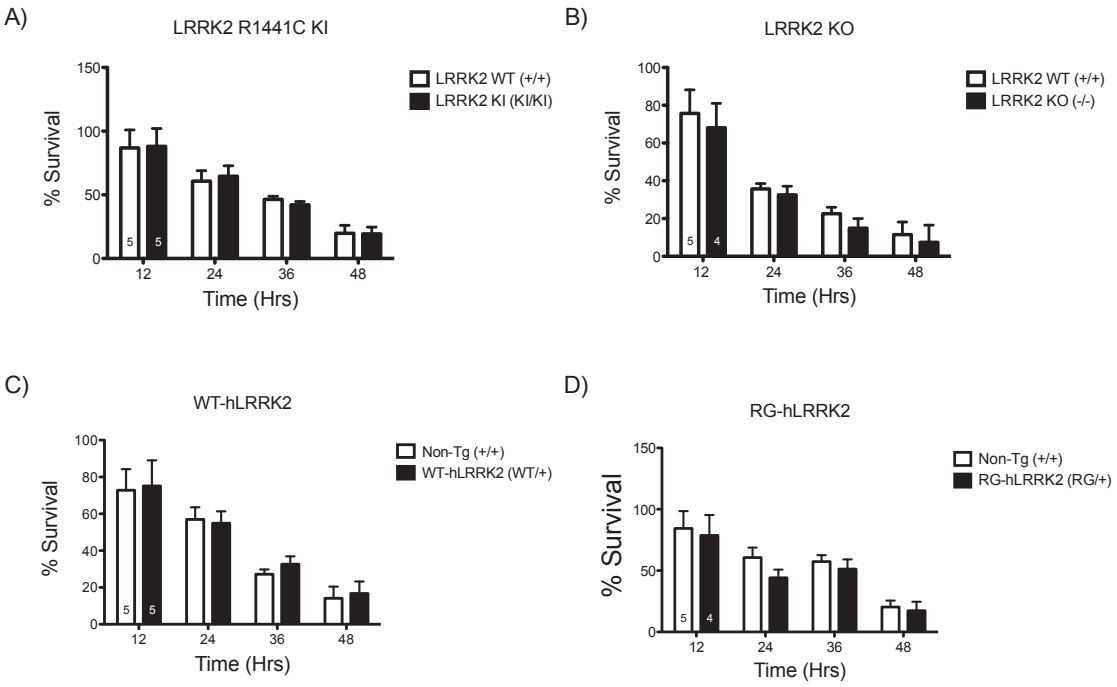

Figure 3.2. Assessment of primary cortical neuron survival following MPP^+ treatment, *in vitro*. Assessment of primary, embryonic cortical neuron survival in cultures harvested from E14.5-15.5 gestational day old embryos from: (**A**) LRRK2 R1441C KI, (**B**) LRRK2 KO, (**C**) WT-hLRRK2 and (**D**) RG-hLRRK2 embryos and their WT or non-Tg littermate controls. Cortical neurons were grown in culture for 6 DIV and treated with either control or 20 μM MPP^+ time-course for the final 12, 24, 36, or 48 hours prior to endpoint and survival analysis. Each bar has been normalized to control treated samples. Note that each strain result came from a single dissection set, as a result of heterozygous crosses, and so all samples were harvested, cultured, treated and analyzed at the same time. Number of embryos (N) indicated in first set of bars.

To determine whether previously reported LRRK2-linked toxicity was a result of protein over-expression, using the same paradigm we tested the over-expressing WT-hLRRK2 and RG-hLRRK2 BAC transgenic (Tg) colonies (Li et al., 2009). Again, when comparing the Tg WT and RG-hLRRK2 primary cortical neurons to each other and to their respective non-Tg (+/+) littermate controls, we found no alterations in their survival responses to MPP^+ treatment (Fig 3.2C,D). To test whether the results from cortical neuronal cultures held true for the intact, adult dopaminergic (DA) system, we assessed the effects of MPTP treatment in these murine models, *in vivo*. This is important since proper MPTP metabolism and function requires input from the glial cells of the adult central nervous system (CNS) (reviewed in (Dauer and Przedborski, 2003)); and LRRK2 levels and function in CNS glial cells has been described (Choi et al., 2015; Gillardon et al., 2012; Hakimi et al., 2011; Miklossy et al., 2006; Moehle et al., 2012). Therefore, it was also important to test the DA neurons of the Substantia nigra pars compacta (SNc), as they are an inherently vulnerable neuronal population (Hirsch et al., 1988; Surmeier et al., 2012; Welberg, 2011).

Expression of LRRK2 does not alter SNc dopaminergic neuron survival following exposure to the neurotoxin MPTP, *in vivo*.

We began by challenging the Lrrk2 R1441C KI (Lrrk2 KI) mice to the well-established *sub-chronic* MPTP paradigm (Haque et al., 2012; Kim et al., 2005a; Lira et al., 2011; Mount et al., 2007; Mount et al., 2013; Qu et al., 2007). Here, male WT (+/+) and homozygous R1441C KI (KI/KI) littermate mice received 1 i.p. injection of 30 mg/kg body weight of MPTP-HCl /day, or equivalent volumes of saline control, for 5

consecutive days and were sacrificed 14 days following the initial injection. Inspection of the striatal dopamine transporter (DAT) fiber density in these mice, a marker of axon terminals that originate from the DA neurons of the SNc, indicated that there were no differences in the level of DAT immunoreactivity between the WT and Lrrk2 KI expressing mice (Fig 3.3A,B). The same results were observed when tyrosine hydroxylase (TH), the rate-limiting enzyme needed for the production of DA, densitometry was assessed in the striatum of these same animals (Fig 3.3C,D). Although the WT animals demonstrate a significant ($p<0.05$) loss, not reached in the KI littermate animals, the comparison between genotypes remains non-significant. We then quantified the number of TH-positive (TH^+) cells present in the SNc using stereological analysis. Again, the presence of the endogenous pathogenic R1441C mutation did not sensitize the SNc DA neurons to cell death following *sub-chronic* MPTP treatment (Fig 3.3E,F).

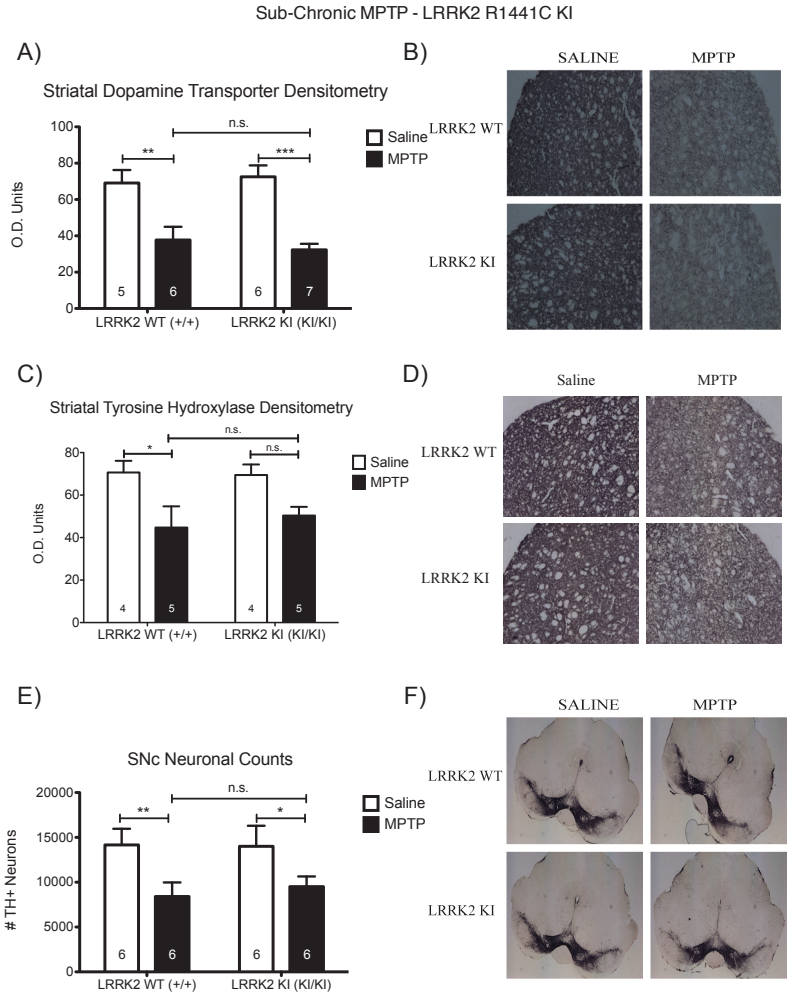

Figure 3.3. Assessment of the nigro-striatal pathway in LRRK2 R1441C KI mice following *sub-chronic* MPTP exposure, *in vivo*. Quantification and representative images of striatal dopamine transporter (DAT) densitometry (**A**, **B**) and tyrosine hydroxylase (TH) densitometry (**C**, **D**) of 14 µ striatal sections normalized to the corpus callosum (c.c.). (**E**, **F**) Stereological DA-SNc neuronal counts from 40 µ sections from~10 week old KI and WT littermate animals following the *sub-chronic* MPTP injection paradigm. Number of animals (N) per group is listed in each bar.

We then tested the Tg WT-hLRRK2 and RG-hLRRK2 over-expressing mice. Since these mice are on a FVB/N genetic background, which is reported to be less susceptible to MPTP treatment (Liu et al., 2003), we proceeded with an *acute* MPTP treatment paradigm (Jackson-Lewis and Przedborski, 2007). Briefly, 8-10 week old male mice receive 4 i.p. injections of 20mg/kg of MPTP-HCl in a single day, separated by 2 hour intervals, followed by sacrifice 7 days post-treatment (Jackson-Lewis and Przedborski, 2007). We observed no significant difference in the sensitivity of the pathogenic mutant RG-hLRRK2 when compared to WT-hLRRK2 over-expressing mice. Neither did the Tg animals show a difference when compared to their respective non-Tg (+/+) littermate controls. These results were observed when both the DAT striatal densitometry (Fig 3.4A,B) and the TH^+ SNc neuronal quantifications were compared (Fig 3.4C,D). Although the RG-hLRRK2 (RG/+) mice display a non-significant trend of having a basal lower SNc neuronal count, this was not exacerbated by MPTP exposure. To ensure that our results were comparable across all the murine strains utilized, we repeated the *acute* MPTP paradigm using the R1441C KI colony. In this case, littermates were not used to allow for rapid assessment. As expected, the WT (+/+) and R1441C (KI/KI) mice reacted identically to *acute* MPTP exposure at all levels assessed. Interestingly, this treatment showed significant loss in the striatal DAT immunoreactivity (Fig 3.5A,B) but lacked significance at the level for either genotype (Fig 3.5C,D).

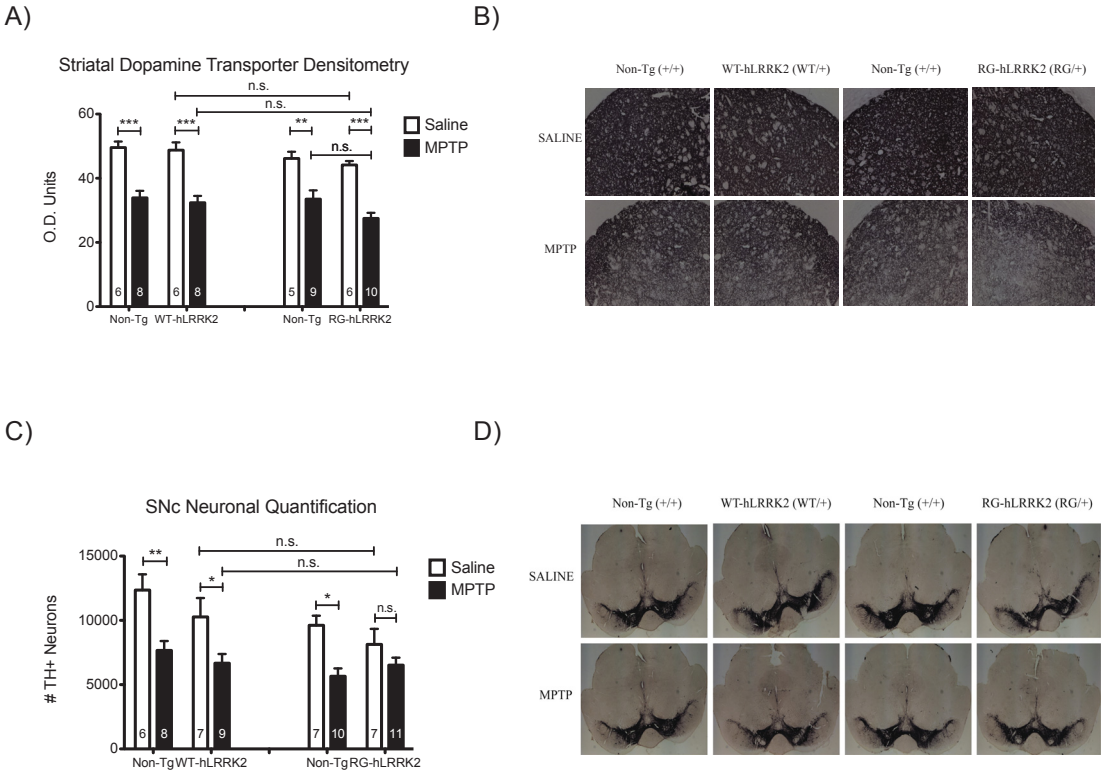

Figure 3.4. Assessment of the nigro-striatal pathway in WT and R1441G-hLRRK2 over-expressing transgenic mice following *acute* MPTP exposure, *in vivo*. (**A**) Quantification and (**B**) representative images of striatal dopamine transporter (DAT) densitometry normalized to the corpus callosum (c.c.). (**C**) Stereological quantification of tyrosine hydroxylase positive (TH+) SNc neuronal counts in ~9 week old WT (WT/+) and R1441G (RG/+) LRRK2 over-expressing Tg animals and their respective non-Tg (+/+) littermate controls following *acute* MPTP exposure. (**D**) Representative SNc images.

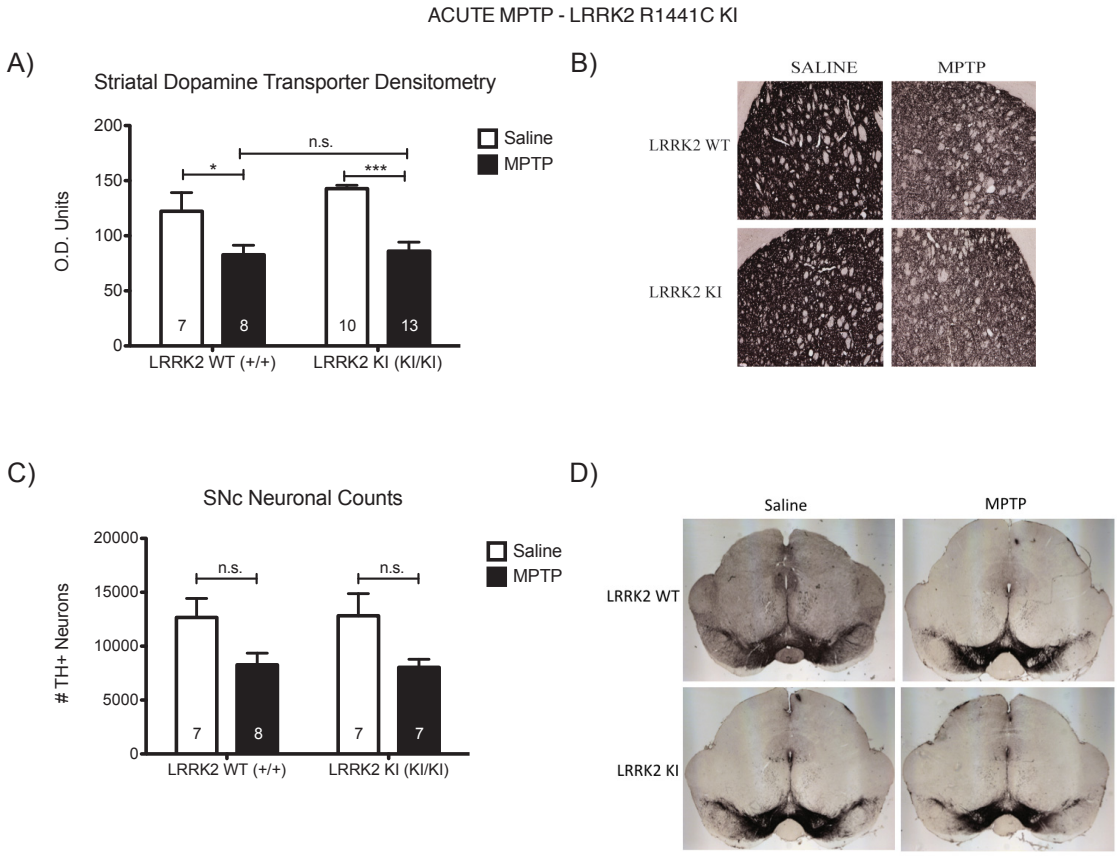

Figure 3.5. Assessment of the nigro-striatal pathway in LRRK2 R1441C KI mice following *acute* MPTP exposure, *in vivo*. Quantification and representative images of (**A**) striatal dopamine transporter (DAT) densitometry normalized to the c.c., and (**B**) stereological DA-SNc neuronal counts in 9 week old homozygous KI animals and their age-matched WT (+/+) controls following acute MPTP exposure.

LRRK2 murine models do not display impaired metabolism of the neurotoxin MPTP, *in vivo*.

To validate that our results were not modulated by any differences in toxin bioavailability, we tested each LRRK2 model's ability to metabolize the systemically injected MPTP compound into its active, toxic MPP^+ metabolite (Irwin and Langston, 1985; Langston et al., 1984c; Ransom et al., 1987). We collected micro-punch tissue samples from both the striatum (2.0 mm) and the SNc (1.0mm) brain regions 1.5 hours following a single injection of 20mg/kg of body weight of MPTP-HCl. At this time point, MPP^+ levels have peaked following a single injection of MPTP (Przedborski et al., 1996). We analyzed the concentrations of the MPP^+ compound using HPLC, as previously employed (Kim et al., 2005a; Smith et al., 2003; Vila et al., 2001). There was no difference in the bioavailability of the toxic MPP^+ metabolite. This was the case across all of the various strains tested: R1441C KI (Fig 3.6A,), WT-hLRRK2 and RG-hLRRK2, and their respective WT or non-Tg (+/+) controls (Fig 3.6B). These results demonstrate that there is no difference in the ability of the various LRRK2 models to metabolize the MPTP compound. Therefore, one of the remaining avenues to investigate was the possibility that subtle impairments existed, perhaps at the neurotransmitter level.

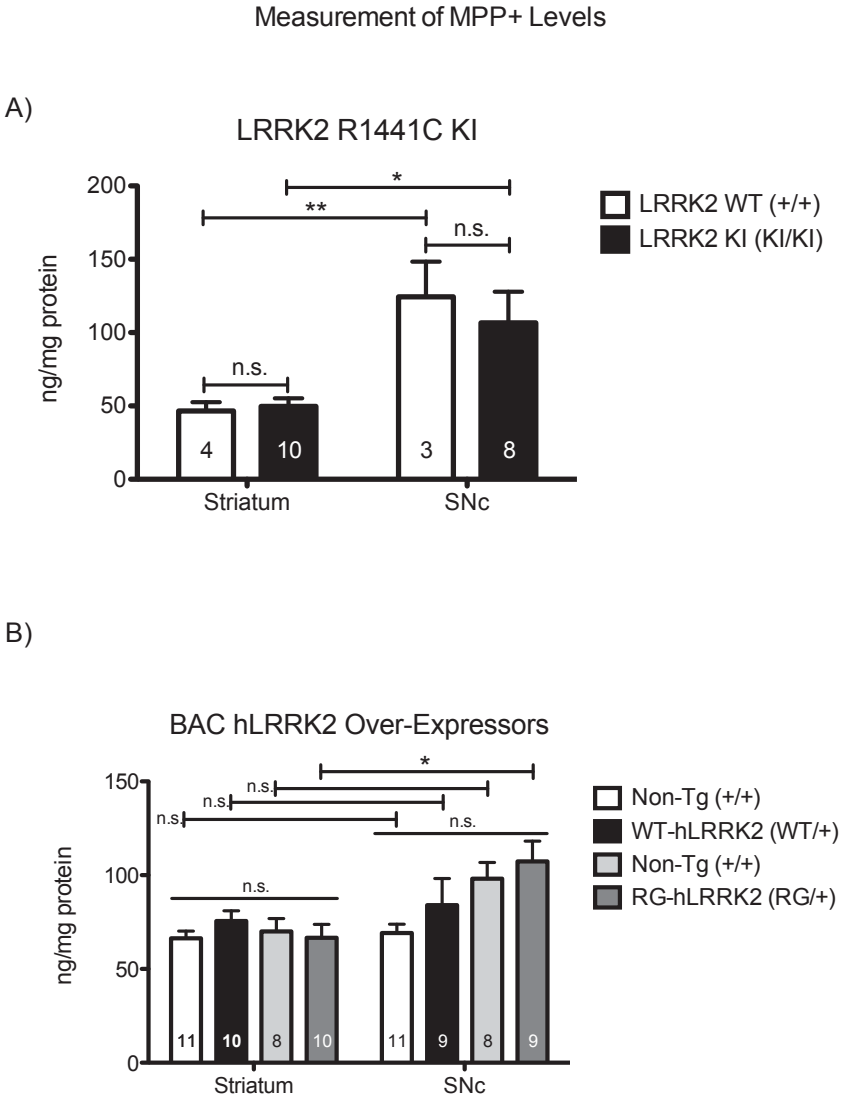

Figure 3.6. Metabolism of MPTP and bioavailability of the toxic MPP^+ metabolite in LRRK2 mice following MPTP injection, *in vivo*. (**A**) Measurements of MPP^+ concentrations (ng/mg protein) in micropunch samples from the striatum and SNc of LRRK2 KI and WT animals sacrificed 1.5 hours following treatment with 1 i.p. injection of 20mg/kg MPTP-HCl using HPLC analysis. (**B**) Equivalent studies in the WT and R1441G hLRRK2 over-expressing animals. Please note that each colony's respective non-Tg littermate controls are displayed adjacent to it.

LRRK2 murine models do not display impaired levels of dopamine or its metabolites following exposure to the neurotoxin MPTP, *in vivo*.

PD-associated motor impairments are caused by a hypo-dopaminergic striatal phenotype amounting as a result of dying DA neurons in the SNc (Cotzias et al., 1967; Sourkes and Poirier, 1965). Therefore, we analyzed the levels of striatal dopamine (DA) and its major metabolite 3,4-dihydroxyphenylacetic acid (DOPAC) following MPTP treatment at the same time point that corresponded to our immunohistological analysis. In this manner we could assess whether the availability of DA is compromised, suggesting that pre-symptomatic DA dysfunction is occurring. Following the *acute* MPTP paradigm, we collected fresh tissue samples from the striatal region on day 7, to assess the levels of DA (Fig 3.7A,B) and DOPAC (Fig 3.7C,D). As expected, we consistently found that there was no significant difference in the levels of DA or its metabolites across the murine models assessed: Lrrk2 R1441C KI (Fig. 3.7A,C) and both the over-expressing WT-hLRRK2 and RG-hLRRK2 models (Fig 3.7B,D). Interestingly, again we found that the RG-hLRRK2 mice displayed a basal decrease in the levels of DA, DOPAC, which as in the case of the SNc neuronal quantification remained a trend.

