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Interactions of LRRK2 in a *Drosophila*  
*melanogaster* model of Parkinson's disease

Ghassan Kabbach

This thesis is submitted as a partial fulfillment of the M.Sc. program in Neuroscience

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## Abstract

Parkinson's disease is the most common movement disorder. A complex neurodegenerative disease, its cause and progressive nature are of unknown roots, making a final cure currently unattainable. Recently, mutations in LRRK2 have been deemed the most common cause of both familial and sporadic forms of Parkinson's disease. Itself a mysterious protein, it harbors pathogenic mutations in all of its complex functional domains. Here, we present a *Drosophila melanogaster* model of LRRK2 by creating four different human LRRK2 transgenic flies. Wild type LRRK2, and LRRK2 mutants I1122V, Y1699C, and I2020T have each demonstrated Dopamine neuron loss, complex behavioral and life span alterations, and a complex eye phenotype. Lastly, we have used the eye phenotype to conduct both a biased screen against recessive Parkinson's disease genes, and an unbiased screen against the *Drosophila* genome.

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## List of Abbreviations

°C – Degrees Celsius  
4E-BP - eIF4E-binding protein  
Ab – Antibody  
AD – Alzheimer`s disease  
ANOVA – Analysis of Variance  
AR-JP - Autosomal Recessive Juvenile Parkinson`s disease  
cDNA - complementary DNA  
*C. elegans* - *Caenorhabditis elegans*  
CHIP – carboxyl terminus of the heat shock cognate protein 70-interacting protein  
CNS – Central Nervous System  
COMTi - Catechol-O-Methyltransferase inhibitor  
COR – C-terminal of Roc  
C-terminus – carboxy-terminus  
CyO – *Curly*  
Da – *Daughterless*  
DA – Dopamine/ Dopaminergic  
DAB – diaminobenzidine  
*D. melanogaster* – *Drosophila melanogaster*  
dPink1 – *Drosophila* Pink 1  
*E. Coli* - *Escherichia coli*  
DBS – Deep Brain Stimulation  
dLRRK – *Drosophila* leucine-rich repeat kinase  
DNA - Deoxyribonucleic acid  
DLB – Dementia with Lewy bodies  
DMV – dorsal motor nucleus of the vagus  
eIF4E – Eukaryotic Translation Initiation Factor 4E  
Elav – embryonic lethal abnormal visual system  
ERK - extracellular-signal-regulated kinase  
ERM - ezrin/radixin/moesin  
FLP - Flippase recombination enzyme  
FRT - Flippase Recognition Target  
GBA – glucocerebrosidase  
GDP - guanine diphosphate  
GFP - Green Fluorescent Protein  
Girk2 - G protein-activated inward rectifier potassium channel 2  
GMR – *glass multimer reporter*  
GPi – Globus pallidus interna  
GTP - guanine triphosphate  
GTP[S] - Guanosine 5 -O-(3-Thiotriphosphate)  
h – hour  
H<sub>2</sub>O<sub>2</sub> – Hydrogen peroxide  
hDJ-1 – Human DJ-1  
HEK-293 - Human Embryonic Kidney 293

hParkin – Human Parkin  
hPink1 – Human Pink1  
Hsp90 – Heat shock protein 90 kDa  
IRAK - Interleukin 1 receptor activating kinase  
kb – kilo-base pair  
kD – kilo Dalton  
LB – Lewy body  
L-Dopa – levodopa  
LRK-1 – leucine-rich kinase 1  
LRRK1 – Leucine-rich repeat kinase 1  
LRRK2 – Leucine-rich repeat kinase 2  
MAO-Bi - Monoamine Oxidase-B Inhibitor  
MAPK – Mitogen-activated protein kinase  
MAPKKK – Mitogen-activated Protein kinase kinase kinase  
MBP - myelin based protein  
min – minute  
MLK - Mixed Lineage Kinase  
MPTP - 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine  
mRNA – Messenger RNA  
MSA-P Multiple System Atrophy Parkinsonism  
N-terminus – amino-terminus  
PCR – Polymerase Chain Reaction  
PD – Parkinson`s disease  
PDD - Parkinson`s disease Dementia  
Pink1 - PTEN induced putative kinase 1  
PPL1 - protocerebral posterior lateral 1  
PPL2 - protocerebral posterior lateral 2  
PPM1/2 – protocerebral posterior medial 1/2  
PPM3 - protocerebral posterior medial 3  
PSP-P Progressive Supranuclear Palsy Parkinsonism  
RIPK - receptor interacting protein kinase  
RNA - Ribonucleic acid  
RNAi – RNA interference  
ROC – Ras-of-Complex GTPase  
RT – Room Temperature  
RT-PCR – Reverse Transcriptase-PCR  
s – second  
SEM – Scanning Electron Microscopy  
SNc – Substantia nigra pars compacta  
STN - Subthalamic nucleus  
TH – *Tyrosine Hydroxylase*  
TUNEL - Terminal deoxynucleotidyl transferase mediated dUTP Nick End Labeling  
UAS - Upstream Activation Sequence  
UCH-L1 - ubiquitin carboxyl-terminal esterase L1  
WT – wild type