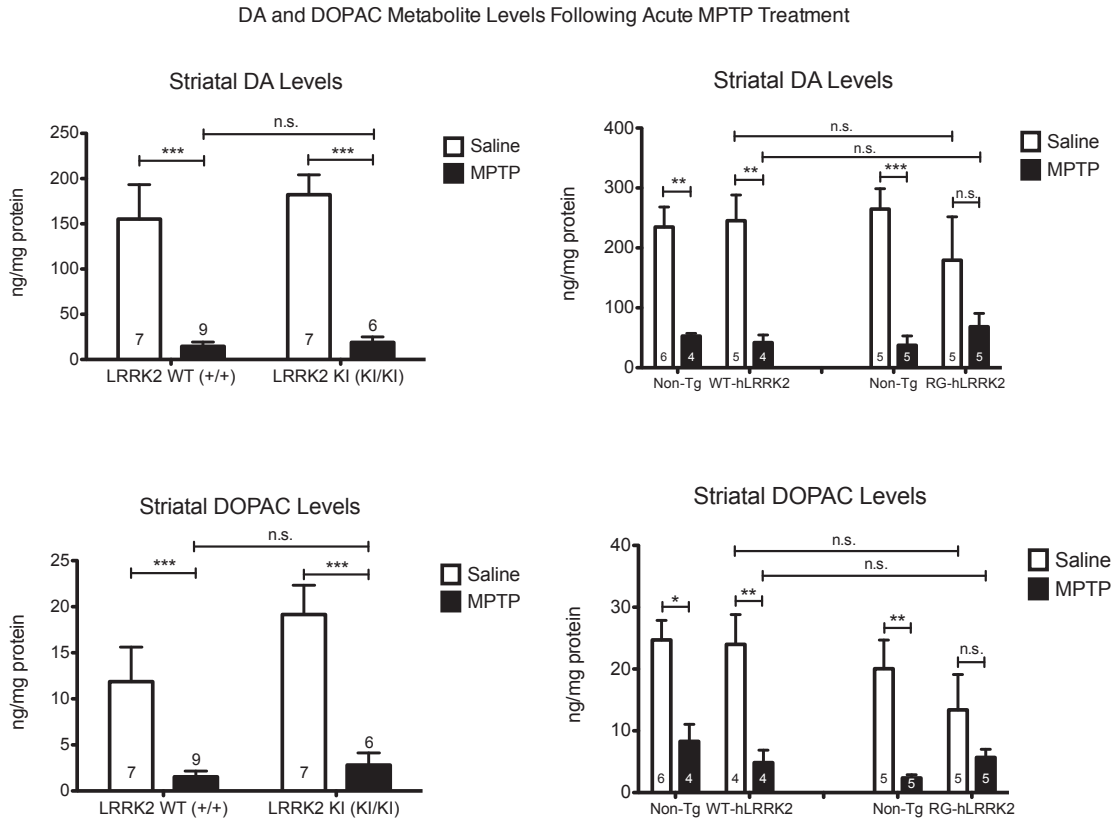

Figure 3.7. Assessment of SNc DA neurotransmitter status and turnover rates. HPLC measurements of DA, DOPAC and HVA concentrations (ng/mg protein) in micropunch samples from the striatum of *Left Panel:* LRRK2 KI and WT animals and *Right Panel*: WT and R1441G hLRRK2 over-expressing animals and their respective non-Tg littermate controls. Samples were collected 7 days following *acute* MPTP treatment, corresponding to the same point when all *acute* striatal immunohistochemistry and SNc neuronal survival assessments were performed.

Discussion

The goal of this study was to investigate the effects of the pathogenic R1441-LRRK2 GTPase mutant in the context of MPTP/MPP^+ treatment. The MPTP/MPP^+ mechanism of action is similar to that of certain environmental toxins (rotenone and paraquat), which are associated with an increased risk of developing PD (Tanner et al., 2011). These agents affect mitochondrial health and are believed to augment the already heightened oxidative stress states of SNc neurons, which are hypothesized to contribute directly to their increased vulnerability (Jenner and Olanow, 1996; Yuan et al., 2007). Our data illustrates that the average mitochondrial length remains unchanged, however, a higher percentage of shorter mitochondria can be detected in primary cortical neurons with R1441C-hLRRK2 over-expression and from those that lack Lrrk2 (KO). We also found that mild MPP^+ treatment exacerbates this phenotype in Lrrk2 KO and WT cortical neurons. However, the ability of MPP^+/MPTP to alter the survival phenotype in murine models of LRRK2 *in vitro* and *in vivo* is lacking.

The field portrays a general consensus that over-expression of WT or mutant LRRK2 sensitizes cells to death (Greggio et al., 2006; Ho et al., 2009; Iaccarino et al., 2007; Smith et al., 2006; Stafa et al., 2012; West et al., 2007). However, there are a few points that can explain the lack of any phenotypic differences in the paradigms assessed. Firstly, a large portion of the data in the field has been generated with an emphasis on the G2019S LRRK2 mutation, which was not included in this study, and all pathogenic *LRRK2* mutations may not share a common pathway/effect on function. Secondly, the use of transient, ectopic, over-expression may produce non-physiologically relevant outcomes, which skews our understanding of a protein's biological function. Lastly,

primary neuronal cultures employ embryo-derived material and *in vivo* studies, including our own, are commonly representative of a juvenile population. These models are counter-intuitive when studying a disease of ageing. These points in the context of our studies are elaborated below.

Our data supports the observations that LRRK2 has subtle effects on mitochondrial morphology, a phenotype that has been described in various systems (Cooper et al., 2012; Hindle et al., 2013; Mortiboys et al., 2010; Niu et al., 2012; Saez-Atienzar et al., 2014; Su and Qi, 2013; Wang et al., 2012b). Although the R1441C GTPase mutation has been assessed using ectopic over-expression (Cherra et al., 2013; Wang et al., 2012b) and neural cells derived from pathogenic human iPSCs (Cooper et al., 2012), an assessment in primary cortical neurons harvested from germline Lrrk2 KO murine models was lacking. We found that expression of R1441C hLRRK2 or Lrrk2 KO cells had no difference in average mitochondrial length, compared to WT or control, but did have an increased percentage of their mitochondrial population with a shorter (fragmented) morphology. The distribution differences observed are significant ($p<0.001$), however, unlike other reports our morphology phenotype is not robust. This may be due to differences in: model systems employed (iPSC, SH-SY5Y), expression methods (ectopic or stable overexpression) and readout measures (% cells with fragmented mitochondria/mitochondria content versus length measurements) (Cherra et al., 2013; Cooper et al., 2012; Wang et al., 2012b). It is also possible that the phenotype develops and progresses with ageing, and so it is not observed in embryonic neuronal cultures. This is supported by evidence of alterations in mitochondrial morphology in aged G2019S KI mice, although no mitochondrial phenotype has been reported in aged

tissue of R1441C/G expressing mice (Yue et al., 2015). The results from our R1441C-hLRRK2 experiments align with the current reports; however, the data from the Lrrk2 KO neurons does not. Based on the current literature, LRRK2-related fragmentation phenotypes are the product of alterations in Drp1-mediated fission mechanics (Su and Qi, 2013; Wang et al., 2012b). This fragmentation phenotype has been demonstrated in pathogenic human G2019S and R1441C fibroblasts and iPSC-derived neurons (Cooper et al., 2012; Su and Qi, 2013). Since R1441C and G2019S reportedly exacerbate the recruitment and phosphorylation of Drp1 while GTP-binding deficient and kinase dead LRRK2 blocked fragmentation, then one would expect Lrrk2 KO neurons to display the converse mitochondrial phenotype (Niu et al., 2012; Su and Qi, 2013; Wang et al., 2012b). However, Lrrk2 KO neurons have the same distribution shift as the R1441C expressing neurons, which is exacerbated by MPP^+ treatment. It is interesting that the shift following MPP^+ treatment is more pronounced in the Lrrk2 WT cells than in the KO neurons. The Lrrk2 KO phenotype is supported by data demonstrating that SH-SY5Y cells treated with LRRK2 inhibitors still demonstrate Drp1 recruitment and a higher percentage of cells with fragmented mitochondria (Oude Groote, 1989). It is important to note that human pathogenic fibroblasts (G2019S) have also been reported to display networked mitochondrial morphology (Mortiboys et al., 2010). Additionally, modulation of Drp1, not LRRK2, was used to rescue the fragmented mitochondrial fibroblast phenotype reported (Su and Qi, 2013). Cumulatively, the data suggests that the nature of this phenotype is more complex than originally presumed. The investigation of the LRRK2-Drp1 interaction and confirmation of a pathogenic phosphorylation phenotype in

various *LRRK2*-linked and healthy control human tissue would help validate the mechanism and address some of the outstanding questions.

We then assessed whether MPP^+ treatment could manifest differential survival responses between pathogenic R1441C/G and WT forms of LRRK2. We found no difference in the sensitivity of each LRRK2 model in response to MPP^+ treatment *in vitro* with endogenous Lrrk2 KO, R1441C KI, nor over-expressing WT and R1441G-hLRRK2. Initially, this was perplexing, since our previous work demonstrated that both WT and R1441C hLRRK2 transient, ectopic overexpression was toxic in primary cortical neurons, *in vitro*, and that rotenone, which inhibits complex I of the mitochondria, is toxic to hLRRK2-expressing drosophila, *in vivo* (Venderova et al., 2009). However, confidence pursued as consistent data emerged across all four independent colonies and we began to explore whether others had reported similar results. Our findings are supported by data demonstrating that 5 μM MPP^+ treatment in LRRK2 expressing iPSC-derived neurons displayed no significant alterations in lactate dehydrogenase release (LDH), suggesting no mitochondrial damage (Cooper et al., 2012). More recently, evidence from pathogenic human LRRK2 G2019S fibroblasts, which demonstrate differential survival outcomes following treatment with MPP^+, suggests that different mutations may illicit differential response to similar treatments (Yakhine-Diop et al., 2014). Confirmation of these findings in Lrrk2 G2019S KI primary neuronal cultures would help resolve this point.

We then demonstrated that in *in vivo* exposure to MPTP does not sensitize dopaminergic neurons of murine R1441C/G LRRK2 expressing models to death. The assessment of an *in vivo* paradigm is critical since MPTP metabolism requires glial

contributions (Przedborski and Jackson-Lewis, 1998). Thereby we can assess the function of an intact, multi-cell type governed system. This is directly relevant to the emerging role of LRRK2 in non-neuronal CNS cell types (reviewed in (Dzamko and Halliday, 2012; Russo et al., 2014)). Our results do not corroborate studies of LRRK2 contribution to survival following exposure to other mitochondrial-targeting toxins reported in invertebrate models, such as *D. melanogaster* (rotenone) and *C. elegans* (paraquat and rotenone) *in vivo* (Ng et al., 2009; Saha et al., 2009). In fact, our own Drosophila-based screen indicated LRRK2 sensitivity to environmental rotenone and identified genetic interactions between LRRK2 and Parkin, Pink1 and DJ-1 (Venderova et al., 2009). By extrapolation, this provided a mechanistic commonality - mitochondrial-related function. The conflicting results between murine and invertebrate LRRK2 studies may be the result of compensation from the mammalian LRRK1 (Biskup et al., 2007; Dachsel et al., 2010b; Westerlund et al., 2008). In addition, the ability of MPTP to manifest differential survival responses in models of genetic PD is variable. Studies demonstrate that both Dj-1 KO and Pink1 KO models are sensitive to MPTP treatment (Haque et al., 2012; Kim et al., 2005a). These two genes have mitochondrial and oxidative stress related roles (Gandhi et al., 2009; Greene et al., 2012; Heeman et al., 2011; Irrcher et al., 2010; Joselin et al., 2012; Parsanejad et al., 2014b; Poole et al., 2008). Conversely, a lack of hypersensitivity to MPTP-induced DA neuronal death has been demonstrated in other germline altered genetic PD models, such as the Parkin KO (Aguiar et al., 2013; Thomas et al., 2007), while the α-synuclein mouse models have generated inconsistent results at the neurochemical and survival levels (Dauer et al., 2002; Dong et al., 2002; Drolet et al.,

2004; Klivenyi et al., 2006; Nieto et al., 2006; Perez-Sanchez et al., 2010; Rathke-Hartlieb et al., 2001; Thomas et al., 2011). Together, this suggests that certain forms of PD are susceptible to MPTP-induced death and relevant pathways. We tested two commonly used and well-characterized MPTP treatment paradigms, *sub-chronic* and *acute*, which induce apoptotic (Tatton and Kish, 1997) or rapid necrotic cell death (Jackson-Lewis et al., 1995), respectively. Within both paradigms, no difference in the sensitivity of these animals in comparison with their respective WT controls is observed, even though LRRK2-induced cell death has been reported to occur via an apoptotic mechanism (Iaccarino et al., 2007). However, our results do corroborate an earlier report demonstrating that Lrrk2 KO mice are not sensitized to MPTP treatment (Andres-Mateos et al., 2009).

In addition, the MPTP paradigms tested employ relatively juvenile (8-10 week old) animals, a caveat when studying a disease of ageing, particularly one with a late age of onset. Interestingly, reduced levels of basal striatal DAT immunoreactivity, TH^+ SNc neuronal counts, and altered levels of DA and its metabolite DOPAC in the R1441G-hLRRK2 mice, albeit non-significant, are observed in these 9-11 week old mice. We cannot conclusively determine whether a R1441G-hLRRK2-linked phenotype will develop with age or whether it is the result of an incomplete penetrance effect that is masked by a pooled population (Healy et al., 2008; Latourelle et al., 2008). However, a quick assessment for the latter indicates that penetrance is not an issue. Perhaps performing MPTP on aged animals would have led to observable differences in their ability to manage MPTP-induced oxidative stress, a question that can be pursued in future studies.

Taken together, our results highlight the possible mechanistic differences between different forms of PD. Perhaps MPTP/MPP^+-induced mitochondrial impairments are more relevant in the context of limited forms of *Parkinsonism*, such as certain recessive genetic cases; or that its presence in *LRRK2*-linked PD is not observable in at such juvenile time points. Conversely, it is possible that in the case of LRRK2 R1441C/G mutations, death caused by the MPTP/MPP^+ mechanism of action, is not a critical factor leading to disease pathogenesis. Importantly, our results do not negate the involvement of environmental toxins in eliciting neuronal damage, but rather they highlight the specificity in the toxin models employed. Cumulatively, the evidence in the field suggests that although rotenone, MPTP and paraquat are similar, each has a unique and selectively differential mechanism of action. Understanding these differences may be the key to discerning the mechanism of action causing genetic susceptibility, or governing gene-environment interplay, in various forms of PD. Therefore, based on our results we conclude that *in juvenile contexts, exposure to varying amounts of MPTP/MPP^+ does not further sensitize pathogenic R1441C/G-LRRK2 expressing murine models to neuronal death.*

Materials & Methods

Animal Care and Handling:

All care, handling and procedures involving the use of animals were performed under the supervision of the University of Ottawa Animal Care and Veterinary Service in accordance with the University of Ottawa's Animal Care Committee; a registered body

under the Animals in Research Act, which adheres strictly to the Canadian Council on Animal Care Standards. All animals were provided standard rodent chow and hydration *ad libitum* and housed under the standard reversed 12hr-light/12hr-dark cycle.

Murine Models Employed:

Viral Over-Expression: Wild-type outbred CD-1 ® IGS timed pregnant females (Charles River - #022) were employed for *in vitro*, ectopic, viral over-expression studies.

Murine LRRK2 Models: The LRRK2 R1441C Knock In (LRRK2 KI) mice on a pure C57BL/6J background (Tong et al., 2009b), and the LRRK2 Knock Out (LRRK2 KO) strain on an ~90% C57BL6/J partial 129SV mixed background (Tong et al., 2010) were obtained directly from, Dr. Jie Shen, Harvard University. Both lines were maintained using heterozygous crosses and littermates were used for *in vitro* and *in vivo* studies, unless otherwise stated.

Transgenic hLRRK2 Models: The human LRRK2 (hLRRK2) BAC transgenic (Tg) models described in (Li et al., 2009) were purchased from The Jackson Laboratory (JAX Mice). Wild-type hLRRK2 over-expressing animals, (FVB/N-Tg(LRRK2*G2019S)1Cjli/J #009610), herein referred to as WT-hLRRK2 and denoted as (WT/+), and the pathogenic R1441G hLRRK2 over-expressing animals (Tg(LRRK2*R1441G)135Cjli/J - #009604), herein referred to as RG-hLRRK2 and denoted as (RG/+). These animals are on a FVB/NJ background and each colony was bred separately. Breeding consisted of pairing a hemizygote transgenic (WT/+ or RG/+) with a non-transgenic (non-Tg) FVB/NJ (+/+) control (#001800). For LRRK2 analytics, WT/+ and RG/+ were used for comparison. In addition, all non-Tg (+/+) littermates were included as internal controls, to account for each colony-specific background.

***In vitro* Experiments:**

Neuronal Cultures:

Mouse primary embryonic cortical neurons were performed as previously described in (Zhang et al., 2006). Briefly, embryonic neurons were collected at E14.5-15.5 from the various strains described above. Individual brain cortices were dissociated then plated on poly-D-lysine hydrobromide (Sigma) coated tissue culture plates and grown in Complete Neurobasal Medium (Neurobasal ® Medium (Gibco) supplemented with B27 (Gibco) and N2 (Gibco) Supplements, Penicillin – Streptomycin Solution (HyClone) and L-Glutamine (Gibco)). Neurons received warm, half volume replenishment of fresh Complete Neurobasal Medium 72 hours post-plating and were grown for either 3 days in vitro (DIV) – for viral work or 6DIV for murine model studies. Neuronal cultures were then fixed, lysed or collected for their respective analytical purposes, as described below.

Survival Experiments:

Cortical neurons were plated at a density of 250,000 neurons/well in a 48-well cell culture plate for 6DIV. Neurons were time-course treated at 48, 36, 24, and 12 hrs prior to end point with either control or 20 μM of 1-methyl-4-phenylpyridinium (MPP^+) iodide (Sigma: M0896-10MG). This concentration has been previously employed (Amini et al., 2013; Parsanejad et al., 2014b). Neurons were then lysed with 1X Cell Lysis Solution (10X PBS, 10% Triton X-100, 1M $MgCl_2$, and 5% cetyldimethylethylammonium bromide). Blinded quantification of surviving nuclei was performed as previously described in (Galehdar et al., 2010).

Mitochondrial Experiments:

Neurons were grown on 1X poly-D-lysine coated coverslips in 24-well culture plates. Approximately 500,000 neurons were plated per well. Fixation was performed using 4% PFA. Cells were stained for TOM-20 (Santa Cruz Biotechnology, 1:200) and Hoechst (Sigma, 1:2000). Fluorescence images were acquired on a Zeiss LSM 510 META confocal microscope. Blinded manual tracing of mitochondrial lengths (>1000/group/treatment) was performed using NIH ImageJ software and presented as average or binned percentages, as previously reported ((Irrcher et al., 2010)).

Murine Models: On DIV6, neurons were treated with control or 10μM 1-methyl-4-phenylpyridinium (MPP^+) iodide (Sigma: M0896-10MG) for 6hrs prior to fixation, as established and reported by our lab in (Joselin et al., 2012). Each point represents an N of WT=10 and KO=15, N= number of randomly selected fields from 2 and 3 independent littermate embryo experiments obtained from the same individual dissection, respectively.

Viral Over-expression: At 24hrs post-plating, cultures were infected with Helper-Dependent Adenoviral (HDAd) constructs expressing control (LacZ), WT hLRRK2 or R1441C-hLRRK2, previously employed by our lab (Venderova et al., 2009). Only viable, infected (green) neurons were analyzed.

***In vivo* Experiments:**

MPTP Administration:

8-10 week old male mice received intra-peritoneal (i.p.) administration of fresh 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine hydrochloride (MPTP-HCl) (Sigma: M0896-10MG)

in 0.9% Saline. *Sub-Chronic Paradigm:* Mice received 1 injection of 30 mg/kg body weight of MPTP-HCl or the equivalent volume of 0.9% saline once a day for 5 consecutive days. Animals were sacrificed 14 days following the initial injection, as previously performed (Qu et al., 2007; Tatton and Kish, 1997). *Acute Paradigm:* Mice received 1 injection of 20 mg/kg body weight of MPTP-HCl or the equivalent volume of 0.9% saline every 2 hours for a total of 4 doses in one day and were sacrificed 7 days later, as characterized in (Jackson-Lewis et al., 1995).

Neurochemical Analysis:

MPP+ Concentrations: Animals received a single injection of 20 mg/kg MPTP-HCl and were sacrificed 1.5 hours later. Brains were rapidly dissected out and placed in a 2.0 mm coronal-segmented dissection block. Micropunch tissue samples from both hemispheres were collected from the striatum (2.0 mm diameter) and SNc (1.0 mm diameter), flash frozen and stored at -80°C until sent for HPLC analysis as described in (Przedborski et al., 1996).

Dopamine and Metabolite Concentrations: Animals were sacrificed 7 days post-Acute MPTP injections. Fresh 2.0 mm thick x 2.0 mm diameter micropunch tissue samples were collected from both striatal hemispheres and placed in a homogenizing solution, as described in (Litteljohn et al., 2010) and stored at -80°C until sent for HPLC analysis, as described in (Litteljohn et al., 2010; Przedborski et al., 1996).

Immunohistochemistry:

Animals were anesthetized and transcardially perfused using 0.9% saline followed by 4% PFA in 1X PBS. Brains were extracted and placed in 4% PFA on a shaker at 4°C for 24 hours followed by washes in a 10% sucrose in 0.1 M PB with sodium azide solution

every 12 hours for the next 3 days. Brains were frozen by submission in cooled (-35°C) iso-pentane and stored at -80°C. Sectioning was performed at -22°C and tissue was collected as free-floating coronal sections from the striatal region (14 μm) and the SNc (40 μm). Tissue sections were stained for Tyrosine Hydroxylase (ImmunoStar, 1:10,000; Millipore, 1:5,000) and Rat X Dopamine Transporter (Millipore 1:2000). Tissue preparation and immunoreactivity were developed using the glucose oxidase 3,3'-diaminobenzidine protocol previously described in (Xu et al., 1997). Striatal sections were imaged on a 10X optical microscope and densitometry was performed using NIH ImageJ software, whereas, stereological assessment of TH+ SNc neurons was performed using Stereo Investigator 10 software, as summarized in (Crocker et al., 2003b). Investigator remained blinded during quantification.

Statistical Analysis:

Statistical significance was determined using either a one or two-way ANOVA, depending on nature of data analysis, followed by Bonferroni Post *hoc* test to determine significance. All data are presented as mean ± standard error of the mean (SEM). Significance is denoted as * = $P<0.05$, ** = $P<0.01$, *** = $P<0.001$, and n.s. = non significant.

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Conflict of Interest Statement

The authors report no conflict of Interest.

Abbreviations

BAC – Bacterial Artificial Chromosome

c.c. – corpus callosum

CNS – central nervous system

DA – Dopamine

DAT – Dopamine Transporter

DIV – Days in vitro

DOPAC – 3,4-Dihydroxyphenylacetic acid

Drp1 – Dynamin-related protein 1

GOF/LOF – gain/loss of function

hLRRK2 – human LRRK2

HPLC – High Performance Liquid Chromatography

i.p. – intraperitoneal

IHC – Immunohistochemistry

iPD – idiopathic Parkinson's disease

iPSC – induced pluripotent stem cell

LDH – lactate dehydrogenase

LRRK2 – Leucine-Rich Repeat Kinase 2

LRRK2 KI – R1441C LRRK2 Knock In

LRRK2 KO – LRRK2 Knock Out

MPP+ – 1-methyl-4-phenylpyridinium

MPTP-(HCl) – 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (hydrochloride)

n.s. – non significant

PBS – phosphate buffer solution

PD - Parkinson's disease

PFA – paraformaldehyde

PINK1 – PTEN-induced Kinase 1

RG – R1441G

RG-hLRRK2 – R1441G-hLRRK2

SEM – standard error of the mean

SNc – Substantia Nigra pars compacta

Tg – transgenic

TH($^{+}$) – Tyrosine Hydroxylase (positive)

TOM-20 – Translocase of Outer Membrane 20

WT – wild type

WT-hLRRK2 – Wild Type hLRRK2

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Chapter 4: General Discussion

Chapter 4: General Discussion

With the growing number of reports indicating LRRK2-related pathways and functions, one thing is evident - LRRK2 has many *potential* candidate interactors and roles, yet very few have been confirmed and/or replicated. For this reason, we created a *Drosophila melanogaster* human *LRRK2* (h*LRRK2*)-linked model of PD as a tool to perform an unbiased, genome saturated screen (results reported in Chapter 2). In addition, we sought to better understand the nature of the gene-environment interaction and test whether MPTP-induced stress altered cellular and survival responses in neurons from various Lrrk2 and transgenic hLRRK2 murine models (results reported in Chapter 3). Together, these studies help provide insight to the biological role and function of LRRK2 and its link to PD pathogenesis.

4.1. Screening for LRRK2 Genetic Interactors

By over-expressing PD-linked hLRRK2 in *D. melanogaster* (fruit flies) and exposing them to environmental rotenone, our studies have demonstrated that drosophila: 1) lose TH-positive CNS neurons; 2) display locomotor impairments; and 3) have a decreased lifespan basally and following exposure to environmental toxins (rotenone) (Venderova et al., 2009). This is more clinically representative than alterations seen in the genetic PD mouse models (reviewed in (Crabtree and Zhang, 2012; Dawson et al., 2010)). Importantly, the drosophila systems allowed us to perform a genetic interaction screen, the ultimate goal behind creating the *LRRK2*-linked PD drosophila model system.

Healthy skepticism highlighted the concern that ectopic, over-expression screening methods would produce false-positive interactors, especially given the large

complex, multi-domain hLRRK2 protein. Nevertheless, of the chromosomal regions tested, which spanned ~80% of the entire *D. melanogaster* genome, the screen produced 36 single gene interactors of over-expressed hLRRK2 (manuscript in preparation). However, there are screening limitations that need to be highlighted. Firstly, genetic interactors were likely missed at stages when larger chromosomal deletion regions were screened, since it is the summation of the individual gene interactions that are observed. Secondly, the chromosomal deficiency (single allelic deletion) assumes that all genes react in a haploinsufficient manner, and therefore deletions of haplosufficiency or incomplete dominance would proceed undetected. Regardless, this model system provided a means to perform a screen in an *in vivo*, unbiased, high-throughput manner. As such, the drosophila suppressor/enhancer genetic screening tool has helped identify and map genetic interactions for various purposes (reviewed in (Lenz et al., 2013; St Johnston, 2002)). PD/LRRK2-relevant findings from my interacting region will be overviewed.

I identified the stock line #7876 hit, which corresponds to the *Df(2R)Exel7131* region (BDSC, Indiana University: http://flystocks.bio.indiana.edu/bloomhome.htm; (Cook et al., 2012b). Interestingly, a separate *D. melanogaster* screen conducted with Parkin and Pink1 RNAi lines also reported an interaction (suppression) with this chromosomal deletion region (Fernandes and Rao, 2011). Subsequent screening by colleagues (G. Kabbach and P. Marcogliese) isolated and identified the following 3 candidate genetic interactors: 1) CG8561 - drosophila convoluted gene; 2) CG8523 - drosophila Multi Drug Resistance 50 (Mdr50) gene; and 3) CG8542 - drosophila Heat Shock Protein cognate 5 (Hsc70-5) gene.

The *D. melanogaster* CG8561 Convoluted gene is involved in development, with reported effects on the tracheal system and motor neuron guidance (Beitel and Krasnow, 2000; Kurusu et al., 2008; Swanson et al., 2009). CG8561 has no reported human orthologue; however, its corresponding amino acid sequence reveals similarity to the human insulin-like growth factor binding protein, acid labile subunit protein (IGFALS). The *IGFALS* gene (OMIM: 601489) contains LRR repeats and is required for insulin growth factor 1 (IGF1) accumulation and half-life extension (Guler et al., 1989; Zapf et al., 1995). Increased amounts of serum insulin-like growth factor (IGF) have been reported in PD patients, leading some to suggest its use as a biomarker (Godau et al., 2010; Godau et al., 2011; Ma et al., 2015; Picillo et al., 2013). Interestingly, an increase in IGF serum concentrations is observed in Lrrk2 KO rats relative to WT animals (Ness et al., 2013). Conversely, brain concentrations of IGF are reportedly reduced in PD (Tong et al., 2009a), and IGF is protective in cell culture and animal toxin-induced models of PD (Ebert et al., 2008; Offen et al., 2001).

The second genetic interactor is the *D. melanogaster* CG8523 Multi Drug Resistance 50 (Mdr50) gene, which is: highly expressed in gut tissue, predicted to produce a transporter involved in the shuttling of drugs across membranes, and its mRNA levels respond to exogenous toxin exposure (rotenone, paraquat, virus etc.) (Attrill et al., 2016). In humans, the Multi-Drug Resistance 1 gene (*MDR1* OMIM:171050) (p-glycoprotein) is ubiquitously expressed and is an important regulator of toxin (endogenous or exogenous) efflux from tissues (Schinkel et al., 1996; van der Valk et al., 1990). It is required for CNS protection from certain drugs and even pesticides (Kim et al., 1998; Peterson, 1977; Schinkel et al., 1994); and is found at various environmental

protective junctions, such as the lungs, gut and BBB (Cordon-Cardo et al., 1989; van der Valk et al., 1990). Certain *MDR1* mutations are said to occur more frequently in specific PD subpopulations; however, the gene is not linked to disease (Furuno et al., 2002). Linkage would provide a connection between genetics, the environment and susceptibility to PD; predicted to be critical in *LRRK2*-linked cases (Gao and Hong, 2011; Kitada et al., 2012). *MDR1* (*ABCB1*)'s association with inflammatory bowel disease (IBD) is also interestingly similar to *LRRK2*'s genetic susceptibility with Crohn's disease (Barrett et al., 2008; Brant et al., 2003; Brinar et al., 2013; Ho et al., 2006; Umeno et al., 2011).

The third interactor is the *D. melanogaster* CG8542 - Heat Shock Protein cognate 5 (Hsc70-5) gene, which is involved in mitochondrial health, functions, and quality control (Banerjee and Chinthapalli, 2014; Zhu et al., 2013). Its human orthologue is the Heat shock 70kDa protein 9 (*HSPA9)* gene (mortalin) (OMIM: 600548). Mutations in *HSPA9* have been identified in PD populations (Burbulla et al., 2010; De Mena et al., 2009). In addition, there is a decrease in the levels of mortalin in the SNc of PD cases, which may correlate to disease severity (Jin et al., 2006; Shi et al., 2008). Interestingly, mortalin interacts with DJ-1 and its loss has negative effects on mitochondrial ROS, morphology and membrane potential (Burbulla et al., 2010). Pink1 and Parkin can rescue the deficits of mortalin knockdown on mitochondrial unfolded protein response (mtUPR), mitochondrial degradation and autophagy, and cell survival (Burbulla et al., 2014). This was of interest to us, since our biased screening results suggest a genetic interaction between LRRK2 and the Parkin, Pink1 and Dj-1 genes, which play a role in mitochondrial health (Venderova et al., 2009). This genetic interaction is corroborated by

evidence indicating a direct interaction between LRRK2 and Hsc70 (Orenstein et al., 2013) and C-terminal Hsp70 Interacting Protein (CHIP) (Ding and Goldberg, 2009; Ko et al., 2009). These interactions support a role for LRRK2, mitochondria, protein degradation and toxins (investigated in Chapter 3 & Appendix II., respectively).

4.2. Gene-Environment Interplay and Related Cellular Responses

Given the evidence in the literature that environmental toxins, mitochondrial dysfunction and protein mismanagement are associated with PD, we investigated these aspects in the context of Lrrk2 KO, WT or pathogenic R1441C/G LRRK2 (reviewed in (Warner and Schapira, 2003)). This information coupled with the overall incomplete penetrance (~30%) observed in *LRRK2*-linked PD suggested that LRRK2 mutations may sensitize neurons to an exogenous insult, and that additional trigger mechanism(s) is needed to manifest toxicity (Hakimi et al., 2011). Therefore, I sought to investigate the ability of the mitochondrial-targeting MPTP/MPP^+ compound to manifest cellular responses and neuronal sensitivity in murine R1441-LRRK2 models. I found no differential response in mitochondrial length, or survival of cortical neurons *in vitro* or in dopaminergic neurons *in vivo*, following MPP^+/MPTP exposure (results of Chapter 3).

4.2.1. Mitochondrial Observations Summarized

No difference was observed in the average mitochondrial length phenotypes when WT and R1441C hLRRK2 were over-expressed, and between Lrrk2 WT and Lrrk2 KO cortical neurons under basal and MPP^+ conditions. However, a significant difference is seen when the percentage of mitochondria is binned into length ranges. In this case,

R1441C-hLRRK2 phenocopies Lrrk2 KO, which could indicate a LOF effect. Confirmation of this phenotype in R1441C Lrrk2 KI germline primary neurons would help validate these results. Currently, reports confirm decreased length morphology, but not interaction (with Drp1) or mediation of the phenotype by LRRK2 modulation, in pathogenic human-derived tissues (Su and Qi, 2013; Wang et al., 2012b). Therefore, when considering the entire body of data, the mechanism presented is inconclusive and should be cautiously interpreted (Niu et al., 2012; Su and Qi, 2013; Wang et al., 2012b). Interestingly, the fragmented mitochondrial phenotype coincides with subsequent effects on autophagy and cell viability (Cherra et al., 2013; Su and Qi, 2013; Wang et al., 2012b). Likewise, I tested these parameters in murine models of R1441-LRRK2 following MPP^+ treatment (unpublished data – Please refer to Appendix II.), and will briefly discuss below.

4.2.2. Absence of Robust Autophagy Alterations in murine models of LRRK2

I also began studies to assess altered autophagy downstream of LRRK2 genotypes. A role for LRRK2 in autophagy has been shown in the literature (Bravo-San Pedro et al., 2012; Bravo-San Pedro et al., 2013; Orenstein et al., 2013; Plowey et al., 2008; Schapansky et al., 2014; Tong et al., 2012). Preliminary data assessing general autophagic protein markers (LC3-II/I ratios and p62) in neurons following control and 10 μM MPP^+ time-course (3, 6, 9 hours) treatment did not seem to suggest any robust differential responses in Lrrk2 KO and WT-hLRRK2 neurons (Appendix II - Fig. S.3, S.4.) (Bjorkoy et al., 2009; Mizushima, 2004). I then assessed all murine models of LRRK2 under the mitochondrial conditions (10 μM MPP^+ for 6hrs). Analysis indicated

no significance in the Lrrk2 R1441C KI and Lrrk2 KO models. A significant ($p<0.05$) alteration in LC3-II/I ratios is seen with the Tg over-expressing colonies following MPP^+, raising the question of ectopic, over-expression artifacts (Appendix II - Fig. S.8). Additionally, since the non-Tg (+/+) controls of each colony responded conversely to an identical treatment, the data remain inconclusive. Moreover, the lack of a p62 phenotype is interesting, considering p62 levels reportedly increase with R1441C expression (Alegre-Abarrategui et al., 2009). Regardless, without complementary changes in LC3 ratios and p62 levels, we cannot conclude that there are differences in autophagic flux (Klionsky et al., 2012). Other forms of autophagy, such as CMA, were not assessed and therefore cannot be commented on (Orenstein et al., 2013). The above data was extracted from a set of experiments simultaneously testing the presence of: a) an autophagy inducer - PP242, and b) PP242 combined with an autophagy inhibitor - Bafilomycin A1, to amplify any subtle differences (Appendix II – Fig. S.6,7). However, due to variability and a lack of significant induction by PP242, the data remains inconclusive. Confirmation would require further studies, an investigation that warrants follow-up. However, as certain publications suggest that mitochondrial fragmentation precedes autophagy dysregulation and subsequent death then the lack of mitochondrial fragmentation in the germline models may underlie the fact that these consequential phenotypes (autophagy and death) were not observed (Wang et al., 2012b).

4.2.3. LRRK2 Neuronal Survival Analysis

When I assessed the survival paradigms in primary cortical neurons, I did so in a comprehensive manner. Each survival experiment represents a single, individualized,

heterozygous pairing dissection, thus controlling for the experimental error from sources such as: parental genetic background, development, dissection quality, growth media and conditions, drug preparation and stock, lysing and neuronal quantification. The lack of a LRRK2-linked phenotype in the *in vitro* studies were corroborated when I investigated the vulnerability of the intact, *in vivo*, dopaminergic system in these models. Note that the Lrrk2 KO model was not assessed *in vivo*, since it was not on a pure (C57BL/6) strain. However, given the results from an independent Lrrk2 KO strain and our *in vitro* studies, I predict that no difference would be observed (Andres-Mateos et al., 2009).

When the lack of hypersensitivity in Lrrk2 KO mice following MPTP was published, many considered this as more evidence to support the GOF hypothesis and refute the LOF hypothesis of LRRK2 (Andres-Mateos et al., 2009). However, in combination with our R1441C/G LRRK2 data, it could be argued that either 1) MPTP has no effect on these LRRK2 mice, or 2) since R1441C/G phenocopy Lrrk2 KO, they act in a similar LOF manner. The R1441C and Lrrk2 KO phenocopying was also observed in our mitochondrial studies. However, the latter is only speculative and would require further studies to validate. Interestingly, studies using human G2019S fibroblasts report hypersensitivity to MPP^+ treatment, *in vitro* (Yakhine-Diop et al., 2014). However, a critical piece of missing evidence in the literature is the assessment of the G2019S Lrrk2 Knock In mice (Longo et al., 2014; Yue et al., 2015). Testing of hyper-kinase G2019S KI mice would help establish whether MPTP-mediated death is irrelevant to *LRRK2* biology, or pertains to specific mutations that can mediate sensitization via independent mechanisms.

In the context of the MPTP data presented herein, there are a few considerations to highlight before conclusions can be drawn. Firstly, my *in vivo* MPTP analysis was focused at the pre-synaptic level (nigral axon terminal DAT/TH densitometry and neuronal quantification). However, LRRK2 midbrain expression is relatively low compared to other regions; higher concentrations of striatal LRRK2 warrant assessment of dysfunction in dopamine-innervated regions (Galter et al., 2006). To support this notion, MPTP treatment in primates increases striatal LRRK2 mRNA levels that correlate with L-dopa-induced dyskinesia severity, suggesting alterations in the post-synaptic response and output (Hurley et al., 2007). Since LRRK2 has been implicated in various cellular roles including transcriptional control (NF-κB, NFAT), it may have downstream effects on striatal/post-synaptic responses following MPTP (Gardet et al., 2010; Liu et al., 2011). It is possible that LRRK2 can alter post-synaptic neuronal processing following insult, and dysfunction commences at this level. Evidence of this nature would help delineate whether terminal dysfunction causes axonal retrograde oriented dysfunction and whether somatic death is consequential or precedes it, a topic of debate (Raff et al., 2002; Schulz-Schaeffer, 2015; Wang et al., 2012a).

Secondly, an investigation of the contributions of the supporting cells in the context of MPTP studies was not evaluated in my studies. Indirectly, astrocyte function was assessed via measurement of metabolized MPP^+ concentrations (Dauer and Przedborski, 2003). However, given the mounting evidence of LRRK2's involvement in immune, specifically microglial function, it is an avenue worth pursuing (reviewed in (Schapansky et al., 2015)). It has been demonstrated that the attenuation of immune responses are protective against MPTP-induced toxicity (Lira et al., 2011; Mount et al.,

2007). Microglia having inhibited Lrrk2 display a dampened immune response, which is protective against LPS insult, whereas R1441G expressing microglia induce neuronal toxicity (Gillardon et al., 2012; Moehle et al., 2012). This further supports the notion that immune-mediated responses contribute to, or exacerbate, the death of neurons following insult (Kannarkat et al., 2013). It also provides evidence that alternative exogenous, immune-mediated (bacteria, viruses) triggers of cellular toxicity may be more relevant in the context of LRRK2 or PD linked neuronal toxicity (Hakimi et al., 2011; Woulfe et al., 2014). This does not negate a neuronal role for LRRK2, but highlights another avenue of investigation that contributes to neuron viability and could be extremely insightful.

With regards to the MPTP model, the literature indicates differential responses between the various PD-linked genetic murine models (Aguiar et al., 2013; Dauer et al., 2002; Dong et al., 2002; Drolet et al., 2004; Haque et al., 2012; Kim et al., 2005a; Klivenyi et al., 2006; Nieto et al., 2006; Perez-Sanchez et al., 2010; Rathke-Hartlieb et al., 2001; Thomas et al., 2011). Although the recessive PD-linked cases are often considered phenotypically (clinically) similar and play mitochondrial-related roles, only DJ-1 and Pink1 KO mice show MPTP-induced sensitivity, whereas Parkin KO does not (Aguiar et al., 2013; Haque et al., 2012; Kim et al., 2005a). However, over-expression of Parkin or DJ-1 rescues the MPTP deficits in Pink1 KO mice, suggesting that Parkin has an MPTP-independent protective mechanism (Haque et al., 2012). Hence, the MPTP model sensitizes specific mechanistic pathways that are only relevant in specific PD sub-types. This of the MPTP model limitation is not concerning, so long as the manner in which findings are extrapolated are handled with reasonable caution; namely, its relevance to iPD.

MPTP exposure is a model of acute DA toxicity, although its toxicity is not completely selective (Javitch et al., 1985; Mayer et al., 1986). Simply stated, if MPP^+ was structurally altered so that it had a higher affinity for a separate transporter/cell type, then it would be employed as a model of death-related syndromes in that context. The MPTP model gained relevance to parkinsonism due to its specificity and reproducibility, and the fact that it provided evidence that exogenous substances could target the system in a similar manner (Tieu, 2011). However, the results obtained using the MPTP model do not necessarily translate in clinical settings. One clear example of such discordance is with fetal stem cell graft surgeries that were performed on a subset of MPTP affected patients, who demonstrated very gradual, but marked clinical improvements (Aydinlik and Lachnit-Fixson, 1977; Widner et al., 1993). Although this procedure was originally reported to demonstrate clinical improvement in early open clinical trial studies (Lindvall et al., 1989; Lindvall et al., 1992; Sawle et al., 1992); it failed to show significant improvement in subsequent double-blinded clinical trials when performed on patients suffering from iPD (Andrews et al., 1992; Freed et al., 2001). This calls into question the fundamental differences between MPTP-induced parkinsonism and iPD.

Although some may interpret my data to mean that environmental toxins are not involved in *LRRK2*-linked PD, I would not encourage such bold conclusions. We can only conclude that at juvenile ages, R1441C/G LRRK2-linked murine models are not susceptible to MPTP-induced death. This does not rule out the possibility that the LRRK2 G2019S mutation would be susceptible or that at advanced ages, mice will respond differentially to exposure. This is more relevant for late-onset *LRRK2*-linked PD, as MPTP-mediated effects are enhanced on aged animals versus juvenile ones (Irwin

et al., 1992; Ohashi et al., 2006). The arguments against environmental (pesticide and herbicide) association with increased risk of developing PD is that, these substances have been more commonly employed within the last few decades, and evidence of PD has been noted dating back to ancient times (reviewed in (Goetz, 2011)). Therefore, compounds with a similar mechanism of action as MPTP may exist naturally in our environment, such as rotenone, which has been used as an "organic" piscicide for centuries (Metcalf, 1948). However, rotenone's actions are broad and reproducibility is inconsistent (discussed in (Tieu, 2011)). Conversely, the herbicide paraquat has been around for a number of decades and has been reported by numerous independent groups to be associated with increased risk of developing PD (Wirdefeldt et al., 2011). Unlike MPTP and rotenone, paraquat does not inhibit complex I; rather it facilitates the production of superoxide radicals and increases cellular oxidative stress (Fukushima et al., 2002). This mechanism of action aligns well with the oxidative stress hypothesis of PD (Fahn and Cohen, 1992). Therefore, I recommend the testing of paraquat in the context of *LRRK2*-linked PD models; regardless of its inability to convincingly reduced striatal DA levels (McCormack et al., 2002; Thiruchelvam et al., 2000). Given the broader pathology in PD, perhaps paraquat or rotenone, actual environmental toxins, are better models to employ and that cellular dysfunction (protein aggregation) rather than death should be the readout measure. Alternatively, the impact of naturally occurring biological triggers, such as bacteria and viruses, may be more relevant for future studies (Starakis et al., 2011; Woulfe et al., 2014).

Taken together, the *D. melanogaster* model of LRRK2-linked PD successfully recapitulated hallmark features of PD, including TH-positive neuronal loss and motor

deficits (Venderova et al., 2009). It also provided a system to perform an in vivo, functional, genetic suppressor/enhancer interaction screen. This screen identified a discrete number of genetic interactors (36) of LRRK2 including: 1) CG8561 - drosophila Convoluted gene, human *IGFALS*; 2) CG8523 - drosophila Mdr50 gene, human *MDR1*; and 3) CG8542 - drosophila Heat Shock Protein cognate 5 (Hsc70-5) gene, human *HSPA9* (manuscript in preparation). These candidate gene interactor have opened the door for subsequent investigations of objectively identified LRRK2 pathways that show relevance to PD biology and can be pursued in future investigations. I also believe that my mammalian investigations highlight cautiousness when investigating *LRRK2* biology and re-iterate the limitations of the MPTP model.

Appendices. Supplemental Information

Appendix I. References

Appendix II. Additional Data and Results

Appendix III. Additional Scientific Contributions

Appendix I. References

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Appendix II. Additional Data and Results

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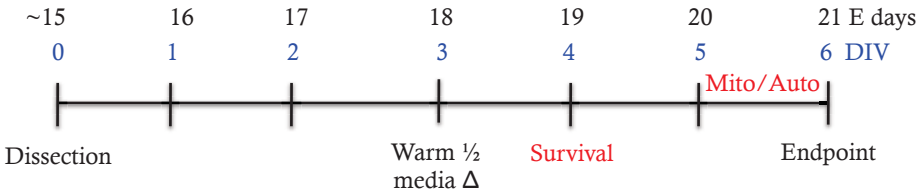
Table S.1. Identifiable genes deleted or disrupted in Primary Hit #7876.
Summary of the identifiable genes interrupted in the Bloomington Deficiency Kit stock #7876 of the 2R chromosome corresponding to *Df(2R)Exel7131*.