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Ghassan Kabbach. Ottawa, 2010

## Chapter 1 – General Introduction

### 1.1 Introduction to Parkinson's disease

A modest English family physician, James Parkinson was the first to write a detailed monograph of the disease then called the Shaking Palsy. In 1817, he described in impressive elegance six cases as having “Involuntary tremulous motion, with lessened muscular power, in parts not in action and even when supported; with a propensity to bend the trunk forwards, and to pass from a walking to a running pace: the senses and intellects being uninjured.” (Parkinson, 2002) In honor of his work, the disease was recognized as a distinct clinical entity and termed Parkinson's disease some sixty years later. (Lees et al., 2009)

Today, Parkinson's disease (PD) is recognized as being part of a broad spectrum of Parkinsonian syndromes, but remains the second most common neurodegenerative disorder after Alzheimer's disease (Gasser, 2009a). It is pathologically characterized by the severe loss of nigrostriatal dopaminergic fibers and the presence of cellular inclusions termed Lewy Bodies. It is clinically diagnosed by the presence of three hallmark features, bradykinesia, rigidity, and tremor. Age remains the most important susceptibility factor for the disease although genetic contributions are now known. It continues to elude a cure, with L-Dopa being the first line treatment option.

### 1.1.1 Clinical Features

It is usually difficult to pinpoint the exact onset of the disease. The symptoms start tame and progress very slowly mostly unnoticed by the patients and their families. After initial diagnosis, patients can recall early signs dating back an average of 2-3 years, which might be a conservative figure considering one case for which the first symptoms occurred 8 years earlier (Lees, 2009). Physical symptoms start as a unilateral weakness, stiffness, and trembling in one of the limbs, mostly the hand. They would increase in intensity and then emerge at another location. As the disease advances, the patient's face becomes expressionless, the voice monotonous, and the trunk slightly bent with reduced arm swing. The patient can start to notice difficulty in fine hand movements and walking will require more attention to prevent falls due to gait freezing, leaning forward, and adopting smaller slower steps. Within 10 years of the disease, postural instability with falls and visual hallucination can develop. Eventually and due to excessive leaning forward, the patient is forced to adopt a running pace with short steps. Sleep disturbances, depression, cognitive decline, and autonomic dysfunction are also not rare. Dementia is a common occurrence in PD, developing in 75% of patients who survive for more than 10 years (Aarsland and Kurz, 2010).

The Queen Square Brain Bank, a UK-based brain archive, now considers bradykinesia with either one of muscular rigidity, 4-6 Hz rest tremor, or postural instability as sufficient to clinically diagnose a Parkinsonian syndrome (Lees et al., 2009). Criteria such as a unilateral onset, a progressive course, hyposmia, excellent

response to L-Dopa, and visual hallucinations will support a definite diagnosis of Parkinson's disease. On the other hand, other criteria such as a history of repeated strokes, head injuries, early severe autonomic dysfunction and dementia with memory problems can suggest a different diagnosis.