Gene Annotation	Gene Symbol / Name	Human Protein Ortholog
CG12505	Arc1: Activity-regulated cytoskeleton associated protein 1	activity-regulated cytoskeleton-associated protein (26%)
CG13941	Arc2: Activity-regulated cytoskeleton associated protein 2	activity-regulated cytoskeleton-associated protein (26%)
CG8468	N/A	solute carrier family 16, member 14 protein
CG8485	N/A	Sucrose Non-Fermenting (SNF) Related serine/threonine-protein kinase
CG8503	N/A	histone methyltransferase
CG8531	N/A	DnaJ (Hsp40) homolog, subfamily C, member 11
CG8547	N/A	N/A
CG8613	N/A	Spermatogenesis-associated protein 20 (SPATA20) isoform 3
CG8617	N/A	CKLF-like MARVEL transmembrane domain-containing protein 3
CG34184	N/A	zinc finger protein 609, isoform CRA_a
CG34185	N/A	hCG1816874
CG34442	N/A	N/A
CG34443	N/A	INO80 complex homolog 1 (S. cerevisiae), isoform CRA_a PREDICTED: histone-lysine N-methyltransferase EZH2 isoform X16
CG34444	N/A	PREDICTED: protein AF-17 isoform X2
CG8561	conv: convoluted	Insulin-like growth factor binding protein, acid labile subunit
CR10102	N/A	N/A
CG8542	Hsc70-5: Heat shock protein cognate 5	Hsc 70 Protein 9 Precursor (mortalin) heat shock 70kDa protein 9B
CG8585	Ih: I_h channel	Ion Channel BCNG-2 Transmembrane protein Jagged

CG8523	Mdr50: Multi drug resistance 50	multidrug resistance protein 1 p-glycoprotein
CR42951	mir-308: mir-308 stem loop	N/A
CR42981	mir-1016: mir-1016 stem loop	N/A
CG30067	Obp50a: Odorant-binding protein 50a	N/A
CG30073	Obp50b: Odorant-binding protein 50b	N/A
CG30072	Obp50c: Odorant-binding protein 50c	N/A
CG30074	Obp50d: Odorant-binding protein 50d	N/A
CG13939	Obp50e: Odorant-binding protein 50e	N/A
CG8479	Opa1: Optic atrophy 1 ortholog (H. sapiens)	mitochondrial dynamin-like 120 kDa protein Optic atrophy 1 (autosomal dominant)
CG8415	RpS23: Ribosomal protein S23	40S ribosomal protein S23
CG8553	SelD: Selenide, water dikinase	Selenophosphate Synthetase; selenide, water dikinase 1 isoform 1
CG34379	Shroom: Shroom	Shroom3 Protein PREDICTED: protein Shroom3 isoform X2
CG8404	Sox15: Sox box protein 15	SOX17 protein
CG8589	tej: tejas	TDRDS protein
CG8151	Tfb1: TFB1 ortholog (S. cerevisiae)	general transcription factor IIH subunit 1
CG8494	Usp20-33: Ubiquitin specific protease 20/33	ubiquitin carboxyl-terminal hydrolase 33 isoform X1 pVHL-interacting deubiquitinating enzyme 1 type II
CG8394	VGAT: Vesicular GABA Transporter	vesicular inhibitory amino acid transporter SLC32A1 protein
CG8536	β4GalNAcTA: β4GalNAcTA	β-1,4-galactosyltransferase 2 isoform b

IN VITRO OXIDATIVE STRESS PARADIGM

- MPP$^+$-Induced PD Model
  - Treatment Paradigm used:
    - Survival: 20uM MPP$^+$ (Time-Course – 48, 36, 24, 12 hrs)
    - Mitochondria: 10uM MPP$^+$ (6hrs)
    - Autophagy: 10uM MPP$^+$ (Time-Course – 9, 6, 3 hrs)



IN VIVO OXIDATIVE STRESS PARADIGMS

- MPTP-Induced PD Model
  - Sub-Chronic Paradigm:
    - 5 i.p. injections 30mg/kg (1 injection/day for 5 consecutive days)
  - Acute Paradigm:
    - 4 i.p. injections 20 mg/kg (1 every 2 hrs) in 1 day

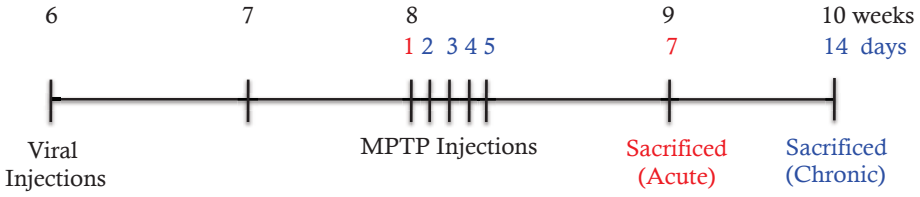


Figure S.1. Schematic representations of the original experimental paradigms assessed, *in vitro* and *in vivo*.
Note that this does not include the stringent autophagy assessments that have been performed and are also presented here in Appendix II.

IN VIVO ECTOPIC OVER-EXPRESSION OF hLRRK2 VIRAL CONSTRUCTS

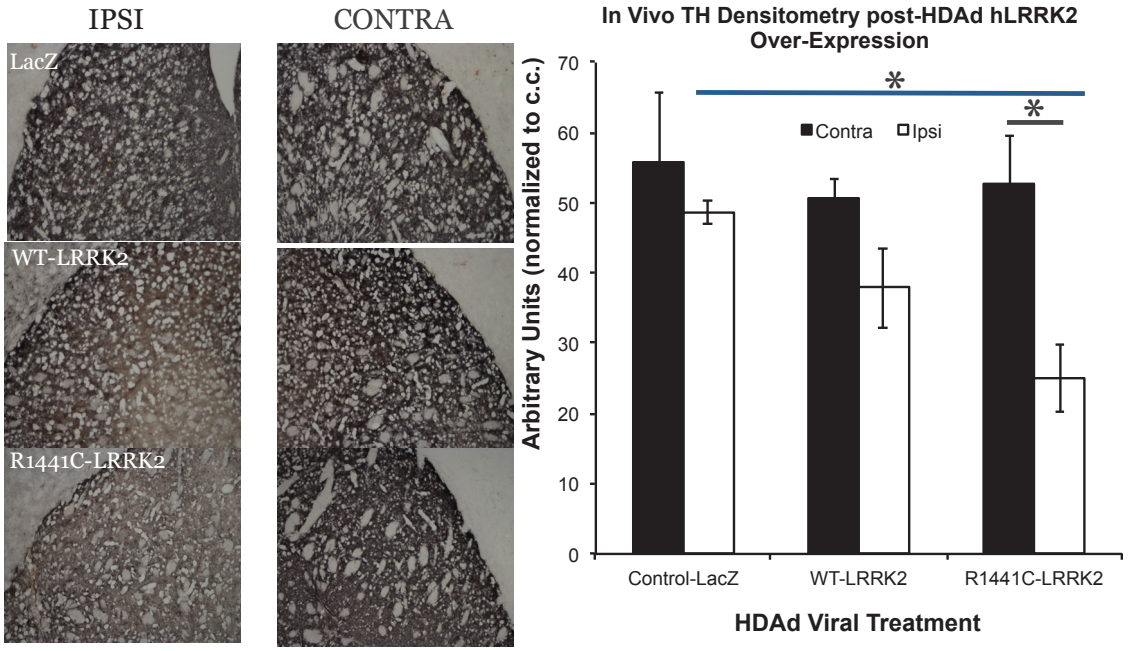


Figure S.2. *In vivo* ectopic over-expression of hLRRK2 viral constructs
(left) Striatal sections of C57BL/6 mice injected with control-LacZ, WT or R1441C–hLRRK2 HDAd for 2 weeks, stained for anti-tyrosine hydroxylase (TH). Note the differences in staining intensity of the ipsilateral (injected) side versus the contralateral (non-injected) hemisphere of the brain. ***(Right)*** Quantification of the densitometry of striatal sections following injections of control-LacZ (n=3), WT (n=3) or R1441C–LRRK2 (n=5) HDAd. * $p < 0.05$.

Methodology: Male C57BL/6 mice ~8 weeks of age received unilateral, intra-striatal, stereotaxic viral injections of Helper-Dependent Adenoviruses (HDAd) custom-made by our collaborator, Dr. Robin J. Parks (OHRI). 2µL of 1.0x107 pfu/µL were injected into the striatum at a rate of 0.5µL/min of vectors carrying: LacZ, GFP, $LRRK2^{WT}$, $LRRK2^{R1441C}$, $LRRK2^{I2020T}$.

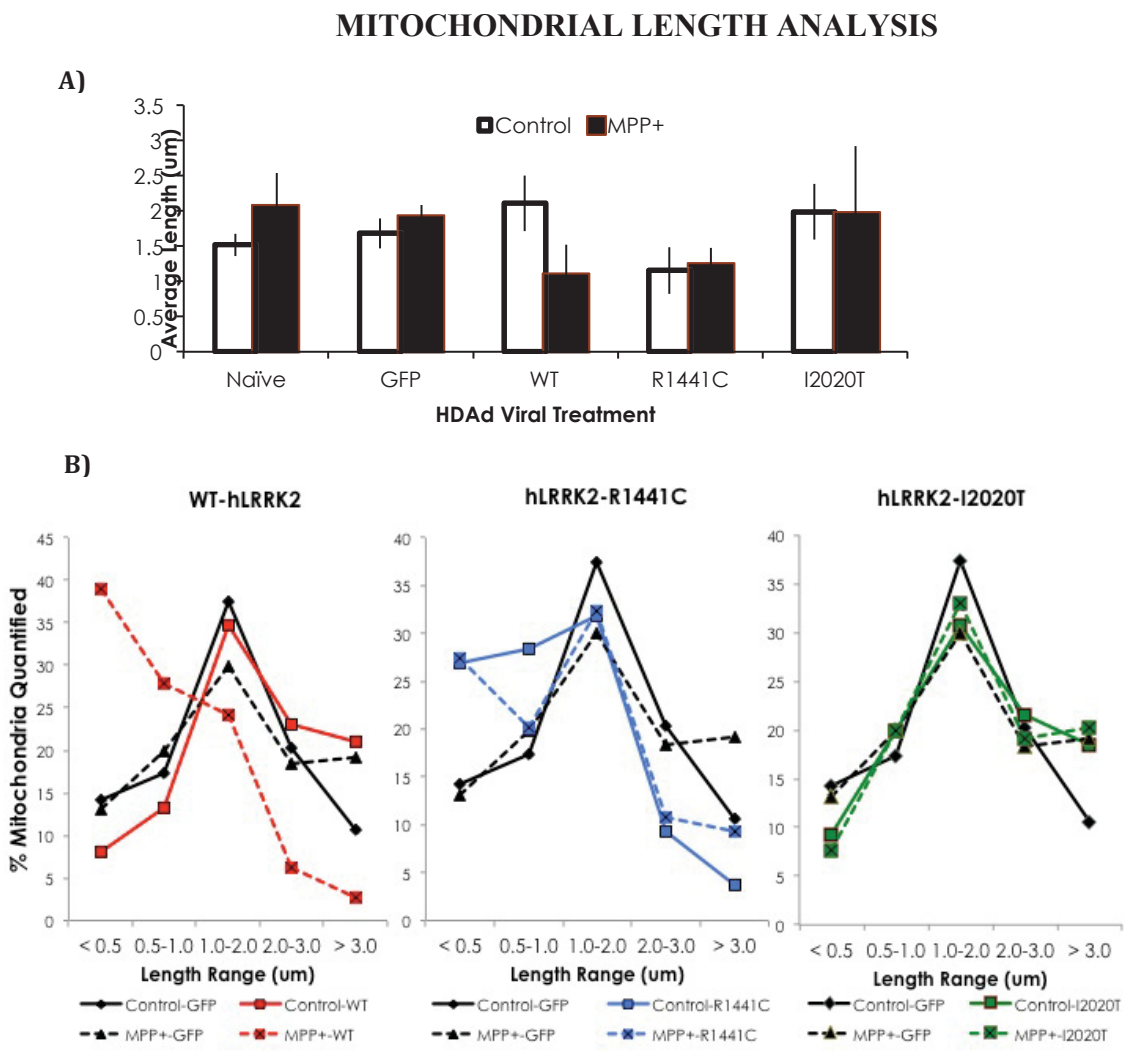


Figure S.3. Mitochondrial length with hLRRK2 following stress conditions
A) Average mitochondrial lengths in neurons over-expressing control, WT or mutant R1441C hLRRK2 exposed to either control or 10 μM MPP^+ treatment for 6 hrs. **B)** Distribution of the mitochondrial lengths measured in the same experiment, shown in a separate style to simplify the data being displayed, Note: Controls are all the same (repeated). This experiment is representative of N of 1.

Methodology: Cortical neurons from WT CD-1 mice are harvested at ~E.15 and grown in culture for 72 hours prior to HDAd infection for 72 hours. In the final 6 hours cultures were treated with control or 10 μM MPP^+. Fixation was achieved with 4% PFA, and the mitochondria stained anti-TOM20. GFP-positive, viable nuclei (Hoechst) were quantified.

ASSESSMENT OF AUTOPHAGY IN LRRK2 KO CORTICAL CULTURES

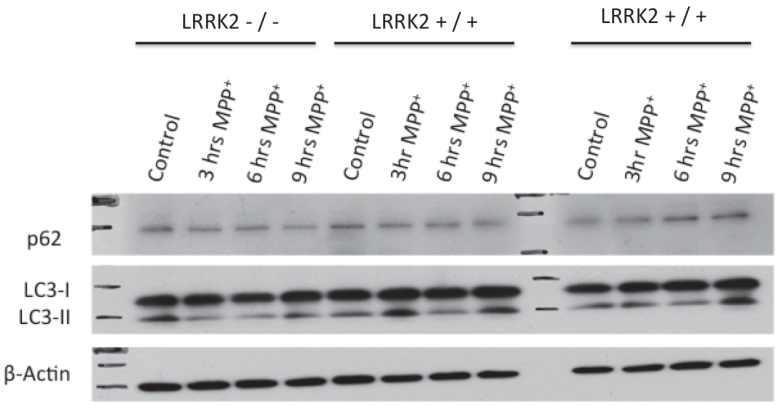


Figure S.4. Assessment of autophagy in Lrrk2 KO cortical neuron cultures
Protein blots looking at the induction of autophagy in basal or oxidative stress conditions, as induced by 3, 6 or 9 hrs of 10 μM MPP+ treatment in Lrrk2 WT or KO cortical cultures. These time points were chosen to parallel the paradigm used in the *in vitro* mitochondrial studies.

ASSESSMENT OF AUTOPHAGY IN WT hLRRK2 EXPRESSING CORTICALS

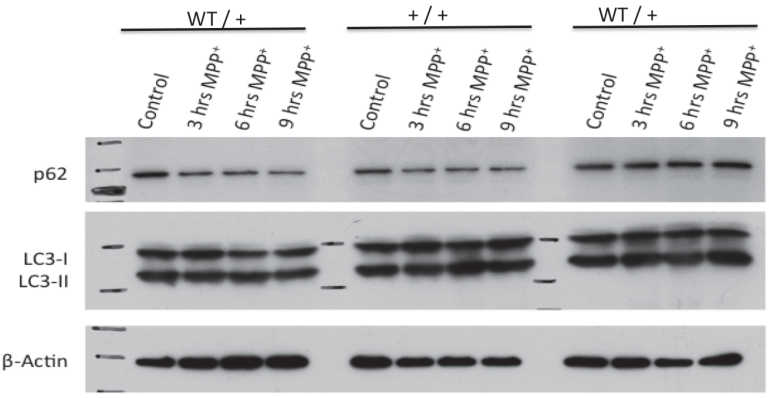


Figure S.5. Assessment of autophagy in hLRRK2 WT over-expressing cortical cultures
Protein blots looking at the induction of autophagy in basal or oxidative stress conditions, as induced by 3, 6 or 9 hrs of 10 μM MPP+ treatment in Lrrk2 WT or KO cortical cultures. These time points were chosen to parallel the paradigm used in the *in vitro* mitochondrial studies.

Methodology: Cortical neurons from littermate embryos were harvested at ~E.15 and grown in culture for 6 DIV. In the final 9, 6 and 3 hours cultures were treated with control or 10 μM MPP^+. Cells were collected and protein samples were run on a 15% SDS-PAGE gel and immunoblotting for β-actin, LC3 and p62 were performed. This figure represents one experiment loaded onto a single gel with N= 1or 2.

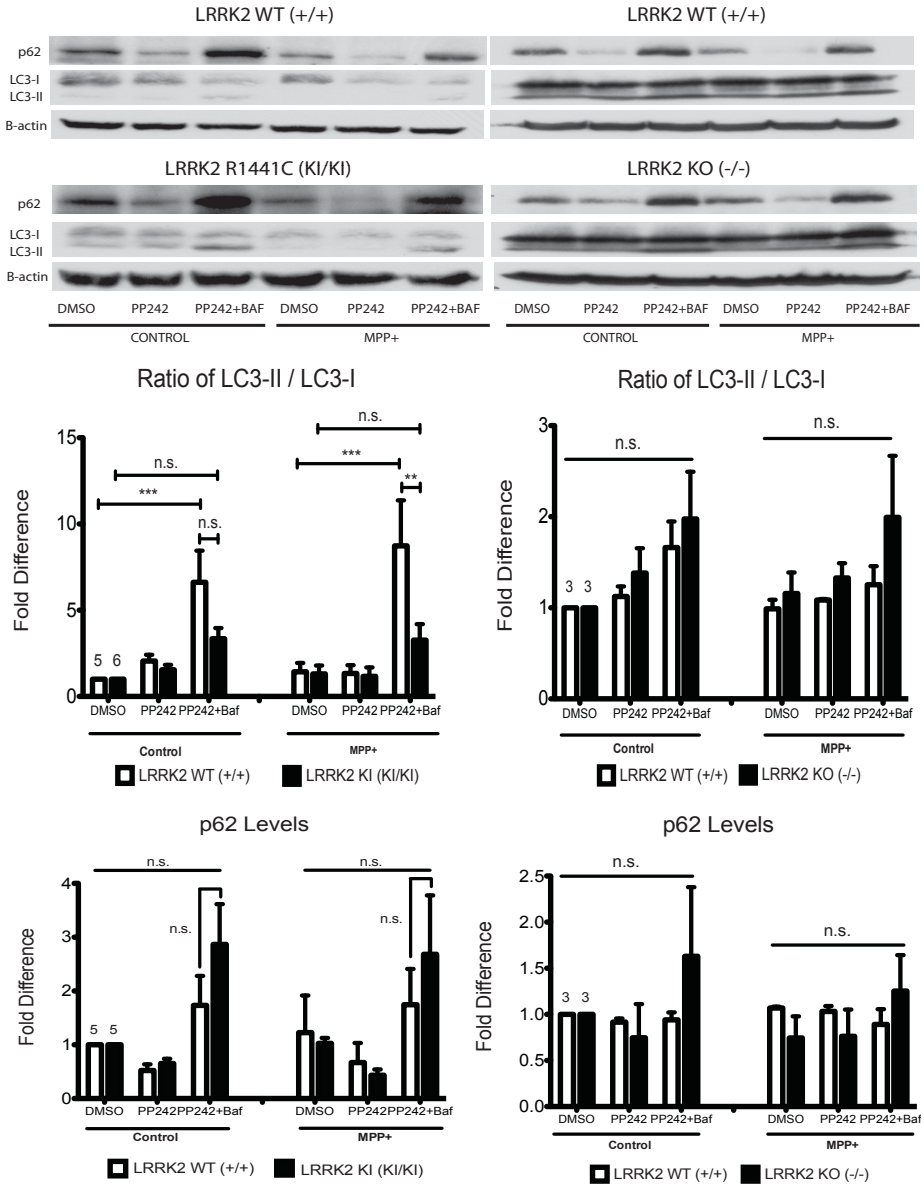


Figure S.6. Cortical neuron autophagy markers in Lrrk2 R1441C KI and Lrrk2 KO and WT samples, *in vitro*.

Assessment of LC3-II/I ratios and p62 proteins levels as markers of autophagy in primary, embryonic cortical neuron cultures harvested from E14.5-15.5 day old littermate embryo samples. Neurons were cultured for 6DIV and treated with either control; PP242 alone; or PP242 in combination with Bafilomycin A1, in the presence or absence of 10 μM MPP^+ for 6 hours. SDS-PAGE gels and immunoblotting performed by M. Parsanejad, PhD.

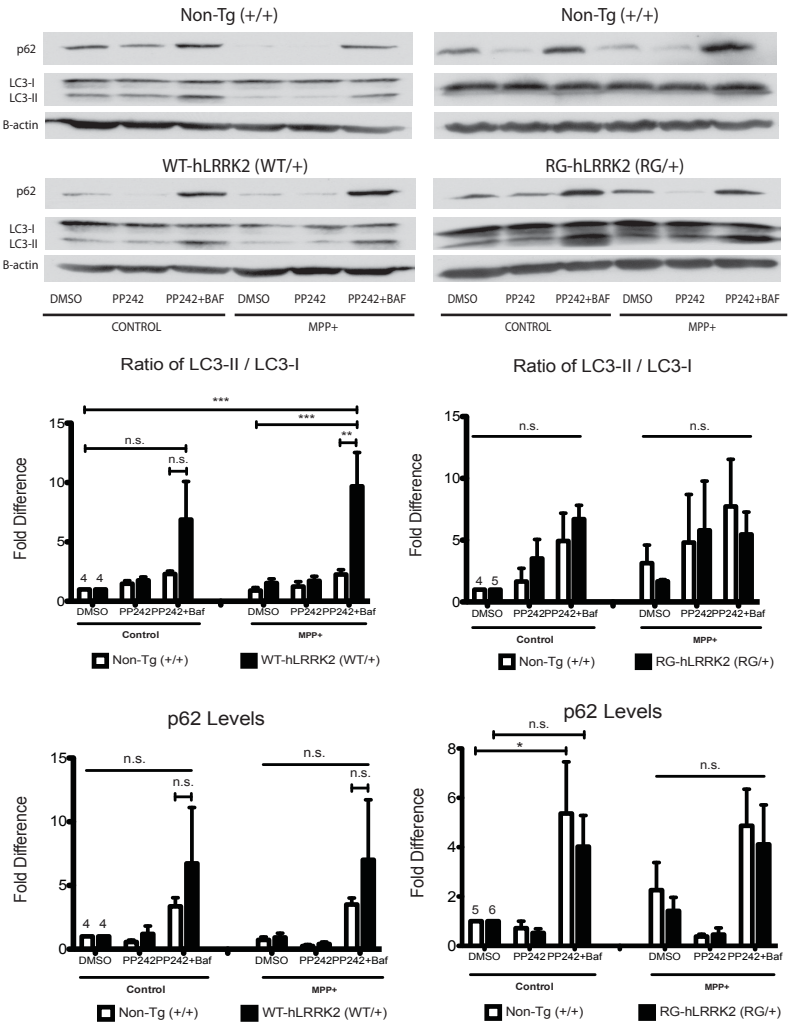


Figure S.7. Cortical neuron autophagy markers in non-Tg or WT and RG-hLRRK2 samples, *in vitro*.

Assessment of LC3-II/I ratios and p62 proteins levels as markers of autophagy in primary, embryonic cortical neuron cultures harvested from E14.5-15.5 day old littermate embryo samples. Neurons were cultured for 6DIV and treated with either control; PP242 alone; or PP242 in combination with Bafilomycin A1, in the presence or absence of 10 μM MPP^+ for 6 hours. Western gels and immunoblotting were performed by M. Parsanejad, PhD.

Methodology: Neurons were treated with control (DMSO), 1 μM PP242 (Sigma) or 1 μM PP242 + 300 nM Bafilomycin A (Cayman Chemical, 300nM) for 20hrs prior to endpoint (DIV4-6). Then in the last 6hrs before collection, neurons from each treatment group were exposed to either control or 10 μM 1-methyl-4-phenylpyridinium (MPP^+) iodide (Sigma: M0896-10MG). Samples were collected, stored and later run on a 15% SDS-PAGE gel. Immunoblotting for β-actin (Sigma, 1:50,000), p62 (American Research Products, 1:1000) and LC3 (Novus Biological, 1:1000) were performed, followed by densitometric analysis using NIH ImageJ software, as previously reported.

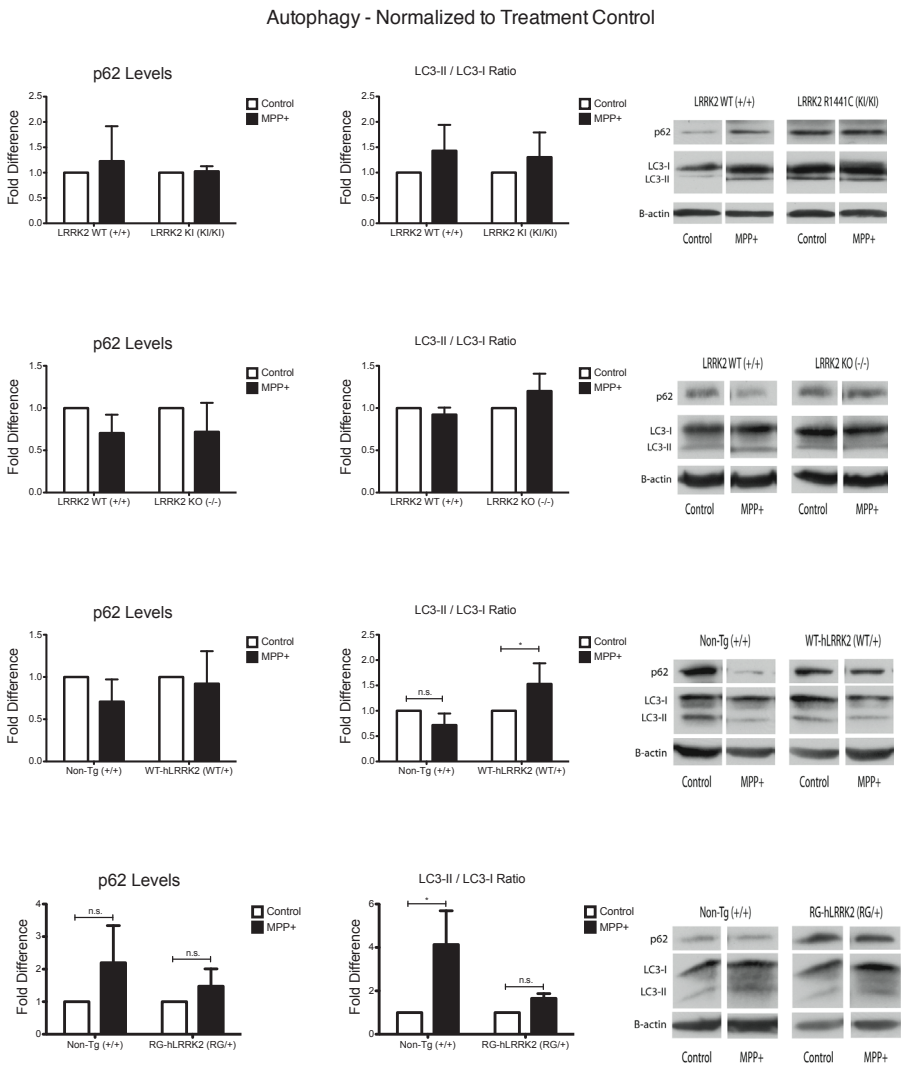


Figure S.8. Assessment of the impact of mild MPP$^+$ treatment on cortical neuron autophagy in various murine models of LRRK2, *in vitro*.
Simplified presentation of the assessment of LC3-II/LC3-I ratios and p62 protein levels as markers of autophagy in primary, embryonic cortical neuron cultures. Non-induced or inhibited data was extracted from the previous results (**Fig. S.6,7**) and normalized to treatment control for each genotype. From left to right, each colony is presented with p62 protein level densitometry, LC3-II/I and representative blots. Assessment was performed in (A) Lrrk2 R1441C KI, (B) Lrrk2 KO, (C) WT-hLRRK2, (D) RG-hLRRK2 and respective WT or Non-Tg littermate (+/+) control cortical neuron samples. Number of embryos (N) is indicated on graphs. Western gels and immunoblotting performed by M. Parsanejad, PhD.

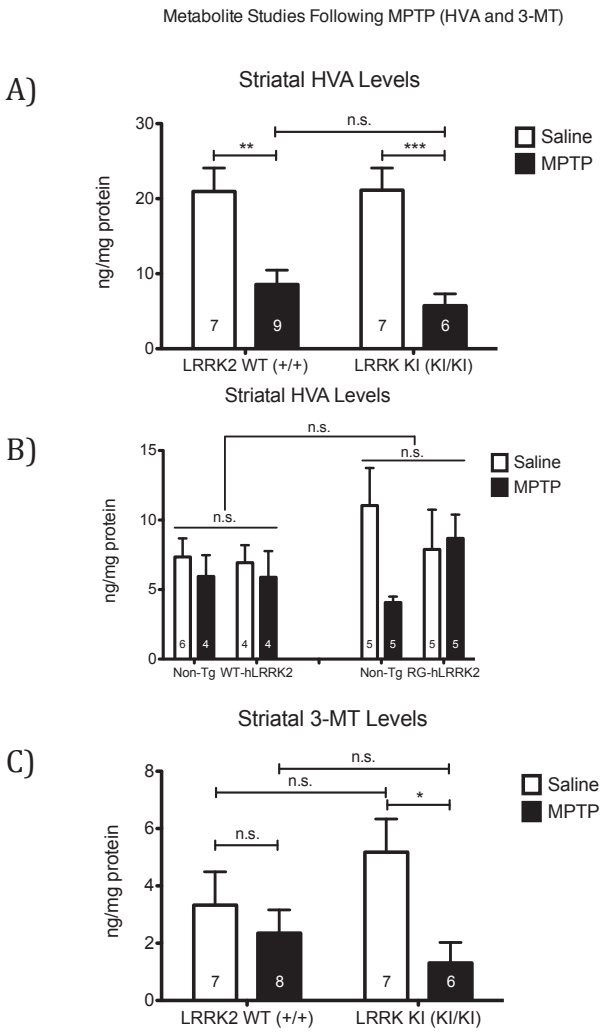


Figure S.9. Assessment of HVA and 3-MT metabolite levels.
HPLC measurements of HVA concentrations (ng/mg protein) in micropunch samples from the striatum of A) Lrrk2 R1441C KI and B) WT or R1441G hLRRK2 over-expressing animals and their respective WT or non-Tg (+/+) littermate controls; and C) 3MT levels in the Lrrk2 R1441C KI model. Samples were collected 7 days following *acute* MPTP treatment, corresponding to the same point when all *acute* striatal IHC and SNc neuronal survival assessments were performed.

Methodology:
Animals were sacrificed 7 days post-Acute MPTP injections. Fresh 2.0mm thick x 2.0 diameter micropunch tissue samples were collected from both striatal hemispheres and placed in a homogenizing solution, stored at -80°C until sent for HPLC analysis, by J. Kulczycki in the lab of our collaborator, Dr. Hymie Anisman, Carleton University.

Appendix III. Additional Scientific Contributions

Inactivation of Pink1 gene in vivo sensitizes dopamine-producing neurons to 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and can be rescued by autosomal recessive Parkinson disease genes, Parkin or DJ-1

This research was originally published in the Journal of Biological Chemistry. Haque ME, Mount MP, Safarpour F, Abdel-Messih E, Callaghan S, Mazerolle C, Kitada T, Slack RS, Wallace V, Shen J, Anisman H, Park DS. Inactivation of Pink1 gene in vivo sensitizes dopamine-producing neurons to 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and can be rescued by autosomal recessive Parkinson disease genes, Parkin or DJ-1. *J Biol Chem. 2012 Jun 29;287(27):23162-70.*

Personal Contributions

Abdel-Messih, E contributed to the project and assisted project lead Haque, ME in his absence, by performing perfusions, extractions, fixation and cryoprotection of viral injected brain samples. Abdel-Messih, E supplied prepared samples, with instructions to Departmental Technician, who sectioned the samples on behalf of Haque ME.

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Inactivation of *Pink1* Gene *in Vivo* Sensitizes Dopamine-producing Neurons to 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and Can Be Rescued by Autosomal Recessive Parkinson Disease Genes, *Parkin* or *DJ-1*

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Background: Mutations in *Pink1* are associated with Parkinson disease.
Results: Mouse *Pink1* deficiency results in hypersensitivity to MPTP-induced dopaminergic neuronal loss, which can be rescued with expression of human *Parkin* or *DJ-1*.
Conclusion: *Pink1* gene can regulate response to exogenous stress.
Significance: These results indicate how endogenous *Pink1* plays an important role in management of exogenous stress in mouse brain.

Mutations in the mitochondrial PTEN-induced kinase 1 (*Pink1*) gene have been linked to Parkinson disease (PD). Recent reports including our own indicated that ectopic *Pink1* expression is protective against toxic insult *in vitro*, suggesting a potential role for endogenous *Pink1* in mediating survival. However, the role of endogenous *Pink1* in survival, particularly *in vivo*, is unclear. To address this critical question, we examined whether down-regulation of *Pink1* affects dopaminergic neuron loss following 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) in the adult mouse. Two model systems were utilized: virally delivered shRNA-mediated knockdown of *Pink1* and germ line-deficient mice. In both instances, loss of *Pink1* generated significant sensitivity to damage induced by systemic MPTP treatment. This sensitivity was associated with greater loss of dopaminergic neurons in the Substantia Nigra pars compacta and terminal dopamine fiber density in the striatum region. Importantly, we also show that viral mediated expression of two other recessive PD-linked familial genes, *DJ-1* and *Parkin*, can protect dopaminergic neurons even in the absence of *Pink1*. This evidence not only provides strong evidence for the role of endogenous *Pink1* in neuronal survival, but also supports a role of *DJ-1* and *Parkin* acting parallel or downstream of endogenous *Pink1* to mediate survival in a mammalian *in vivo* context.

Parkinson disease (PD)[3] is a neurodegenerative disorder characterized by loss of dopamine neurons and movement deficits (1). Several recessive genes (*Pink1*, *Parkin*, and *DJ-1*) have been linked with familial forms of the disease (2). How mutation of these genes leads to PD pathology is unknown. *Parkin* possesses an E3 ubiquitin ligase activity, and its loss is associated with mild mitochondrial defects (3). Expressed *Parkin* translocates to defective mitochondria in response to the loss of the mitochondrial membrane potential and mediates mitophagy (4–6). In contrast to *Parkin*, *DJ-1* has an atypical peoxyredoxin like peroxidase activity (7). Its expression or loss has been associated with cell survival, particularly in response to oxidative stress (8, 9).

Pink1, the third and most recent recessive PD gene identified, contains a mitochondrial targeting motif and a serine-threonine kinase domain (10). The physiological substrate(s) of *Pink1* are not fully defined. *Pink1* can phosphorylate two mitochondrial proteins, *Trap1* and *HtrA2* (11, 12). However, the physiological relevance of this phosphorylation needs clarifica-

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[3] The abbreviations used are: PD, Parkinson disease; AAV, adeno-associated virus; DA, dopamine; DAT, dopamine transporter; ISH, *in situ* hybridization; MPP^+, 1-methyl-4 phenylpyridinium; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; MTN, medial terminal nucleus; *Pink1*, PTEN-induced kinase 1; SNc, Substantia Nigra pars compacta; TH, tyrosine hydroxylase.

tion. Growing evidence also suggests that *Pink1* is required for the proper maintenance and regulation of mitochondrial morphology and function (13–16). Germ line deletion of *Pink1* in *Drosophila* causes mitochondrial defects in indirect flight muscles and results in complete disruption of mitochondrial cristae (13, 14). However, this does not occur with *Pink1* germ line-deficient mice. Similarly, *Pink1* has been shown to be essential for the translocation of expressed *Parkin* to the mitochondria in response to mitochondrial depolarization (17). However, whether this pathway of mitochondrial quality control is critical for the pathology of PD is ultimately unknown.

At a more fundamental level, *Pink1* may be an important modulator of cell survival (18). *Pink1* deficiency by itself does not appear to induce neuronal loss (19, 20). However, whether *Pink1* is essential in response to exogenous stress is an intriguing possibility. Indeed, it has been shown that expression of *Pink1 in vitro* is protective in response to exogenous stress such as 1-methyl-4 phenylpyridinium (MPP^{+}), rotenone, and staurosporine (21–23). However, these studies suffer from the caveats of overexpression, and the role of endogenous *Pink1* in dopamine neurons *in vivo* is unknown. Here, we show that knockdown or knock-out of *Pink1* in the SNc area is more sensitive to 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) insults. We also find that this sensitization is reversed by other PD-associated genes such as *Parkin* or *DJ-1* and that these genes can protect even in the absence of *Pink1*. This evidence suggests that there are functional interactions among these three recessive PD genes that have a demonstrable impact on environmental stress.

EXPERIMENTAL PROCEDURES

Mice—All procedures were approved by the University of Ottawa Animal Care Committee, and the animals were maintained in strict accordance with the Guidelines for the Use and Treatment of Animals put forth by the Animal Care Council of Canada and endorsed by the Canadian Institutes of Health Research. Germ line-deleted *Pink1* mice were a generous gift from Dr. J. Shen and were back-crossed to C57BL/6 for more than seven generations (19).

Generation of Mouse sh-Pink1 Adenovirus—The *Pink1* siRNA oligonucleotides (Ambion ID nos. 180640, 180641, and 180642) (directed to silence mouse *Pink1* as well as negative control siRNA (nontargeting siRNA) were purchased from Ambion and cloned into the pSilencer 3.0-H1 siRNA vector as reported previously (23). The shRNA fragment, including the H1 promoter, was subcloned to pAdTrack-CMV vector to generate adenovirus as previously described and validated (23). We used the siRNA (ID no. 180640) in the subsequent experiment because it showed the highest knockdown efficiency.

Generation of Human DJ-1 Adenovirus—We generated adenovirus-harboring human *DJ-1* as mentioned earlier (8). Briefly, adenovirus vector-expressing human *DJ-1* were generated by subcloning into pAdTrack-CMV vector (8) in which the expression of GFP and *DJ-1* is driven by two separate CMV promoters. Adenovirus was produced and titered as described previously (8).

Generation of Human Parkin Adeno-associated Virus (AAV)—GFP-*Parkin* AAV was a generous gift from Dr. Edward A. Fon (University of McGill, Canada). AAV was generated and purified as described previously (24).

Viral Gene Delivery in Vivo—Male, 8–10 week-old C57BL/6 mice were purchased from Charles River Laboratories. The mice were individually housed and were acclimated to the new environment before receiving the recombinant adenoviruses that expressed sh-*Pink1* or scrambled DNA as control. Adenoviruses (2 μl; 1×10^7 particles/μl per construct) were stereotaxically injected into the striatum (coordinates from bregma: anterior-posterior, +0.5 mm; medial-lateral, −2.2 mm; dorsal-ventral, −3.4 mm) at an infusion rate of 0.5 μl/min using a syringe pump (PHD2000; Harvard Apparatus). The reduction of *Pink1* message is as described before (23). In addition, we performed *in situ* hybridization (ISH) to show the down-regulation of *Pink1* in the virus-injected area especially in the SNc area as described below. Similarly, human *DJ-1* or GFP control adenoviruses were injected to the midbrain of *Pink1* KO and WT mice. The mice that received sh-*Pink1* or *DJ-1* were challenged with MPTP or saline as mentioned in the MPTP injection section.

AAVs harboring human *Parkin* or GFP control were diluted with 20% mannitol to obtain 1.3×10^5 virus particles/injection. The mannitol premixed viruses (2 μl) were stereotaxically injected into the SNc area (coordinates from bregma: anterior-posterior, −3.0 mm; medial-lateral, −1.6 mm; dorsal-ventral, −4.1 mm) at an infusion rate of 0.5 μl/min using a syringe pump as mentioned earlier. The mice were kept for 2 weeks for complete expression of *Parkin* or GFP, and then the animals were challenged with MPTP as mentioned below.

ISH of Mouse Pink1—ISH was performed as described previously (25) using digoxigenin-labeled antisense RNA riboprobes prepared by *in vitro* transcription from linearized plasmids containing partial sequence of the mouse *Pink1* gene. In brief, mouse *Pink1* cDNA was generated from mouse RNA by RT-PCR. The generated cDNA was used as a template to synthesize a 500-bp DNA fragment by PCR and cloned into pcDNA 3.1(+) vector in sense and antisense direction. Sense and antisense vectors were then used to generate digoxigenin-labeled RNA probes where sense is used as a negative control. Mouse brains were sectioned at 14-μm thickness and incubated with the above mentioned probe overnight and then processed for staining. The stained sections were analyzed on an Axioskop 2 microscope and images were captured using a Microfire camera. The ISH signal was assessed by densitometric analyses using ImageJ software (National Institutes of Health). Briefly, the average densities of striatal fields or individual neurons in the SNc area in both contralateral and ipsilateral sites were determined. Percentage signal was then calculated as ratio of the signal in the ipsilateral *versus* contralateral site and multiplied by 100.

MPTP Administration in Vivo—Mice were challenged with MPTP once a day for 5 consecutive days (25 mg/kg, intraperitoneal, measured as free base; MPTP-HCl; Sigma-Aldrich) 1 week after adenovirus injection to permit sufficient time for retrograde transport and expression of the adenoviral-derived proteins (26, 27). In the case of AAV,

MPTP injection started 2 weeks after initiation of viral injection. Mice used as control received an equivalent volume of saline (0.9%) once daily. Assessment of dopamine neuron survival was performed 2 weeks after the start of the MPTP dosing regimen.

Immunohistochemical Analysis of Tyrosine Hydroxylase (TH)-positive Neurons—Brain tissues from mice injected with MPTP or saline were collected for immunohistochemical analyses as described previously (26, 27). Antibodies used were TH (1:10,000; Immunostar), and immunoreactivity was visualized by using an avidin-biotin complex peroxidase reaction.

Assessment of Dopamine Neuron Loss in Vivo—For viral experiments, the loss of neurons in the SNc was determined by serial section analysis of the total number of TH neurons in the medial terminal nucleus (MTN) region. Intrastriatal administration of adenovirus results in the highest retrograde expression of the gene at this level (26). Briefly, mouse brains were collected and sectioned into 14-μm slices for TH staining. The total numbers of TH positive neurons in the MTN region (-3.08 to -3.28 mm of bregma) in the ipsilateral and contralateral hemispheres were counted separately from at least three sections for each animal. The average numbers of TH neurons in each site of the brain were calculated and presented in the graph. We counted the same subpopulations of TH neurons in the case of AAV injections because they were directly delivered in the MTN region. Cresyl violet staining and counting were similarly performed to validate the result of TH immunostaining as reported previously (26). Briefly, sections at the levels of the MTN as described above were assessed for healthy cells with the morphology and size of dopamine neurons. Quantitation is similar as described for TH counts.

For the evaluation of TH neurons of *Pink1* KO mice only, we employed optical fractionation (28) using Stereo Investigator (version 6; MicroBrightField, Williston, VT), as described previously (29). In brief, 40-μm brain sections were examined within the rostal and caudal limits of the SNc (-2.54 to -3.88 mm of bregma). For each brain, seven coronal sections were examined. After immunoblotting, mounting, defatting, and coverslipping, the thickness of the sections was measured with a *z*-axis microcreator according to the manufacturer's instructions. Sections were analyzed using a $\times 100$ lens. Total number of TH-positive neurons was determined using the optical fractionator. Cresyl violet staining for *Pink1* KO mice was performed at one of the levels of the SNc area (-2.54 to -3.88 mm of bregma) where TH population is highest. The results are presented in number and percentage.

HPLC Analysis of MPP^+—Analysis of MPP^+ was carried as described previously (30). Briefly, *Pink1* KO and WT mice were injected with a single dose of MPTP (25 mg/kg, intraperitoneal, measured as free base; MPTP-HCl; Sigma-Aldrich). 90 min after MPTP injection, the mice were killed, and striata were collected and processed for HPLC analysis (31).

Statistical Analyses—Data analysis was carried out using independent two-tailed *t* test. Significance was marked by * when $p < 0.05$, ** when $p < 0.01$.

RESULTS

Dopamine Neurons of Pink1 Knock-out Mice Are Sensitive to DA Toxin MPTP in Vivo—We demonstrated previously that ectopic expression of WT *Pink1* in the SNc area protected the TH neurons against the dopaminergic toxin MPTP (23). The metabolite of MPTP, MPP^+, is transported to dopaminergic neurons by the dopamine transporter (DAT) where it blocks mitochondrial complex I function and results in degeneration (32). However, to demonstrate the role of *Pink1* in degeneration more definitely in the adult *in vivo* context, loss-of-function studies are required. Our first approach in this regard was to utilize *Pink1*-deficient mice. Recently, several groups generated germ line-deleted *Pink1* mice (19, 33). These *Pink1* KO mice do not show basal loss of DA neurons. However, subtle defects such as decrease in DA release have been observed (19). Accordingly, we initially challenged either WT or *Pink1*-deficient animals with a subchronic paradigm of MPTP as described under "Experimental Procedures" and examined dopaminergic neuron survival. We evaluated the entire SNc region (-2.54 to -3.88 mm of bregma) by optical fractionation/stereology for healthy TH-immunopositive neurons. Importantly, DA neurons of *Pink1*-deficient mice were more sensitive to MPTP than their WT littermates as shown in Fig. 1, *A* and *B*. We also performed cresyl violet staining at one of the level of SNc area as mentioned under "Experimental Procedures" to corroborate the loss of dopamine neurons further. We observed a trend similar to that obtained for TH immunohistochemical analysis (Fig. 1*B*). The TH-positive neurons of SNc project their processes to the striatum. These terminal fibers are enriched with the DAT. Therefore, we examined whether the loss of DA neurons in the SNc area correlated with terminal loss as evaluated by DAT staining. As expected, we found a significant loss of DAT staining in *Pink1*-deficient animals compared with littermate controls (54.76% striatal density in MPTP-treated WT animal *versus* 31.80% in *Pink1* KO animals when normalized with saline-treated WT animals) (Fig. 1, *D* and *E*). To ascertain that the greater loss of DA neurons or decrease in DAT density is not due to the increased production of MPP^+ from MPTP, we measured the MPP^+ in the striatum. Indeed, we found that equal amounts of MPP^+ available in both WT/KO mouse striatum (Fig. 1*F*).

shRNA-mediated Knockdown of Mouse Pink1 in SNc Area Sensitizes DA Neurons to MPTP—The use of germ line deficiencies brings up potential concerns of unforeseen confounds brought on by developmental compensation that may have little to do with the original function of the ablated gene. Because of these concerns, a more transient approach to *Pink1* knockdown was explored in conjunction with the deficient mice. We first generated shRNA viral vectors to *Pink1* which could be used in our *in vivo* adult MPTP model. We employed the shRNA sequence which we had previously shown to be effective in down-regulating mouse *Pink1* expression *in vitro* (23). We subsequently generated sh-*Pink1* adenovirus or a control shRNA virus (scrambled sequence with no known homology with mouse gene). The effectiveness of our sh-*Pink1* vector to silence *Pink1* in the SNc was evaluated utilizing ISH. This method was chosen due to the absence of reliable *Pink1* anti-

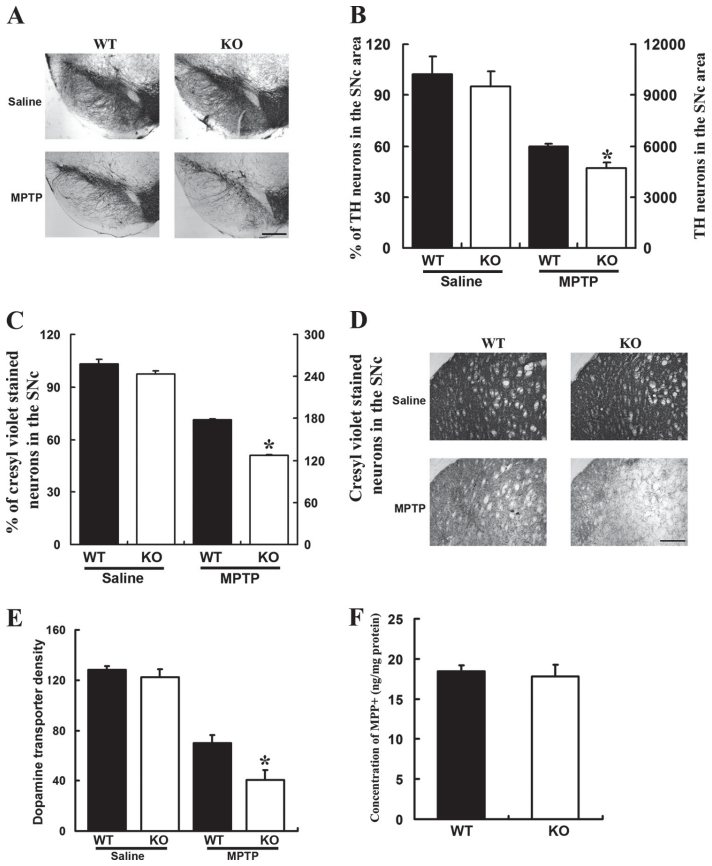

FIGURE 1. **Dopamine neurons of germ line-deficient *Pink1* mice are more sensitive to neurotoxin MPTP.** *A*, representative photomicrographs illustrating TH immunoreactivity in the ventral midbrain SNc. *Scale bar*, 250 μm. *B*, quantification of TH-immunoreactive neurons in SNc area of *Pink1* WT/KO animals by stereology, as described under "Experimental Procedures." *C*, cresyl violet-stained neurons in SNc area. *D*, representative photomicrographs illustrating DAT immunoreactivity in the striatum of *Pink1* WT/KO animals. *Scale bar*, 500 μm. *E*, quantification of optical density of striatal DAT-stained fiber density of *Pink1* WT/KO animals. *F*, HPLC analysis of striatal MPP^+ levels in *Pink1* WT/KO animals. Values are means $\pm$ S.E. (*error bars*; $n = 3$–5). *, $p < 0.05$ compared with WT animals treated with MPTP.