### 1.1.2 Pathological Features

PD is marked by a progressive loss of selected brain-stem nuclei and the presence of Lewy Bodies. The most affected population of neurons are the neuromelanin-containing dopaminergic (DA) neurons of the substantia nigra pars compacta (SNc). This results in regional loss of striatal DA levels particularly in the putamen. The pattern of cell loss in the SNc is different from that seen in normal aging on both the regional and cellular levels. Regionally, it is the ventrolateral and then medial ventral segments of the SNc that are affected in PD which is opposite of the dorsal pattern seen in normal aging (Fearnley and Lees, 1991). Within the pars compacta, it is the calbindin-poor Girk2-rich pockets, termed nigrosomes, that are most devastated, and not the calbindin-rich cells affected mostly in normal aging (Damier et al., 1999b, a). Extensive cell loss in the SNc, estimated at 30%, would have already occurred before the first signs of PD are noticed. The attrition rate continues at 7% per year increasingly worsening the severity of bradykinesia and rigidity (Lees, 2009). Other important catecholaminergic nuclei affected in PD are the locus coeruleus, the raphe nuclei, and nuclei of the ventral tegmental area. Non-catecholaminergic neurons are also affected, such as the cholinergic neurons of the

dorsal motor nucleus of the vagus (DMV), the nucleus basalis of Meynert, the olfactory bulb, and even the parasympathetic neurons of the gut.

A number of intraneuronal inclusions accompany the cell loss in PD: the Lewy body (LB), the pale body, and the Lewy neurite (Lang and Lozano, 1998; Dickson et al., 2009; Kovari et al., 2009). LBs are consequently divided into two categories: Classical (brainstem), and Cortical. Classical LBs are eosin and hematoxylin positive spheres with a dark core and a pale peripheral halo. Found mostly in the somata of DA neurons in the substantia nigra, they are also present in the locus coeruleus, the DMV, raphe nuclei, the nucleus basalis of Meynert, and the olfactory system among other structures. The core is predominantly composed of ubiquitin while  $\alpha$ -synuclein is rich in the periphery. Cortical LBs on the other hand lack the inner core and halo and are present in limbic and neocortical regions such as layer V cells in the cortex. They are mostly linked with the clinical entity Dementia with Lewy Bodies (DLB) (McKeith, 2006). However, they are not considered a pathological correlate of dementia in PD as they can be present in many non-demented patients (Colosimo et al., 2003). It is a matter of controversy whether or not LBs contribute to the disease state in any negative or positive manner or if they are simply an innocent bystander unrelated to the main disease mechanism. For example, LBs can be present in regions where cell loss has not occurred, and in the brains of the elderly without signs of PD. Nevertheless, LBs are central to the confirmation of a PD diagnosis post-mortem and pathologists will hesitate to confirm a case of PD if LBs were not found (Greenfield and Bosanquet, 1953). The Pale body is seen as a possible precursor of LBs, while the Lewy neurite is a neuronal process with abnormal  $\alpha$ -synuclein filaments. A staging system of LB progression has been put forward by Braak

et al. (Del Tredici et al., 2002; Braak et al., 2003) which proposes a progression of LBs developing caudorostrally from lower brainstem regions and the olfactory bulb, towards the substantia nigra, and finally the neocortex. However, no clinical correlation between the proposed stages and the severity of neuronal degeneration and clinical manifestation of PD has been found, and the staging system did not include brain cases of DLB patients in which Cortical LBs emerge first in the cortex (Jellinger, 2009; Lees, 2009). It has been suggested, however, that younger onset patients have LB distribution more consistent with the Braak staging (Halliday et al., 2008).

### 1.1.3 Etiology and Epidemiology

PD is primarily an idiopathic disorder with an unknown cause. Old age is the most important susceptibility factor as the mean age of onset is 65. The prevalence of PD increases from 1-2% for people over the age of 65, to 4% for people over the age of 85 (Harris et al., 2009). The disease affects men and women similarly and spans an average of 15 years with pneumonia being the most common documented cause of death (Lees et al., 2009). About 10-15% of the cases of PD are early onset (Harris et al., 2009; Lees et al., 2009), and 10% demonstrate Mendelian inheritance termed Familial PD (fPD). PD is considered juvenile if it develops before 20, and early onset if it develops before 50.

Atypical parkinsonism encompasses diseases that have parkinsonian symptoms but are different enough to be considered a separate clinical entity. Most common of

these are Autosomal Recessive Juvenile PD (AR-JP), Multiple System Atrophy Parkinsonism (MSA-P), Progressive Supranuclear Palsy Parkinsonism (PSP-P), and Corticobasal degeneration (Lang and Lozano, 1998; Lees et al., 2009). Postencephalitic PD, vascular PD, and PD caused by head trauma, tumours, and drugs such as MPTP are also known causes of atypical PD (Lang and Lozano, 1998; Lees et al., 2009). Depending on the presence of dementia and the onset of its occurrence, we can have different diagnosis of Parkinson's disease Dementia (PDD) and Dementia with Lewy Bodies (DLB), although some argue these are one clinical entity (Johansen et al.; McKeith, 2009). Classical PD itself has three identifiable variants; a rapidly progressive bradykinetic-rigid form, a slowly progressive tremulous form, and a bradykinetic syndrome with early dementia which closely resembles DLB (Lees, 2009).

Single gene mutations have been discovered recently to contribute to both sporadic and familial cases of PD. These monogenetic causes frequently present with a disease similar to sporadic PD but possibly with a more benign course (Lees, 2009). Parkin, Pink1, and DJ-1 are linked with autosomal recessive, early onset PD, while  $\alpha$ -synuclein and LRRK2 are linked with autosomal dominant PD. Monogenetic contributions to PD increase with decreased age of onset reaching 67% for Parkin's contributions to cases of PD with age of onset less than 20 years (Periquet et al., 2003). Interestingly and contrary to the other genes which are by far limited to Mendelian forms of PD, LRRK2 mutations were also found in sporadic cases in high prevalence depending on the population (Benamer and de Silva, 2010). More on the genetics of Parkinson's disease will be discussed in subsequent paragraphs.

#### 1.1.4 Treatment of Parkinson's disease

Dopamine replacement therapy is the first treatment option in PD. L-Dopa (levodopa) was released in 1968 with much excitement to its apparent potential, but is now known to be only useful in approximately the first ten years of the disease (Olanow et al., 2009a). It is the PD drug with the highest efficacy, but can produce unwelcomed motor complications after 5-10 years; wearing-off, On-Off phenomena, and dyskinesias. Wearing off is a self-explanatory term for the wearing off of the benefits of levodopa until the next medication administration. The On-Off phenomenon is the sudden oscillation of motor symptoms from being severely parkinsonian to normal. Dyskinesias are involuntary movements that are due to the high plasma concentration of levodopa causing pulse-like activation of the dopamine receptors, and eventually other effects on striatal signaling pathways (Pisani and Shen, 2009). Other unpleasant side effects of levodopa are nausea, sedation, orthostatic hypotension, hallucinations, and depression.

Currently the most common administration of levodopa is in combination with a peripheral dopa decarboxylase inhibitor such as Cabidopa (Rezak, 2007). This can reduce the unspecific effects of levodopa outside the Central Nervous System. The use of Catechol-O-Methyltransferase inhibitors (COMTi's) has recently been introduced to increase the amount of dopamine reaching the brain when levodopa is combined with carbidopa. This drug can combat the wearing-off phenomenon but can cause dyskinesias in vulnerable patients (Rezak, 2007). Monoamine Oxidase-B Inhibitors (MAO-Bi's) can increase the levels of dopamine at the synapse, while Amantadine can enhance the release

of dopamine from presynaptic terminals (Rezak, 2007). Another class of drugs, Dopamine Receptor Agonists, is also a popular substitute for levodopa. It has less efficacy and less motor complications, but some impulse control problems such as pathologic gambling, hypersexuality, and compulsive eating and shopping (Fahn, 2010).

Unfortunately, none of these treatments, single or combined, will prevent or reverse the progress of the disease. Other treatment strategies are thus considered such as cell transplantation and surgery. The most common cell transplantation strategies are the transplantation of fetal nigral cells, fetal porcine nigral cells, retinal pigmented epithelial cells, and dopamine stem cells. However, none of them were able to provide benefits for patients in double-blind, controlled trials (Olanow et al., 2009b). Also when re-examined years later, these cells seemed to be affected by PD pathology (Kordower et al., 2008b; Kordower et al., 2008a), and up to 50% of patients developed a new form of disabling prolonged off-medication dyskinesia (Olanow et al., 2009c). These dyskinesias sometimes necessitate the use of surgery such as Deep Brain Stimulation (DBS) (Olanow et al., 2009b). Surgical intervention in PD by DBS is most commonly of the Subthalamic nucleus (STN) and of the globus pallidus interna (GPI) (Perlmutter and Mink, 2006; Wichmann and DeLong, 2006). It's not more useful for motor symptoms than levodopa treatment, but it is useful for patients with disabling dyskinesias and fluctuations as it can decrease the required drug dose (Fahn, 2010). Like other treatments, it has no effects on disease progression, can target only a portion of the patient base, and is associated with considerable post-operational risks such as suicide (Voon et al., 2009). Physical and cognitive therapy, along with balance exercises and muscle training can prove useful for PD patients (Keus et al., 2007).