bodies needed for immunohistochemical analysis of endogenous *Pink1 in vivo*. The adenovirus was injected into the striatum area which retrogradely transports to the SNc. A week after the injection, the brain tissues were prepared for ISH to detect the *Pink1* signal. As shown in Fig. 2, *A–C*, the *Pink1* message signal was significantly lower (71%) in the striatum of the ipsilateral side, where viral injection was carried out, relative to the contralateral side. Most importantly, this reduction was clear in the area of the SNc in cells with the shape and size of DA neurons (68% reduction). One week after viral transduction with either sh-*Pink1* vector or control, animals were challenged with the same subchronic regimen of MPTP utilized with the *Pink1*-deficient animals as described earlier. Two weeks after the initial MPTP or saline dose, animals were killed, and the midbrain was sectioned. The number of TH-immunopositive neurons in both virus-injected (ipsilateral) and virus-uninjected (contralateral) in the SNc at the level of the MTN was assessed. Consistent with the germ line deficiency data, we found that silencing of *Pink1* in the SNc area led to increased loss of DA neurons in response to MPTP (Fig. 2, *D* and E). Silencing of *Pink1* by itself did not significantly affect TH neuron numbers, at least in the 3-week time frame examined. This result is in line with recent reports indicating that *Pink1* knockdown or knockout does not cause any DA neuron loss (19, 20, 33). To further ensure that the loss of TH neurons was not due to simple loss of TH marker expression and not degeneration, we also carried out cresyl violet staining and analyses of the SNc in adjacent sections at the level of the MTN. As shown in Fig. 2*F*, results similar to TH counts were obtained. Taken together, these results clearly indicate the critical role of endogenous *Pink1* in

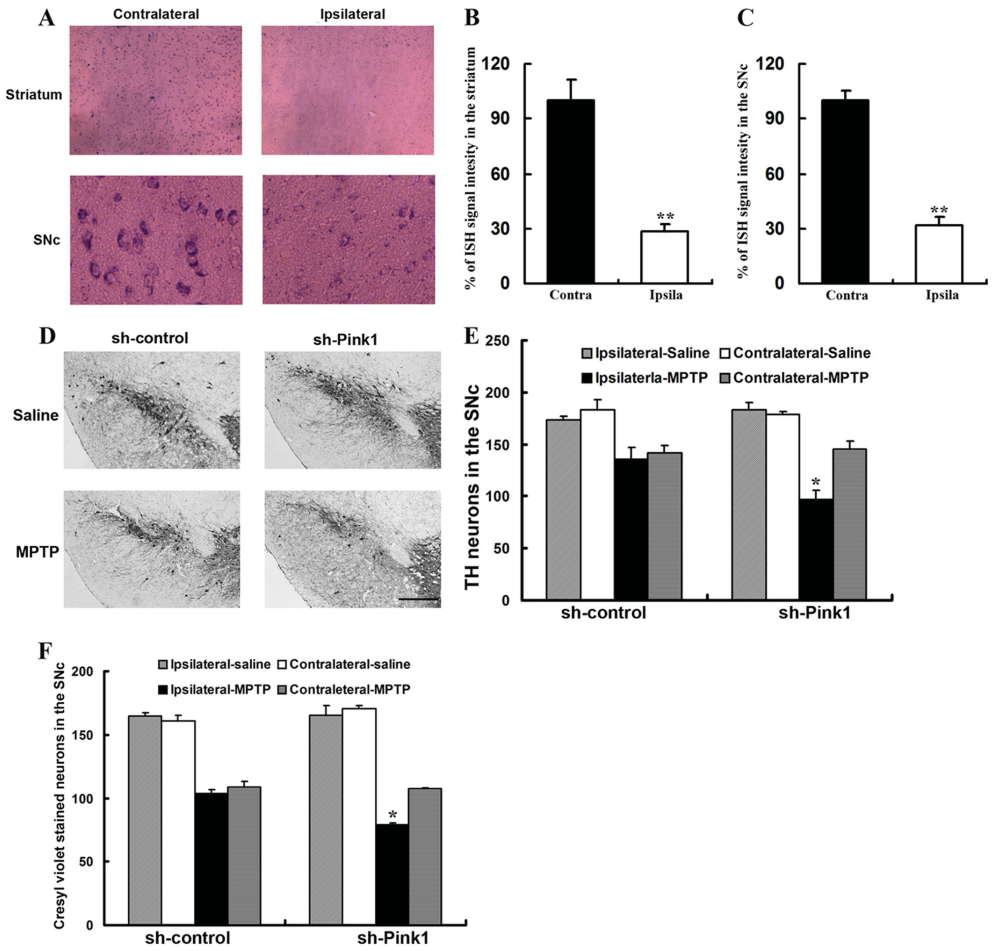

FIGURE 2. **shRNA-mediated knockdown of *Pink1* results in greater loss of dopamine neurons upon MPTP treatment.** *A*, adenoviruses (2 μl, 1×10^7 particles/μl) expressing *Pink1* shRNA or control injected directly into the striatum (ipsilateral side) of the animals. A week after viral injection, the brains were collected, fixed, and sectioned. The brain sections were processed for ISH to examine *Pink1* transcript in striatum and SNc area. *B* and *C*, percentage of ISH signal intensity in the striatum (*B*) and SNc (*C*). **, $p < 0.01$ *versus* contralateral site. *D*, adenovirus-injected mice challenged with MPTP as described under "Experimental Procedures." Brains were collected and sectioned into 14-μm slices for TH immunostaining. Representative images of TH-immunoreactive neurons of the ipsilateral side of the animals, 2 weeks after treatment with MPTP or saline, are shown. *Scale bar*, 250 μm. *E* and *F*, quantifications of TH-immunoreactive neurons from the ipsilateral or the contralateral region of SNc area (*E*) or cresyl violet-stained neurons (*F*). All values are means ± S.E. (*error bars*; $n = 3$–5). *, $p < 0.05$ compared with control virus-injected (ipsilateral) side of MPTP-treated animals.

neurons in mediating neuronal survival to exogenous stress in the adult animal *in vivo*.

Role of Parkin and DJ-1 in Modulating Sensitization Observed with Pink1 Loss—A functional relationship between *Pink1* and the other recessive PD genes, *Parkin* and *DJ-1*, has been suggested. For example, studies in *Drosophila* indicated that *Parkin* acts downstream of *Pink1* to rescue the abnormal wing and mitochondrial defects in indirect flight muscles, a phenotype associated with *Pink1* loss of function, at least in flies. Similarly, *Parkin* accumulates in damaged mitochondria and enhances mitophagy in a *Pink1*-dependent manner (4, 6, 17). However, in this case, the role of this translocation in mediating survival is unknown. Importantly, the capacity of *Parkin* to compensate for *Pink1* loss-mediated sensitization to death or to protect in the absence of *Pink1* is unknown. To test this, we examined whether *Parkin* expression in dopaminergic neurons can also rescue sensitization effects of MPTP due to absence of *Pink1*, *in vivo*. We delivered *Parkin*

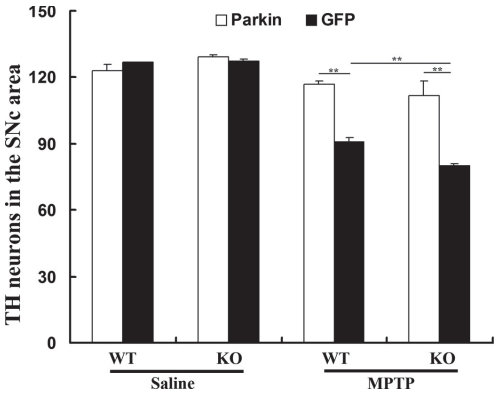

FIGURE 3. **Expression of AAV *Parkin* at the SNc area rescues DA neurons of *Pink1* KO mice from MPTP sensitization.** The AAVs expressing human *Parkin* or control were injected directly into the SNc area of animals 2 weeks before the initiation of MPTP treatment. Brains were collected and sectioned into 14-μm slices for TH immunostaining. Quantifications of TH-immunoreactive neurons from the ipsilateral region of SNc area of mice brain of different groups are shown. All values are means ± S.E. (*error bars*; $n = 3–4$), except GFP-injected saline-treated WT control ($n = 2$).

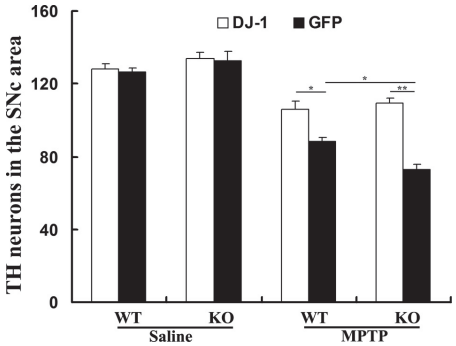

FIGURE 4. **Delivery of adenovirus-expressing *DJ-1* at striatum area rescues DA neurons of *Pink1* KO mice from MPTP sensitization.** The adenoviruses (2 μl, 1×10^7 particles/μl) expressing human *DJ-1* or control were injected directly into the striatum of animals 7 days before the initiation of MPTP treatment. Brains were sectioned into 14-μm slices for TH immunostaining. Quantifications of TH-immunoreactive neurons from the ipsilateral region of SNc area of mice brain of different groups are shown. Values are means ± S.E. (*error bars*; $n = 3–4$).

or GFP control AAV to the SNc 2 weeks prior to a challenge with MPTP. We found that expression of *Parkin* is effective in rescuing the sensitization induced by *Pink1* loss. It is important to note that in this case, the sensitization induced by *Pink1* deficiency was slightly lower (although still significant) than that observed in Fig. 1*B*. This may be due to the effects of viral transduction. Interestingly, *Parkin* provided the same extent of neuroprotection in the absence or presence of endogenous *Pink1* (Fig. 3). This result suggests that *Parkin* not only compensates for *Pink1* deficiency, but also provides further protection even in the absence of *Pink1*. This in agreement with the recent reports showing that ectopic expression of AAV *Parkin* was protective against MPTP under WT backgrounds (34, 35).

DJ-1 is an another PD gene involved in scavenging oxidative stress in multiple model systems (8, 9, 36). We have previously shown that loss of *DJ-1* sensitizes animals to multiple environmental stressors such as ischemia and MPTP *in vivo* (8, 9). Given its roles in processes such as mitochondrial function (37) and survival (9) which are also implicated for *Parkin* and *Pink1*, we next tested whether *DJ-1* might also compensate for *Pink1* loss. In this case, *DJ-1* or GFP controlled adenovirus was delivered stereotaxically to the striatum in either WT or KO *Pink1* animals. We have previously shown *DJ-1* to be effective in promoting survival *in vivo* with this virus (8). As shown previously, GFP-injected *Pink1* KO animals showed hypersensitization to death compared with WT controls in response to MPTP. Importantly, exogenous *DJ-1* expression was effective at overcoming this sensitization. Similar to *Parkin* expression, *DJ-1*-expressing animals showed the same level of survival at the SNc whether or not *Pink1* was present (Fig. 4). These results indicate that the other two recessive PD genes *Parkin* and *DJ-1* can protect even in the absence of *Pink1*. This suggests that they are either downstream of *Pink1* or on parallel pathways to regulate survival.

DISCUSSION

Recent discoveries of several genes associated with familial PD have given us the opportunity to enhance our molecular understanding of this devastating disease. Among these genes, *Pink1* has received much attention due to its clear mitochondrial targeting motif, its well defined kinase domain, and involvement in mitochondrial quality control pathways. Yet, the mechanism by which its loss leads to PD in humans is still unclear.

Role of Pink1 in Survival—One critical aspect of *Pink1* function has to do with its potential role in survival. As stated previously, it has been reported that WT *Pink1* expression blocks death against a number of death stimuli including MPP^+, rotenone, staurosporine and MG-132 (10, 21–23). We likewise observed this phenomenon in primary neurons expressing WT human *Pink1* (23). However, the endogenous role of *Pink1*, particularly in the adult mammalian brain *in vivo*, is less clear. This question is made more significant given recent questions on the relevance of seemingly critical biological processes such as *Parkin*-mediated mitochondrial quality control in the adult animal (38). This reflects a broader question of relevance of *in vitro* findings in the adult *in vivo* context.

Pink1 deficiency in mice does not lead to increased basal DA neuronal loss as observed in human PD. Our present data demonstrating that shRNA knockdown of *Pink1*, at least in the time frame examined, did not lead to increased basal DA loss is consistent with these results. In the fly, *Pink1* deficiency does lead to demonstrable DA loss, but the reason for this difference is unclear. One explanation may be that in the mammalian system, there are other survival pathways that are sufficient to prevent basal DA loss at least under basal conditions in the lifetime of the mouse. These compensatory pathways likely do

not involve *DJ-1* and *Parkin* because *Pink1*/*DJ-1*/*Parkin* triple KO mice also do not show basal DA loss (39).

Rather than focus on basal processes, the effects of exogenous environmental stress might better illuminate the functional role of *Pink1* in survival. Because of the role of *Pink1* in mitochondrial function/quality control and its localization, the effect of *Pink1* loss on mitochondrial stress is particularly interesting. This is even more critical given the increased association of mitochondrial defects in PD and the links of variety of PD genes to mitochondrial processes. Accordingly, we examined how *Pink1* loss may affect DA survival in response to the mitochondrial complex I toxin MPTP, to address the central question of whether endogenous *Pink1* plays a role in DA survival in the adult mammalian brain. Our results support the veracity of these hypotheses as follows: (i) germ line-deficient *Pink1* animals are more susceptible to MPTP and (ii) this is likely not due to developmental compensatory changes because transient knockdown of *Pink1* also shows similar results.

The mechanism(s) by which *Pink1* regulates survival has not been identified as yet. The localization of *Pink1* alone is complex. It has been reported to localize to the inner mitochondrial membrane basally and at the outer mitochondrial membrane following mitochondrial membrane depolarization (4, 6). It is suggested that *Pink1* can exist also in the cytoplasm (23, 40, 41). This last point is particularly intriguing given the fact that although the kinase activity of *Pink1* is critical for its protective effects when expressed, its mitochondrial localization sequence is not required either *in vitro* or *in vivo* in response to mitochondrial stress (23). This finding suggests that although *Pink1* may play a role in mitochondrial quality control, this may not be the primary pathway by which survival is mediated. Alternatively, *Pink1* may act on substrates in the cytoplasm to mediate survival as it is known that when *Pink1* is localized to the outer mitochondrial membrane, its kinase domain faces the cytoplasm (42). Interestingly, it was shown that stable knockdown of *Pink1* in cell culture system promotes autophagy. This autophagic event due to the absence of *Pink1* can be reversed by overexpression of WT *Pink1* or Δ*Pink1* (43). However, the link between autophagy and survival is unclear. In this regard, autophagy has been shown either to promote or inhibit death processes. Finally, *Pink1* has also been shown to phosphorylate *Trap1*, and this phosphorylation event is necessary for survival against oxidative stress (11). Whether this is critical in the adult DA system is unknown.

Interaction of Pink1 with Other Autosomal Recessive PD Genes, DJ-1 and Parkin—There are several common features of the three recessive PD genes *DJ-1*, *Parkin*, and *Pink1*. Loss of all of three genes has in some way been associated with mitochondrial deficits in the mouse tissue (3, 37, 44). Deficiencies also appear to mediate defects in DA uptake and turnover (19, 45, 46). These three genes are also associated with management of free radical damage (7, 9, 23, 34, 35). For example, several reports indicated that *DJ-1* expression protects and its loss sensitizes to oxidative inducers (8). Similar observations have been shown for *Parkin* and *Pink1* (23, 34, 35). In fact, *DJ-1* itself has atypical peroxyredoxin activity, was modified by reactive oxygen species (7), and was shown to regulate the stability of one of the master antioxidant regulator *Nrf2* (47). *Parkin* is also reported to induce genes such as *TFAM*, which is critical for mitochondrial biogenesis (48). How these parameters relate to the direct mechanism of cell-mediated survival is not known. Likewise, whether or not these three recessive genes act together to mediate survival *in vivo* remains to be established. As mentioned previously, ablation of all three recessive genes does not lead to DA neuron loss *in vivo* (39). This argues against the notion that these genes act in parallel to mediate basal DA survival *in vivo*. However, our present work supports the notion that modulation of either *DJ-1* or *Parkin* levels can compensate for the loss of *Pink1*. This is consistent with reports indicating that *Parkin* and *DJ-1* can also compensate for *Pink1*-mediated defects in the fly (49). Moreover, it supports a recent report that *DJ-1* acts in parallel with the *Pink1*/*Parkin* pathways (50).

Our observation that *Parkin* can compensate for *Pink1* deficiency is also interesting in light of the recent observations that *Pink1* is required for *Parkin* translocation to the mitochondria upon mitochondrial depolarization (17). Therefore, one interpretation of this finding is that *Parkin* acts downstream of *Pink1*, and thus the ability of *Parkin* expression to rescue *Pink1* deficiency make sense. However, there are some caveats regarding this interpretation. First, it is thought that *Pink1* is an absolute requirement for *Parkin* translocation. In this regard, our results demonstrating *Parkin*-mediated protection in the absence of *Pink1* are pertinent. The manner by which *Parkin* is mediating survival must be independent of its presence in the mitochondria. In addition, a mouse model defective in respiratory chain components which results in fragmented mitochondria and dissipation of membrane potential did not demonstrate *Parkin* accumulation at the mitochondria (38). They also found that mitophagy in these mice was not impacted in the presence or absence of *Parkin*, arguing against the role of *Parkin* in clearing defective mitochondria. Taken together, these observations call into some question whether or not mitochondrial quality control pathways mediated by *Pink1*/*Parkin* may be critical for DA survival. Much more work is clearly needed to address these essential questions. Nevertheless, our work clearly demonstrates that endogenous *Pink1* is an essential component that prevents neuronal loss in the adult SNc in response to environmental stress. We also provide evidence that modulation of either *Parkin* or *DJ-1* can protect even in the absence of *Pink1*, suggesting that *Pink1* is not a required factor for *DJ-1*/*Parkin*-mediated protection.

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Regulation of ischemic neuronal death by E2F4-p130 protein complexes

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Personal Contributions

Abdel-Messih, E contributed to the project and assisted project lead Iyirhiaro, GO in her absence, by providing technical support alongside Estey C while performing 4VO stroke surgeries, and assisted in performing perfusions, extractions, fixation and cryoprotection of brain samples for analysis for various sets of in vivo studies.

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Regulation of Ischemic Neuronal Death by E2F4-p130 Protein Complexes*

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Background: The contribution of E2F4 to hypoxic/ischemic neuronal death is understood poorly.
Results: Loss of E2F4 leads to an increase in B-Myb and contributes to hypoxic/ischemic neuronal death.
Conclusion: E2F4 is important for survival following hypoxic/ischemic neuronal death.
Significance: Targeting E2F4-repressive functions may be important in maintaining neuronal survival under hypoxic/ischemic conditions.

Inappropriate activation of cell cycle proteins, in particular cyclin D/Cdk4, is implicated in neuronal death induced by various pathologic stresses, including DNA damage and ischemia. Key targets of Cdk4 in proliferating cells include members of the E2F transcription factors, which mediate the expression of cell cycle proteins as well as death-inducing genes. However, the presence of multiple E2F family members complicates our understanding of their role in death. We focused on whether E2F4, an E2F member believed to exhibit crucial control over the maintenance of a differentiated state of neurons, may be critical in ischemic neuronal death. We observed that, in contrast to E2F1 and E2F3, which sensitize to death, E2F4 plays a crucial protective role in neuronal death evoked by DNA damage, hypoxia, and global ischemic insult both *in vitro* and *in vivo*. E2F4 occupies promoter regions of proapoptotic factors, such as B-Myb, under basal conditions. Following stress exposure, E2F4-p130 complexes are lost rapidly along with the presence of E2F4 at E2F-containing B-Myb promoter sites. In contrast, the presence of E2F1 at B-Myb sites increases with stress. Furthermore, B-Myb and C-Myb expression increases with ischemic insult. Taken together, we propose a model by which E2F4 plays a protective role in neurons from ischemic insult by forming repressive complexes that prevent prodeath factors such as Myb from being expressed.

Inappropriate activation of the cell cycle machinery is implicated in a number of neuronal death models induced by NGF deprivation (1), excitotoxicity (2), oxidative stress (3), DNA damage (4), and stroke (5–8). For example, an increase in cyclin D1 levels and its associated kinase activity is observed following DNA damage (an upstream mediator of stroke damage) and ischemic insult (5, 7, 9–11). In addition, treatment with pharmacologic cyclin-dependent kinase (Cdk)[3] inhibitors such as flavopiridol protects neurons from both DNA damage- and ischemia-induced cell death (4, 6, 7). Expression of a dominant negative form of Cdk4, an important regulator of the G_1/S phase of the cell cycle, is protective following DNA damage and global cerebral ischemia (5, 9). Together, these studies reveal a crucial role for Cdk4 in neuronal death induced by DNA damage and ischemic insult. However, the downstream effectors of cell cycle reactivation in neurons under ischemic stress remain unclear. In this regard, members of the E2F transcription factors may play a pivotal role.

The E2Fs consist of eight related family members, generally classified as activators (E2F1, E2F2, and E2F3a) or repressors (E2F3b and E2F4-E2F8) on the basis of their ability to promote or repress gene transcription (12). The activity of E2Fs (E2F1-E2F5) is regulated by their association with the pocket proteins, which include retinoblastoma protein (pRb), p107, and p130 (12, 13). E2F association with pocket proteins can promote or repress the expression of its targets, which include genes involved in cell cycle progression, DNA damage, and apoptosis (12, 13). For example, in dividing cells, pRb association with E2F prevents the expression of genes required for DNA synthesis and cell cycle progression. However, phosphorylation of pRb by Cdk4/6 disrupts its association with E2F and results in the transactivation of its target genes. In addition to transactivation, E2F complexes can also repress gene function. For example, E2F4-p130 can recruit chromatin modification factors, such as histone deacetylases (HDACs), to promoters of target genes to form an active repression complex (13–16). In this scheme, phosphorylation of p130 by Cdks disrupts its associa-

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[3] The abbreviations used are: Cdk, cyclin-dependent kinase; AAV, adeno-associated virus; CGN, cerebellar granule neuron; 4VO, four-vessel occlusion; CA1, cornu ammonis 1.

tion with E2F4, resulting in the derepression of target genes. Thus, E2Fs can function as effectors of Cdk signaling via the transactivation or derepression of target genes.

The involvement of E2F and, in particular, E2F1 in the cell cycle-induced death of neurons under pathologic stress is suggested by a number of key observations. First, phosphorylation of pRb, a preferred partner for activating E2Fs such as E2F1, is observed in neuronal death models, including DNA damage and ischemia (6, 7, 17). Second, the expression of kinase-dead Cdk4 and treatment with flavopiridol attenuates pRb phosphor ylation following DNA damage and ischemia (5, 7, 17). Importantly, the expression of mutant pRB or dominant negative DP-1, a binding partner of E2Fs, prevents neuronal death following DNA damage and/or hypoxia (5, 17). Finally, E2F1 deficiency is protective following ischemic insult both *in vitro* and *in vivo* (8, 18, 19). These observations suggest that cell cycle reactivation in neurons under pathologic stress signals death through pRb inactivation and activation or derepression of E2F1 target genes.

In addition to E2F1 and pRb, other E2Fs may also be important in neuronal death. E2F4-p130 complexes are present basally in postmitotic neurons, suggesting that they may act to repress gene targets important in neuronal survival/function (16, 20). Interestingly, in the absence of insult, siRNA-mediated down-regulation of p130 or E2F4 induced apoptosis of cortical neurons (21). In addition, the expression of wild-type or phosphorylation-resistant p130 is protective following NGF deprivation of PC12 neurons (21). Whether p130-E2F4 complexes play a role in neuronal death *in vivo* is unclear. In this study we examined the potential involvement of E2F4 in neuronal death induced by ischemic stress and DNA damage. The results of our study demonstrate an important role for E2F4 in the survival of neurons, which is in contrast to the role of activating E2Fs such as E2F1/3.