It is thus clear that even though many treatments provide promising avenues for the future, we need to gain more insight into the molecular pathways of neurodegeneration and etiology of PD. For the first time now, the availability of genetic factors, associative or causative, can allow us to look into possible pathways of degeneration and identify players that could be modified to stop, slow down, or possibly reverse disease progression. We will thus examine the genetics of Parkinson's disease, including the molecular and biochemical details of its most common genetic cause.

#### 1.1.5 Genetics of Parkinson's disease

Initial efforts to pinpoint genetic contributions to PD were traditionally overshadowed by a focus on environmental toxins such as pesticides and metals. An early study in 1949 by Henry Mj6nes found evidence for autosomal-dominant transmission, but this was soon discredited due to problems in the methodology (Mj6nes, 1949). Surveys and twin studies championed by Roger Duvoisin and colleagues were unable to confirm any evidence of familial aggregation, and the conclusion by 1987, in the words of Duvoisin himself, was that "the best available data do not support a role of heredity in the etiology of PD" (Duvoisin, 1987). However, evidence of familial segregation resurfaced again. Dedicated work by Duvoisin and colleagues in Italy and the United States culminated in the confirmation of dominant familial inheritance in 1992 (Duvoisin and Johnson, 1992), the discovery of the chromosomal region by 1996 (Polymeropoulos

et al., 1996), and then finally the isolation of mutations in alpha-synuclein as segregating with the disease in 1997 (Polymeropoulos et al., 1997).

$\alpha$ -synuclein was thus the first PD gene to be identified as the causative agent behind the PARK1 locus contributing to autosomal dominant PD. There are three common missense mutations, A53T (Polymeropoulos et al., 1997), A30P (Kruger et al., 1998), and E46K (Zarranz et al., 2004). Also of importance was the discovery of duplications and triplications of the gene as causative factors (PARK4), which were ironically confirmed in the same kindred described by Mjones in 1949! (Mjones, 1949). A53T and E46K mutations tend to cause a wide spectrum of parkinsonian symptoms ranging from typical PD to DLB. Duplications cause a late onset typical PD while triplications lower the age of onset to 35 years suggesting an important role for dosage in the mechanism of  $\alpha$ -synuclein's toxicity.

Development of  $\alpha$ -synuclein antibodies led to the discovery that  $\alpha$ -synuclein is the major component of LBs and is now the most important marker to identify LBs replacing ubiquitin (Spillantini et al., 1997). The protein product is small and is still of unknown function although is thought to play a role in synaptic vesicle trafficking as it can bind brain vesicles (Jensen et al., 1998).

Parkin mutations, identified in 1998 (Kitada et al., 1998), are the most common cause of autosomal recessive early onset PD. Parkin encodes a small E3-ubiquitin ligase of unknown function. However, recent studies have linked Parkin to the clearing of damaged mitochondria (Darios et al., 2003; Greene et al., 2003). Multiple missense and nonsense mutations and deletions have been found with Parkin (Lucking et al., 2000),

and these account for about 5-10% of early-onset PD cases without family history. Parkin homozygous mutations are of high age-dependent penetrance, and the effects of single heterozygous mutations on late-onset typical PD is still unclear (Gasser, 2009b).

Pink1 (PTEN-induced kinase 1) is the second most common cause of autosomal recessive early-onset PD after Parkin (Valente et al., 2004). It has an unknown function and contains an N-terminal mitochondrial targeting sequence. In elegant *Drosophila* studies, Pink1 has been shown to act upstream of Parkin and to be also involved in mitochondrial dynamics (Clark et al., 2006; Park et al., 2006; Yang et al., 2006; Yang et al., 2008). Interestingly, compound heterozygous Pink1 mutations can be found in sporadic late-onset cases (Gelmetti et al., 2008). DJ-1 was initially discovered as an oncogene and plays different proposed roles in oxidative stress (Yang et al., 2005), chaperone activity, and RNA binding (van der Brug et al., 2008). It's a rare cause of AR-JP (Bonifati et al., 2003; Pankratz et al., 2006).

Many excellent discussions have been written on the genetics of PD (Klein and Schlossmacher, 2006; Klein et al., 2007; Gasser, 2009b, a; Hardy et al., 2009; Schapira, 2009). It suffices to say that there are other weak loci or genes that are thought to associate with or increase susceptibility to PD such as glucocerebrosidase (GBA) (Aharon-Peretz et al., 2004) and UCH-L1 (Wintermeyer et al., 2000). All of these recessive genes and loci contribute predominantly to familial PD and are either already rare causes of PD such as DJ-1 or are very rare in sporadic PD, such as  $\alpha$ -synuclein multiplications (Ahn et al., 2008; Brueggemann et al., 2008; Theuns and Van Broeckhoven, 2008; Troiano et al., 2008). We can thus appreciate the extent of the

excitement generated by the discovery of such common genetic mutations as LRRK2's G2019S, contributing on its own to around 40% of sporadic PD cases in such populations as North African Arabs (Lesage et al., 2006) and Ashkenazi Jews (Ozelius et al., 2006).

LRRK2 mutations were discovered in 2004 to contribute to both familial and apparently sporadic cases of PD. It's a large complex protein and mutations linked to PD have been found in most of its functional domains and in many different populations. It contributes to clinically typical PD albeit with some neuropathological variations. The protein remains to be of unknown function although an overactive kinase domain is thought to contribute to its gain-of-function mechanism. Due to the overwhelming commonality of LRRK2 mutations contributing to late-onset typical PD, we have decided to model its toxicity in *Drosophila* in the attempt of understanding its function. What will follow thus is an overview of its genetics, molecular biology, and proposed pathological mechanisms.

## 1.2 Leucine-rich repeat kinase 2

### 1.2.1 Background

The PARK8 locus was mapped to chromosome 12 in 2002 in a large Japanese family (Funayama et al., 2002), and later confirmed in Caucasian populations (Zimprich et al., 2004a). Efforts were taken to discover the culprit gene in the locus that contained 29 possible genes. The largest and last gene to be sequenced (Figure 1) had different missense mutations and was determined to be the most likely candidate in 2004 (Paisan-Ruiz et al., 2004; Zimprich et al., 2004b). Later studies showed that the Japanese family did indeed carry a LRRK2 mutation which was found to be I2020T (Funayama et al., 2005) just adjacent to the G2019S mutation which is now deemed the most common. Although the gene clearly belonged to the ROCO family of GTPases, it was termed Leucine-Rich Repeat Kinase 2, to adhere to the naming of its only human homolog, LRRK1 which was coined earlier (Biskup and West, 2008).

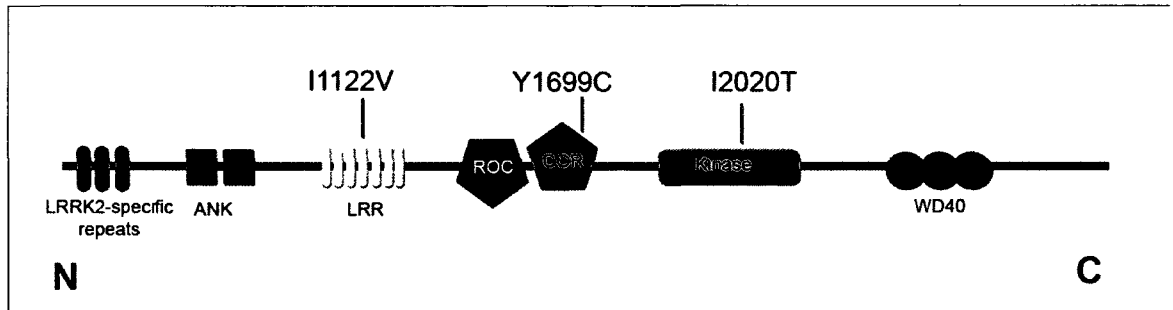


Figure 1. Domains of LRRK2 showing PD-linked mutations. Starting from the N-terminus: a region of LRRK2-specific repeats, Ankryin-like repeats, leucine-rich repeats, ROC GTPase, a C-terminal of ROC (COR) domain, a kinase domain, and a WD40 domain.