EXPERIMENTAL PROCEDURES

Viral Construction—Plasmids containing a human E2F4 cDNA sequence were subcloned into the SpeI sites of the AM/CBA-pI-WPRE-bGH vector and used to generate a recombinant adeno-associated (AAV) virus, as described previously (22). Plasmids containing Bmyb-luciferase with a wild-type E2F site and a mutant construct harboring a mutation that abolishes E2F binding have been reported previously (23). The plasmids were subcloned into the adeno-associated vector for efficient delivery into primary neurons. The adenovirus expressing E2F1 has already been described (24).

Transgenic Mice—E2F1- (25, 26), E2F3- (25, 27), and E2F4-deficient (28) mice have been described previously. Knockout mice were generated from heterozygous breeding pairs and genotyped by PCR using primers that have been published previously (26, 28). E2F1 null mice were genotyped using the following primers: 5′-GGATATGATTCTTGGACTTCTTGG-3′, 5′-CTAAATCTGACCCCAAACGC-3′, and 5′-CAAGT-GCCAGCGGGGCTGCTAAAG-3′.

Cell Cultures and Treatments—Primary cerebellar granule neurons (CGNs) and cortical neuronal cultures were established as described previously (24, 29) from CD1 (Charles River Laboratories, Quebec, Canada) or E2F transgenic mice. CGNs were transfected with E2F4 siRNA, a C-Myb siRNA mixture, or control siRNA (Santa Cruz Biotechnology) using Lipofectamine 2000 (30) 5 days after plating. Alternatively, CGNs were infected with an adenovirus expressing E2F1, E2F4, or GFP at the time of plating at a multiplicity of infection of 50. Cortical neuronal cultures were cotransfected with E2F1, E2F3, or E2F4 along with GFP-containing plasmids using the calcium phosphate method 3 days after plating, as described previously (30, 31). Cortical neurons were treated with 10 μM camptothecin (Sigma) 3–4 days after plating and examined for survival at the indicated times, as described previously (30). Cortical cultures from transgenic mice and CGNs were lysed at the designated times after insult using a mildly disruptive lysis buffer and evaluated as described previously (5). Neurons cotransfected with E2F and GFP plasmids were fixed and stained with Hoechst 33342 (Sigma). Viability was assessed by nuclear integrity in GFP-positive cells in random fields, as described previously (29).

Hypoxia—CGN cultures were subjected to hypoxia at 1% O_2 with 5% CO_2-balanced N_2 in a humidified hypoxia chamber (Coy Laboratory Products, Ann Arbor, MI) after 1 week in culture, as described previously (5). Hypoxia was induced in the presence of 10 μM MK801 (Research Biochemicals, Natick, MA), an NMDA channel blocker, for 18 h, followed by reoxygenation at normoxia for 24 h for survival studies. Alternatively, CGN cultures were subjected to varying durations of hypoxia and reoxygenation for biochemical studies. Control cultures were maintained in a humidified incubator at 37 °C and were not treated with hypoxia.

Viral Injection—Animal experiments were carried out in accordance with Canadian Council for the Use and Care of Animals in Research guidelines with approval from the University of Ottawa Animal Care Committee. Intrahippocampal, recombinant, adeno-associated viral injections in rats have been described previously (5). Briefly, male Wistar rats (80–125 g) were injected unilaterally with the recombinant adeno-associated virus carrying an enhanced green fluorescent protein (EGFP) control or E2F4 (10^{10} genomes/μl) 2 weeks before a four-vessel occlusion insult. 2 μl of recombinant adeno-associated virus diluted in PBS with 1 μl of mannitol (20%) was injected stereotaxically using a Harvard infusion pump (Harvard Apparatus) into the hippocampus (from the bregma, −3.6 mm anteroposterior, +2.1 mm lateral, and −2.75 mm deep).

Global Cerebral Ischemia—The four-vessel occlusion (4VO) method of global ischemia was induced as described previously for 10 min (6, 32). Four days following ischemia, rats were perfused and sacrificed, and the brains were extracted, sectioned, and stained for histological assessment. The neuronal viability of cells in the hippocampal CA1 was assessed as described previously (6, 32).

Immunohistochemistry—Antigen retrieval and deparaffinization was carried out on brain sections as described previously (32). Following permeabilization with 0.3% Triton X-100 for 10 min, rat brain sections were blocked in 10% normal goat serum (Jackson ImmunoResearch Laboratories) diluted in 2% bovine serum albumin (Fisher Scientific) in 0.01 M PBS for 1 h at room temperature. Sections were incubated with mouse mono-

clonal anti-GFP (Abcam) or anti-E2F4 antibodies (Abcam, 1:200) overnight at 4 °C. For visualization, sections were incubated with Alexa Fluor 488 goat anti-mouse (1:200) secondary antibody (Jackson ImmunoResearch Laboratories) for 1 h at room temperature. Neuronal nuclei were stained with Hoechst 33342 (Sigma).

Immunoprecipitation—CGN cultures were harvested and homogenized in immunoprecipitation buffer (50 mM HEPES (pH 7.5), 150 mM NaCl, 1 mM EDTA, 2.5 mM EGTA, 1 mM DTT, and 0.1% Tween 20) supplemented with protease inhibitor mixture (Roche). 500 μg of total protein lysate was incubated with 4 μg of anti-E2F4 (Santa Cruz Biotechnology) or anti-p130 antibody (Santa Cruz Biotechnology) overnight at 4 °C. As a control, samples were incubated with normal rabbit IgG (Santa Cruz Biotechnology). The antigen-antibody complex was captured with anti-rabbit Ig immunoprecipitation beads (eBiosciences) at 4 °C for 4 h. The beads were recovered, and the immunoprecipitation product was resolved on a 10% SDS-polyacrylamide gel.

Western Blotting—Cell and hippocampal tissue samples were collected at the designated times after insult and homogenized in solubilization buffer (0.0625 M Tris, 2.5 mM EDTA, 2.5 mM EGTA, 10% glycerol, 2% SDS, 0.001% bromphenol blue, and 5% β-mercaptoethanol) (24). Alternatively, E2F4 null CGNs or siRNA-transfected cultures were harvested and homogenized in solubilization buffer. Samples were run on SDS-polyacrylamide gels and transferred onto a PVDF membrane (Millipore). Membranes were probed with the following antibodies: anti-E2F4 (Abcam), anti-p130 (BD Transduction Laboratories), anti-phospho-p130 (Santa Cruz Biotechnology), anti-E2F1 (Santa Cruz Biotechnology), anti-E2F3 (Santa Cruz Biotechnology), and anti β-actin (Sigma). Densitometry was performed on Western blots using ImageJ software and normalized to the loading control. The results were expressed as the fold change over sham-operated animals in the 4VO time course and over no hypoxia control for hypoxia/reoxygenation experiments.

Semiquantitative Reverse Transcription PCR—At the indicated times following 4VO, total RNA was extracted from hippocampal tissues using QIAcube (Qiagen) following the protocol of the manufacturer. Alternatively, total RNA was extracted from siRNA-treated CGN cultures. 100 ng of total RNA was used for cDNA synthesis and targeted gene amplification using a SuperScript One-Step RT-PCR kit (Invitrogen). cDNA synthesis and amplification were conducted using the following conditions: 42 °C for 45 min, 94 °C for 2 min, followed by cycles of 94 °C for 1 min, T_m for 30 s, and 72 °C for 1 min. The rat MYB genes were amplified with the following primers: 5′-GGCTG CCGTGGCTACTACTTCTAA-3′ and 5′-CGCGC CGTTTC-TTCTGTCG-3′ for B-Myb at a T_m of 59 °C for 35 cycles and 5′-ATGCCCTGGAAGTGAAC AAC-3′ and 5′-CAGCTTTT-GTAAGCGGGTTC-3′ for C-Myb at a T_m of 54 °C for 35 cycles. Expression of GAPDH mRNA was used as a standard for loading control. GAPDH was amplified using the following primers: 5′-ATCCGTTGTGGATCTGACATGC-3′ and 5′-TGTCATTGAGAGCAATGCCAGC-3′ at a T_m of 52 °C for 28 cycles. The mouse C-Myb genes were amplified using the following PCR primers: 5′-GAGAGGTGGCACAACCATTT-3′ and 5′-GGGAACGTGACTGGAGATGT-3′ at a T_m of 54 °C for 31 cycles. The expression of ribosomal S 12 mRNA was used as a standard for loading control. S 12 cDNA was amplified using the following primers: 5′-GGAAGGCA TAGCTGC-TGG-3′ and 5′-CCTCGATGAC ATCCTTGG-3′ at a T_m of 57 °C for 25 cycles. Densitometry was performed on results and normalized to loading control. The results were expressed as fold change over control.

ChIP Assay—CGNs treated with/without hypoxia and reoxygenation were subjected to a ChIP assay as described previously (33). The mouse B-Myb promoter region was amplified using the following primers: 5′-CCTCCTCCTTC-TCCTCCTTC-3′ and 5-CACTATACCCGTGCGCTTCT-3′. PCR products were resolved on an agarose gel. Densitometry was performed on results and expressed as fold change over no hypoxia control.

Luciferase Assay—One day after plating, CGNs were infected with wild-type or mutant luciferase viruses along with AAV-β-galactosidase as an internal control. After hypoxia/reoxygenation, cells were lysed in buffer provided in the Promega luciferase system (Promega). The luciferase assay was performed according to the instructions of the manufacturer. Relative luciferase activities were obtained by normalizing the luciferase activity against β-galactosidase activity. Results were presented as fold increase in reference to control values.

RESULTS

E2F Family Members Differentially Regulate Neuronal Death Mediated by Genotoxic Stress—Aberrant activation of neuronal cell cycle induced by pathologic stress such as DNA damage and ischemic insult is thought to contribute to cell death through regulation of E2F members (7, 17, 33). However, the relative contribution of different E2F members in neuronal death is not clear. To begin to examine the involvement of different E2F members, we focused initially on the effect of select activating E2Fs (E2F1 and E2F3) and the repressive E2F4 members in neuronal death. Primary cortical neurons null for these E2F members were treated with the DNA-damaging agent camptothecin and evaluated for survival. Neurons null for E2F1 (Fig. 1*A*) and E2F3 (Fig. 1*B*) were more resistant to DNA damage-induced death when compared with those from their littermate controls. E2F1$^{-/-}$ neurons were significantly more resistant to DNA-induced death at 8 h (59% survival, $p < 0.01$) and 12 h (23% survival, $p < 0.05$) compared with the wild-type control (43 and 13% survival at 8 and 12 h, respectively). Similarly, neuronal survival in the E2F3$^{-/-}$ neurons (59 and 27%) was greater than that observed for the wild type (43 and 20%, $p < 0.01$ and $p < 0.05$ at 8 and 12 h, respectively). In contrast, E2F4 deficiency resulted in sensitization to DNA damage-induced death at 12 h (21% survival) compared with the wild-type control (44%, $p < 0.01$, Fig. 1*C*).

We next tested the effect of expression of E2F1, E2F3, and E2F4 on neuronal death induced by DNA damage. As shown in Fig. 1, *D–F*, expression of E2F1 (Fig. 1*D*) and E2F3 (Fig. 1*E*) significantly ($p < 0.01$) reduced neuronal survival compared with neurons transfected with vector only. In contrast, overexpression of E2F4 (Fig. 1*F*) resulted in an increase in neuronal survival (46% in vector- and 65% in E2F4-expressing neurons, $p < 0.01$). Together, these results demonstrate a proapoptotic

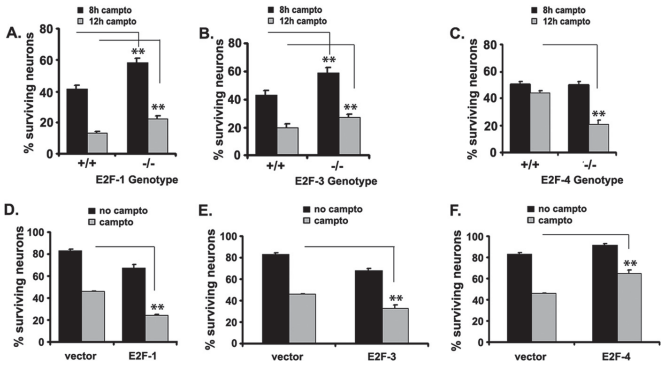

FIGURE 1. **E2F family members play differential roles in neuronal death induced by genotoxic stress.** E2F1 (*A*), E2F3 (*B*), and E2F4 (*C*) wild-type and knockout neurons were treated with 10 μM camptothecin (*campto*, a DNA-damaging agent) and evaluated for survival at 8 and 12 h following treatment. E2F1 (*D*), E2F3 (*E*), and E2F4 (*F*) containing a plasmid or vector control were transfected in cortical neuronal cultures 3 days after plating and treated with 10 μM camptothecin 24 h later. Survival was assessed 24 h following treatment. Results are expressed as percent of control ± S.E. **, $p < 0.01$.

role for E2F1 and E2F3 and a prosurvival role for E2F4 in neuronal death induced by DNA damage.

E2F1 and E2F4 Have Opposing Effects on Neuronal Survival following Hypoxia/Reoxygenation—We next determined the effect of E2Fs in a more physiologically relevant model of ischemic neuronal death *in vitro* with a focus on E2F1 and E2F4. To this end, primary CGNs infected with an adenovirus expressing E2F1, E2F4, or GFP were subjected to 18 h of hypoxia in the presence of MK801, followed by reoxygenation for 24 h. Neurons treated in this fashion die in a delayed manner dependent upon cell cycle activation (5). Consistent with our observation with DNA damage, the expression of E2F1 (Fig. 2*A*) significantly reduced neuronal survival compared with the GFP control (24% survival in E2F1- *versus* 45% in GFP-expressing neurons, $p < 0.01$). In contrast, expression of E2F4 was significantly protective following hypoxia/reoxygenation compared with the GFP control (65% survival in E2F4- *versus* 45% in GFP-expressing neurons, $p < 0.01$) (Fig. 2*A*).

Loss of function studies were consistent with the observations above (Fig. 2, *C* and *D*). In this regard, we focused on the role of E2F4 because loss of function studies with E2F1 following ischemic insult have been shown previously to promote survival (8, 18, 19, 34). CGNs were transfected with siRNA to E2F4 or control siRNA and subjected to hypoxia and reoxygenation. E2F4 knockdown in transfected cultures was verified by Western blot analysis (Fig. 2*B*). Evaluation of other E2Fs, including E2F1 and E2F3, showed that the levels of these proteins were not affected significantly by E2F4 siRNA-mediated knockdown.[4] We observed sensitization of neurons to death induced by hypoxia/reoxygenation when E2F4 was transiently knocked down (3% survival) compared with the siRNA control (29% survival, $p < 0.001$). Interestingly, neurons transfected with E2F4 siRNA were remarkably more vulnerable to neuronal death even in the absence of insult (19% survival in E2F4 knockdown *versus* 84% in siRNA control cultures, $p < 0.001$) (Fig. 2*C*). CGNs from E2F4$^{-/-}$ and E2F4$^{+/+}$ mice were also similarly subjected to hypoxia/reoxygenation. E2F4$^{-/-}$ neurons also showed an increased sensitivity to hypoxia-induced neuronal death (23% survival in E2F4 null *versus* 58% in wild-type neurons, $p < 0.01$) (Fig. 2*D*). These results suggest that, unlike E2F1, E2F4 plays a prosurvival role in neuronal death induced by hypoxia. Interestingly, the increased sensitization of E2F4 siRNA-treated neurons to death in the absence of insult suggests a potential compensatory effect in E2F4 knockout neurons. To examine this possibility, we analyzed E2F1 and E2F3 protein levels in E2F4 wild-type and knockout neurons by Western blot analysis. Our results showed that the E2F1 protein level is unaffected by the loss of E2F4 (Fig. 2, *E* and *F*). However, the E2F3 protein level was reduced significantly ($p < 0.05$) with germ line E2F4 loss (Fig. 2, *G* and *H*). This is in contrast with our earlier observation that E2F3 levels are unaffected by the transient knockdown of E2F4.[4]

E2F4 and p130 Proteins Are Reduced following Hypoxia/Reoxygenation in Vitro—E2F4 is known to form repressive complexes with p130 in neurons, which may be important in neuronal survival (21). Coimmunoprecipitation experiments showed that E2F4 and p130 normally form complexes in CGN (Fig. 3*A*). This is in accordance with previous reports (23, 35). We then asked whether there were perturbations in total E2F4 and p130 protein levels following hypoxia/reoxygenation insult. CGN cultures were subjected to varying durations of hypoxia or 18 h of hypoxia, followed by varying reoxygenation times. Total protein was harvested at the indicated times and analyzed by Western blot analysis (Fig. 3, *B* and *D*). The blots were probed with antibody against E2F4 (Fig. 3*B*) or p130 (Fig. 3*D*). E2F4 (Fig. 3, *B* and *C*) protein levels were diminished significantly ($p < 0.05$) following 18 h of hypoxia (*R0*) and remained reduced for up to 8 h following reoxygenation when compared with untreated cultures. Although there was a reinduction of E2F4 levels at 2 and 4 h of reoxygenation, it did not reach the levels of untreated controls. Similar to E2F4, p130 (Fig. 3, *D* and *E*) protein levels were also reduced drastically ($p < 0.05$) immediately following hypoxia, with a brief reinduc-

[4] G. O. Iyirhiaro, Y. Zhang, F. Safarpour, S. M. Callaghan, R. S. Slack, and D. S. Park, unpublished data.

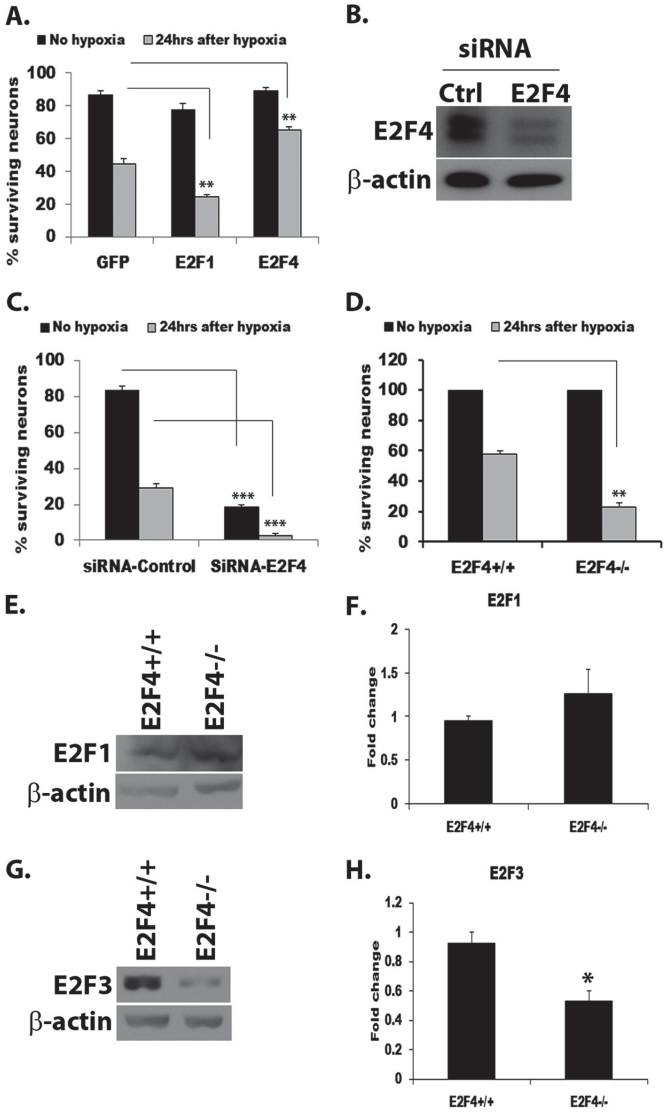

FIGURE 2. **E2F1 and E2F4 have opposing roles in neuronal death induced by hypoxia.** *A*, primary CGN cultures were infected with adenovirus expressing E2F1, E2F4, or GFP control at the time of plating and treated with hypoxia after a week in culture for 18 h, followed by reoxygenation for 24 h. *B*, Western blot analysis showing E2F4 knockdown in CGN cultures treated with E2F4 siRNA mixture. *Ctrl*, control. *C*, CGNs were transfected with E2F4 or control siRNA 5 days after plating. Cultures were treated with 18 h of hypoxia followed by 24 h of reoxygenation 2 days after transfection. *D*, CGNs from E2F4 KO and wild-type mice were similarly treated with 18 h of hypoxia and 24 h of reoxygenation. Neuronal survival was evaluated 24 h after hypoxia. Results are expressed as percent of control ± S.E. *E*, E2F1 protein levels are unaffected by E2F4 deficiency. CGNs from $E2F4^{+/+}$ and $E2F4^{-/-}$ neurons were subjected to Western blot analysis and probed with anti-E2F1 antibody and anti-β-actin as a control. *F*, quantitation of E2F1 levels as in *E*. *G*, E2F3 protein levels are diminished in E2F4 knockout neurons. A Western blot analysis was conducted on $E2F4^{+/+}$ and $E2F4^{-/-}$ CGNs. E2F3 was detected using anti-E2F3 antibody. β-actin was used as a loading control. *H*, quantitation of E2F3 protein levels as in *G* by densitometry. The protein levels of E2F1 (*E*) and E2F3 (*G*) were normalized to β-actin. *Error bars* represents the mean ± S.E. ($n \geq 3$). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

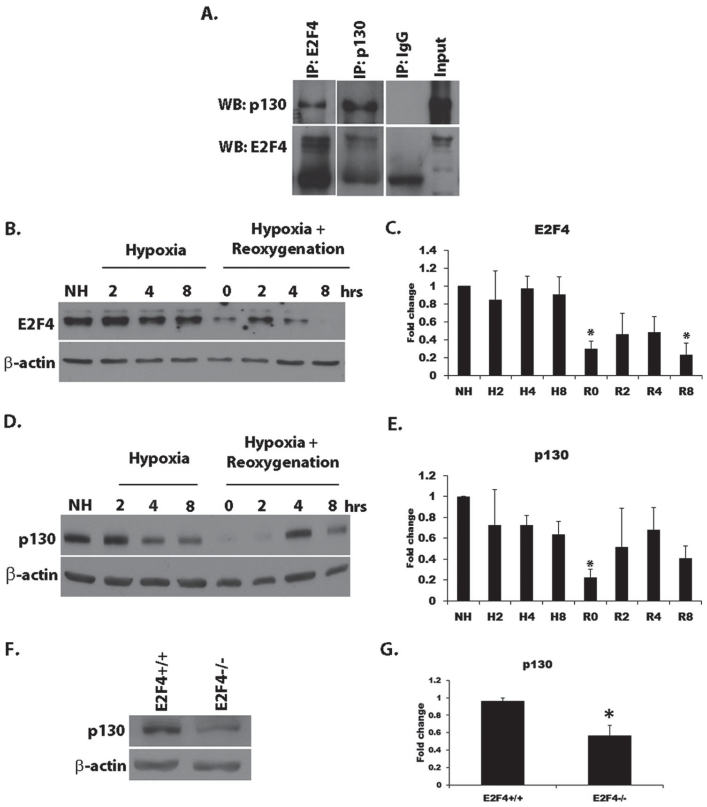

FIGURE 3. **p130 and E2F4 protein levels are down-regulated following hypoxia/reoxygenation of CGNs in culture.** *A*, E2F4 forms a complex with p130 in CGNs. Total cell lysates from CGN cultures were subjected to reciprocal immunoprecipitation (*IP*) with anti-E2F4 and anti-p130 antibodies. Immune complexes were resolved by Western blot (*WB*) analysis and probed with anti-E2F4 and anti-p130 antibodies. *B*, Western blot analysis showing a loss of E2F4 protein in CGNs subjected to varying durations of hypoxia or 18 h of hypoxia with varying durations of reoxygenation. *NH*, no hypoxia. *C*, densitometry was performed on E2F4 blot as in *B*. E2F4 protein levels were normalized to the actin control. *H*, hypoxia; *R*, reoxygenation; *R0*, 18 h of hypoxia and 0 h of reoxygenation. *D*, Western blot analysis showing the loss of p130 following different durations of hypoxia or 18 h of hypoxia with varying reoxygenation times. *E*, densitometry was performed on p130 protein levels as in *D* and normalized to the β-actin control. *F*, basal p130 protein levels were diminished in E2F4$^{-/-}$ CGNs. A Western blot analysis was conducted on total protein lysate obtained from E2F4$^{+/+}$ and E2F4$^{-/-}$ CGN cultures. The blots were probed with anti-p130 antibody and anti-β-actin as a control. *G*, densitometry was conducted on p130 blots as in *F*. p130 levels were normalized to β-actin. *Error bars* represent the mean ± S.E. ($n \geq 3$). *, $p < 0.05$.

tion at 2 and 4 h of reoxygenation. The reason for this induction is unclear at the moment. However, levels appeared to diminish again thereafter. The loss of p130 could be related to the loss of E2F4 following hypoxia and reoxygenation. Indeed, a Western blot analysis performed on E2F4$^{-/-}$ CGN cultures showed that p130 protein levels were diminished significantly ($p < 0.05$) in the absence of E2F4 (Fig. 3, *F* and *G*).

Hypoxia/Reoxygenation Induces a Concomitant Reduction of E2F4 and Induction of E2F1 Binding at the B-Myb Promoter—The previous evidence indicates that p130 is lost following ischemia. We next determined how E2F members such as E2F1 or E2F4 might differ in binding to known E2F sites. In this regard, we focused on B-Myb, a prodeath factor described previously for neurons and regulated by E2Fs (21, 35). We examined the relative promoter occupancy of both E2F1 and E2F4 following hypoxia/reoxygenation *in vitro*. ChIP was carried out on lysates from CGN cultures treated with 18 h of hypoxia and 16 h of reoxygenation using E2F1- and E2F4-specific antibodies. E2F1- and E2F4-associated chromatin was subjected to PCR using B-Myb promoter-specific primers. As shown in Fig. 4*A*, endogenous E2F4 occupancy at the B-Myb promoter was present in the untreated, no hypoxia samples under basal conditions. This occupancy decreased with hypoxia/reoxygenation (Fig. 4, *A* and *B*). This contrasts with E2F1, where occupancy is low under basal conditions and increases with hypoxic stress (Fig. 4, *A* and *C*). Consistent with the loss of E2F4, a ChIP analysis performed using p130-specific antibody also showed that it is lost from the B-Myb promoter following hypoxia/reoxygenation (Fig. 4, *D* and *E*). Interestingly, we

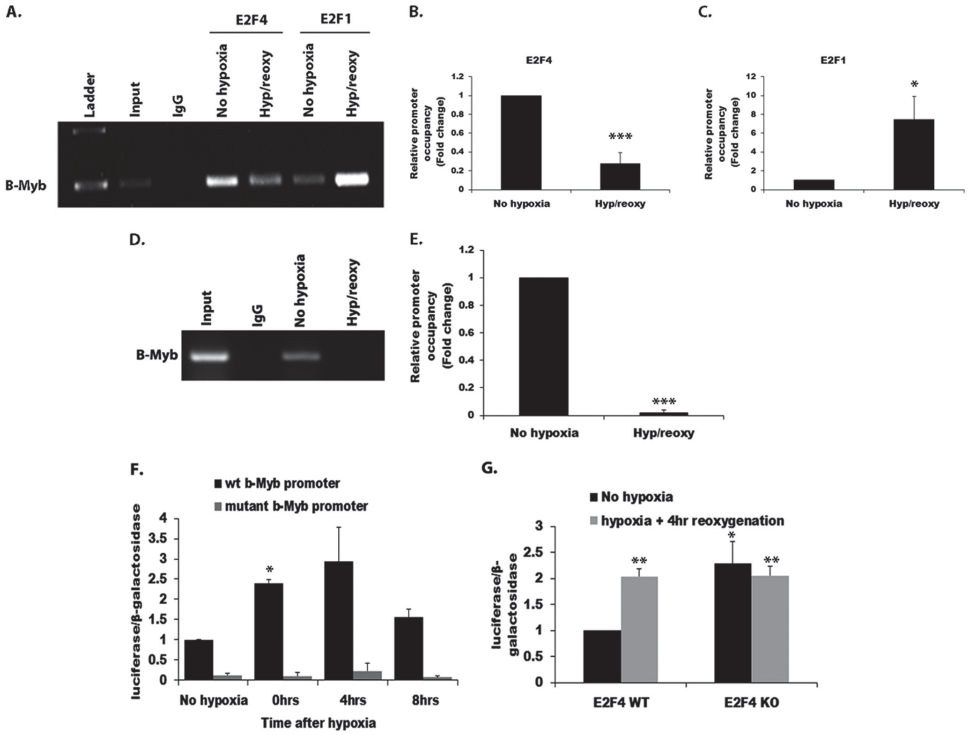

FIGURE 4. **Reduction of E2F4 and induction of E2F1 binding at the B-Myb promoter following hypoxia and reoxygenation.** *A*, ChIP was conducted with E2F4 and E2F1 antibody on CGNs treated with hypoxia (*hyp*) for 18 h, followed by reoxygenation (*reoxy*) for 16 h. *B*, densitometry of the B-Myb signal following ChIP performed with anti-E2F4 antibody as shown in *A*. Loss of E2F4 was observed at the B-Myb promoter following hypoxia and reoxygenation. *C*, quantitation of the B-Myb signal following ChIP with anti-E2F1 antibody. *D*, ChIP was performed with anti-p130 antibody. p130 binding at the B-Myb promoter was lost following hypoxia and reoxygenation. *E*, densitometry of the B-Myb signal following ChIP as shown in *D*. *F*, E2F activity increased after hypoxia and reoxygenation in CGNs. Cells were infected with AAV expressing B-Myb-promoter-luciferase with wild-type E2F or a mutated E2F site and β-galactosidase. Cells were treated with 18 h of hypoxia followed by reoxygenation for up to 8 h. Luciferase activity and β-galactosidase were measured at the indicated times. Data represent values of luciferase/β-galactosidase activity. *G*, a luciferase assay was conducted in E2F4 wild-type and knockout CGNs treated with 18 h of hypoxia followed by 4 h of reoxygenation. *Error bars* represent mean ± S.E. (n ≥ 3). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.005$.

found that, in the absence of E2F4, p130 is lost from the B-Myb promoter under basal conditions.[4] This result indicates that E2F1 and E2F4 may mutually regulate B-Myb in an opposing manner to increase B-Myb expression and promote death. The ChIP experiments were performed at later time points where we could observe robust biochemical changes. To query the effects of E2F regulation at earlier time points, we next examined changes in E2F-mediated activity at the B-Myb promoter utilizing more sensitive, luciferase-based promoter assays. To this end, CGNs were infected with AAV expressing E2F reporter constructs, B-Myb-promoter-luciferase containing wild-type or mutated E2F sites as a control, and β-galactosidase 1 day after plating. The cells were treated with hypoxia and varying duration of reoxygenation after a week in culture. Luciferase and β-galactosidase analyses showed that E2F activity at the B-Myb promoter was induced significantly (2.5-fold, $p < 0.05$) immediately following hypoxia and remained elevated during reoxygenation (Fig. 4*F*). Because E2F4 is known to form repressive complexes and our results indicated that it is lost following hypoxic stress, we next investigated the effects of its deficiency on overall E2F activity at the B-Myb promoter. E2F4 wild-type and knockout CGNs were similarly infected with AAV expressing the same E2F reporter constructs as above, and luciferase activity was measured following 18 h of hypoxia and 4 h of reoxygenation (Fig. 4*G*). Consistent with our earlier results, luciferase activity increased 2-fold ($p < 0.01$) following hypoxia/reoxygenation in the E2F4 wild-type CGNs (Fig. 4*G*). In the E2F4 null CGNs, the basal luciferase activity was elevated (2-fold, $p < 0.05$) compared with E2F4 wild-type cultures and was not increased further with hypoxia/reoxygenation (Fig. 4*G*). This result indicates that E2F4 is important in the basal suppression of B-myb. It is important to note here that, because no death is observed basally (at least with non-stressed E2F4 knockout neurons), this suggests that multiple signals, including Myb, act in concert to promote death following ischemic insult.

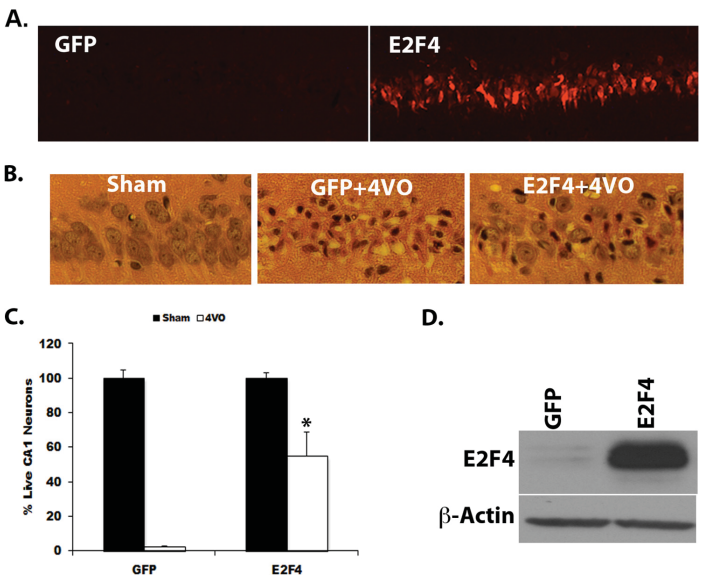

FIGURE 5. **E2F4 expression protects CA1 neurons from global cerebral ischemia.** *A*, immunofluorescence staining showing E2F4 overexpression in hippocampal CA1 neurons of rats injected with AAV expressing E2F4. *B*, hematoxylin and eosin-stained sections of CA1 neurons in sham- and 4VO-operated rats injected with GFP or E2F4 expressing AAV. *C*, quantitation of live CA1 neurons following 4VO in GFP and E2F4 injected rats. Rats were injected with AAV expressing E2F4 or a GFP control and subjected to sham surgery of 10 min of 4VO. Brains were extracted 4 days following reperfusion, sectioned, and stained with hematoxylin and eosin. H&E-stained coronal sections were evaluated for cell survival in the hippocampal CA1 ($n \geq 4$/group, data are mean ± S.E). *, $p < 0.05$ compared with GFP control. *D*, Western blot analysis of E2F4 expression in the hippocampus of GFP and E2F4 injected rats.

E2F4 Expression Is Protective following Transient Cerebral Ischemia in Vivo—To further investigate the role of E2F4 under ischemic conditions, we next examined its effects in an *in vivo* model of stroke induced by global ischemia. AAV expressing E2F4 or GFP control were injected unilaterally into the hippocampal CA1 region 2 weeks prior to insult, as described previously (5). Global ischemia was induced for 10 min using the four-vessel occlusion method, as described previously (6, 32). E2F4 overexpression and efficiency of viral delivery were verified by immunohistochemistry staining of CA1 neurons (Fig. 5*A*) and confirmed by Western blot analysis performed on hippocampal protein lysate from AAV-injected rats (Fig. 5*D*). Analysis of CA1 neurons 4 days following global ischemia showed significantly more live CA1 neurons (55% survival) in E2F4-expressing rats compared with those expressing GFP (3%, $p < 0.05$) (Fig. 5, *B* and *C*). GFP or E2F4 overexpression alone had no effect on neuronal viability in the absence of insult (Fig. 5*C*).

E2F4 and p130 Proteins Are Diminished following Global Ischemia—We also asked whether perturbations in E2F4 and p130 levels occurred *in vivo* following cerebral ischemia. Similar to the results obtained in CGNs, coimmunoprecipitation results showed that E2F4 and p130 also normally form complexes in rat hippocampal lysates.[4] To examine potential changes in E2F4 and p130 protein levels *in vivo* following ischemia, rats were subjected to 10 min of 4VO and sacrificed at various times following reperfusion, as described under "Experimental Procedures." Hippocampal lysates were extracted and subjected to Western blot analysis (Fig. 6*A*). The blots were probed with antibody against p130, E2F4, and actin for a loading control. p130 levels were increased (although not statistically significant) at early time points following ischemia but declined precipitously 24 h following reperfusion (Fig. 6, *A* and *B*). The early increase in p130 could be a compensatory response. Similarly to p130, the E2F4 protein level was reduced dramatically reduced 24 h following ischemia (Fig. 6, *A* and *C*). The biological function of p130 (14, 36) as well as its stability and down-regulation (37) are known to be regulated by Cdk-mediated phosphorylation. p130 has been shown to contain multiple Cdk phosphorylation sites, including Ser-952 (36). Therefore, we examined p130 Ser-952 phosphorylation following ischemia *in vivo*. Cdk-mediated phosphorylation of Ser-952 was increased following cerebral ischemia (Fig. 6, *D* and *E*). These results indicate that p130 is phosphorylated at early time points following ischemic insult and that both p130 and E2F4 proteins are subsequently lost in the death process. These results are similar to those observed with hypoxia/reoxygenation *in vitro*.

B- and C-myb Transcripts Are Induced following Global Ischemia in Vivo—Because our results showed that E2F activity is increased at the B-myb promoter *in vitro*, we examined potential changes in its transcript levels as well as that of the related C-Myb following ischemic insult *in vivo*. We observed that both B-Myb (Fig. 7, *A* and *B*) and C-Myb (Fig. 7, *C* and *D*)

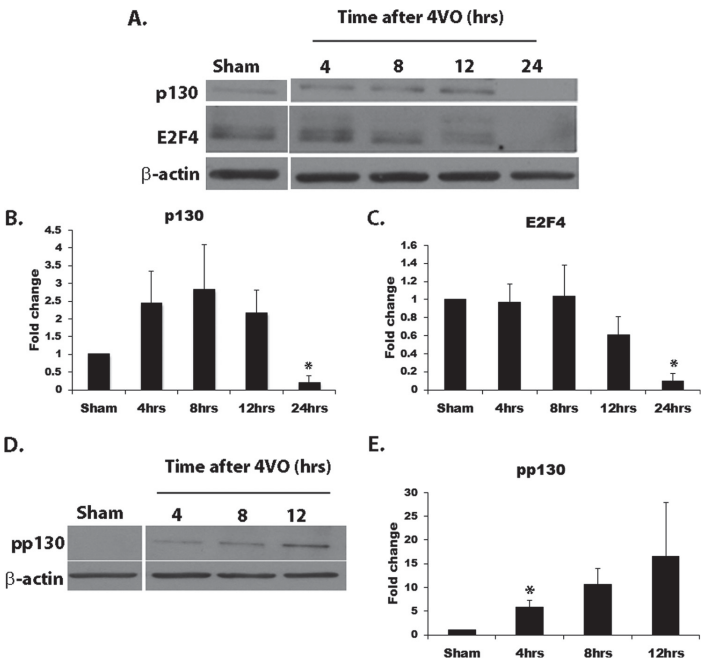

FIGURE 6. **p130 and E2F4 protein levels are decreased following global cerebral ischemia in rats.** *A*, Western blot analyses of p130 and E2F4 expression following 4VO in rats. Animals were subjected to 10 min of 4VO and sacrificed at the indicated times following reperfusion. Hippocampal tissues were extracted and subjected to Western blot analysis. Densitometry was performed on p130 protein levels (*B*) as well as E2F4 protein levels (*C*) and normalized to the actin control. *D*, Western blot analysis of p130 phosphorylation following 4VO. Rats were treated as in *A* and subjected to analysis using antibody directed at p130 phosphorylated at Ser-952. *E*, densitometry of Ser-952-phosphorylated p130. Expression levels were normalized to the actin control ($n \geq 3$). *, $p < 0.05$ compared with sham.

mRNA transcript levels were increased 3-fold ($p < 0.05$) 24 h following global ischemia. Overall, our results suggest that E2F4-p130 complexes provide neuronal protection under ischemic stress by regulating the expression of proapoptotic factors. Consistent with this hypothesis and previous reports (23, 35), we found that siRNA-mediated knockdown of the C-Myb gene provided protection for neurons subjected hypoxia/reoxygenation insult (Fig. 7*F*). CGN cultures were transfected with siRNA to C-Myb or control siRNA 5 days after plating and subjected to 18 h of hypoxia followed by 16 h of reoxygenation. Analysis of neuronal survival showed increased neuronal survival in neurons transfected with C-Myb siRNA (56%) compared with control siRNA (37%) (Fig. 7*F*). Knockdown of the C-Myb message was verified by semiquantitative RT-PCR in siRNA-treated cultures 24 h after transfection (Fig. 7*E*).

DISCUSSION

Cell cycle induced death of neurons has been described in numerous cell death paradigms (1, 20, 38–40). However, the downstream effectors of this pathway in the context of pathologic neuronal death are not fully understood, particularly in the context of ischemic damage. In this regard, a growing body of evidence suggests that death following ischemic insult is mediated via pRb-E2F1.

In line with this, we found that E2F1 deficiency was protective, whereas its overexpression exacerbated death induced by DNA damage and hypoxia/reoxygenation. Our data are consistent with a proapoptotic role demonstrated previously for E2F1 in other contexts, including death induced by staurosporine (41), β-amyloid (42), potassium deprivation (24, 43, 44), kainic acid (34), oxygen-glucose deprivation (OGD) (8), and ischemia (8, 24, 34, 41, 42). Similar to E2F1, E2f3, another member of the activating E2F family, is proapoptotic. Overexpression of E2F3 promoted cell death in response to camptothecin treatment, whereas its down-regulation was protective. This is in line with a previous report demonstrating a protective role for E2F3 deficiency in the developing CNS in response to DNA damage (45).

The role of the repressive E2F4 contrasts that of activating E2F members by promoting survival under conditions of ischemic stress. This is supported by several findings in this study. Acute down-regulation of E2F4 in CGNs using siRNA caused death in the absence of insult. Hypoxia/reoxygenation resulted in death that was exacerbated with E2F4 deficiency and knock-

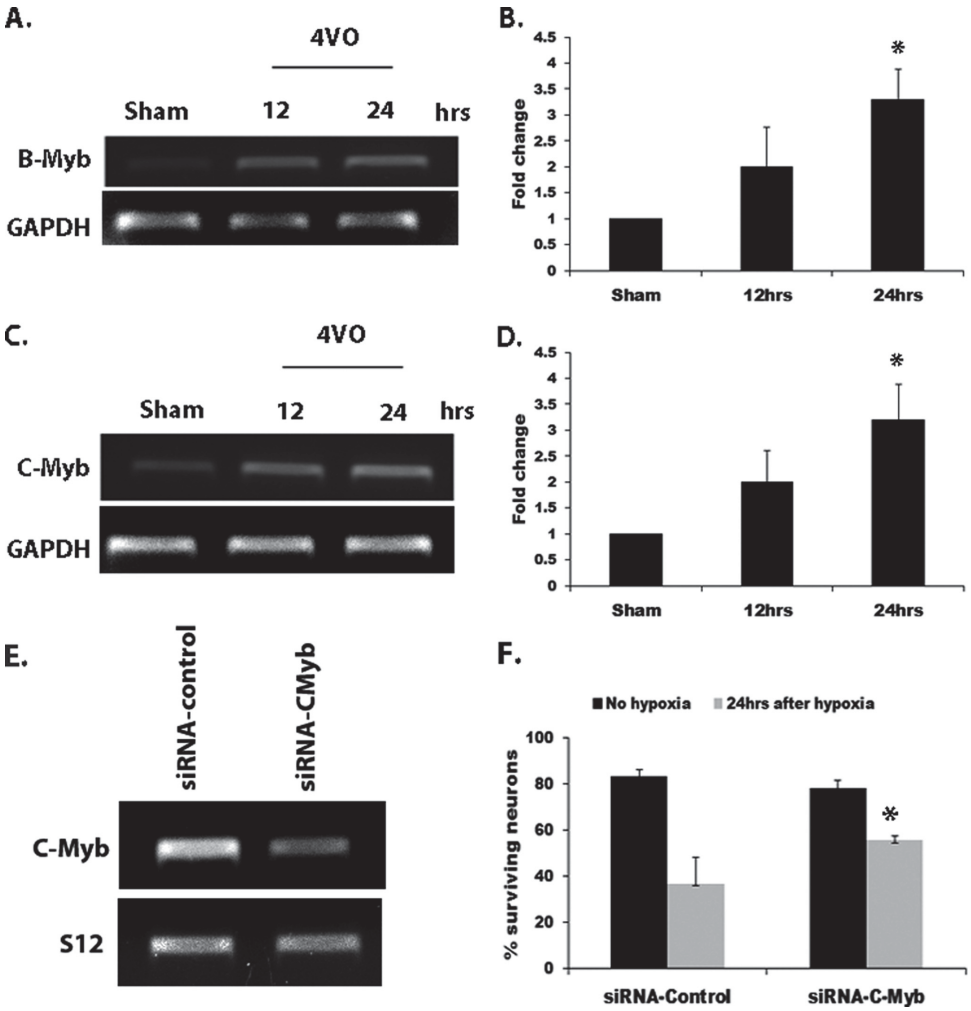

FIGURE 7. **B- and C-Myb mRNA transcript levels are increased following global cerebral ischemia.** *A*, semiquantitative RT-PCR of the B-Myb message following global ischemia. Rats were subjected to 10 min of 4VO followed by 12 and 24 h of reperfusion. Hippocampal tissues were extracted and analyzed by semiquantitative RT-PCR. GAPDH is shown as a control. *B*, densitometry of B-Myb levels. Data are expressed as fold over sham-operated control. *C*, semiquantitative RT-PCR of the C-Myb message following 4VO as described in *A*. *E*, semiquantitative RT-PCR showing siRNA mediated knockdown of the C-Myb message 24 h following transfection. S 12 is shown as a loading control. *F*, C-Myb knockdown protects CGNs from hypoxia/reoxygenation insult. Neurons were transfected with C-Myb siRNA and subjected to 18 h of hypoxia followed by 16 h of reoxygenation. *Error bars* represent the mean ± S.E. ($n \geq 3$). *, $p < 0.05$.

down in culture. In contrast, we found that overexpression of E2F4 protected neurons from death induced by hypoxia/reoxygenation *in vitro* and global cerebral ischemia *in vivo*. The data presented here as well as by others (8, 18, 19) demonstrate that, in the context of cerebral ischemic damage, the activities of both the activating and repressive E2Fs are an important determinant of neuronal survival and death.

The differential roles of activating and repressive E2Fs in neuronal survival is interesting. Although the exact reasoning behind this role is not clear, there are some intriguing hypotheses generated by our data. First, we showed that p130-E2F4 levels are present basally but reduced following ischemic insult. Second, we show that E2F4 binding to the E2F site on the B-Myb promoter is reduced following

hypoxia. This suggests a model where p130-E2F4 exists basally to form active repressive complexes but is lost after death-inducing insult. These repressive complexes may be critical for neuronal survival. In support of this, p130 predominantly occupies E2F sites in cultured neurons (21) and has been shown, along with E2F4, to participate in the mammalian DP, Rb-like, E2F and MuvB-like (DREAM) protein complex (46). Additionally, p130 levels are high in neurons (47, 48), and p130 loss has been suggested to promote death (49). In particular, p130 deficiency leads to strain-dependent lethality in null mice (49), and its down-regulation in cultured neurons promotes apoptosis (21).

In this scenario, we suggest that E2F4 is protective because it would facilitate the formation of repressive complexes, which are lost following ischemic insult. In contrast to E2F4, we propose that E2F1 acts to promote death by directly activating prodeath genes. This is supported in this study by the observation that E2F1 is prodeath and that its occupancy on death-inducing genes is increased following hypoxic insult. Therefore, we propose a model whereby hypoxia/ischemia leads to the loss of E2F4-repressive complexes on target promoters and allows for increase in E2F1 transactivating activity on those genes. Both would then contribute to an increase in expression of targets genes such as Myb, leading to death. Indeed, we show that targets such as B-Myb and C-Myb are increased following ischemia *in vivo*. It will be important to fully test this hypothesis in the future.

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