## 1.2.2 Epidemiology and Clinical Manifestation

The G2019S mutation alone is the most common genetic cause of both familial and sporadic PD. It is found in 5-7% of familial Caucasian PD cases, and 1-2% of sporadic cases (Gilks et al., 2005; Kachergus et al., 2005). The overall frequency of LRRK2 mutations in Europe is 1.5% of sporadic and 4% of familial PD (Gasser, 2009b). Due to the common founder effect, some population display a very high rate of PD due to the G2019S mutation such as North African Arabs reaching 30-41% in familial PD and 30-39% in apparently sporadic PD (Benamer and de Silva, 2010). The high prevalence in sporadic cases suggests reduced penetrance which is estimated at 35-85% (Klein et al., 2007). The average age of onset for LRRK2 mutations is in the 50s ranging from a rare age of onset in the 20s to late 90s (Healy et al., 2008).

The disease is indistinguishable from typical idiopathic PD although one study scanning for G2019S mutations (Healy et al., 2008), with doubts casted on its methodology (Elbaz, 2008), reported that it follows a slower more benign course with less cognitive problems. R4141G, which was discovered in the Basque region of Spain (Paisan-Ruiz et al., 2004), presented as a tremor dominant disease and for that LRRK2 was initially called dardarin from the Basque word “dardara” meaning tremor. On the other hand, the Japanese mutation I2020T presented with less tremor and more gait disturbances (Hasegawa et al., 2009). The other confirmed pathogenic mutation is Y1699C (Paisan-Ruiz et al., 2004; Zimprich et al., 2004b) and is also rather clinically typical. Some mutations are less clearly causative but are known to be associated with PD

such as I1122V and R1441H (Biskup and West, 2008). In Chinese PD patients, G2385R (Di Fonzo et al., 2006; Farrer et al., 2007) is very common reaching 6-9% versus 2-4% in controls.

### 1.2.3 Biochemistry and Cell Biology

LRRK2 is a large gene comprised of 51 exons and yielding a 7.5 kb long cDNA transcript. The protein is 2527 amino acids with an estimated size of 280 kDa. It exists as a homodimer and possibly a heterodimer (Dachsel et al., 2010). Multiple domains are found in LRRK2; a variable N-terminus region with putative ankyrin-repeats homology, a leucine-rich repeats domain, a Ras-of-Complex GTPase (ROC) domain, a C-terminal of ROC linker domain (COR), a kinase domain, and a C-terminal WD40 domain.

In mouse and rat brains, LRRK2 mRNA was found in the striatum and cortex but not in the SNc. However, LRRK2 protein was found to be ubiquitously expressed in the brain including the nigrostriatal dopamine system. There, it was found highest in the striatum and less in the SNc (Higashi et al., 2007a). In control human brains, however, both LRRK2 mRNA and protein were found in PD-relevant brain regions such as the cerebral cortex, caudate putamen, and the SNc. However, protein levels in the SNc are still considered low (Biskup et al., 2006; Greggio et al., 2006; Higashi et al., 2007b).

On the subcellular level, cell culture and rodent studies have shown LRRK2 to localize to both the somata and dendrite of neurons. It's associated with vesicular and membranous structures such as endosomes, lysosomes, synaptic and Golgi transport

vesicles, the microtubule network, and the mitochondria (Biskup et al., 2006; Greggio et al., 2006; Hatano et al., 2007; Gandhi et al., 2008). Some, but not all, studies have suggested a localization of LRRK2 to LBs (Greggio et al., 2006; Higashi et al., 2007b).

#### 1.2.4 Protein-Protein Interaction domains

Leucine-rich repeats are a common occurrence in the human genome and play an important role in protein-protein interactions (Kobe and Deisenhofer, 1994). I1122V is a mutation found in the leucine-rich repeat domain of LRRK2. The WD40 is another protein-protein interaction domain and it contains the G2385R mutation. This domain is known to interact with lipid rafts in other proteins (McArdle and Hofmann, 2008). It has been shown to be required for the dimerization, autophosphorylation, and consequently neuronal toxicity of LRRK2 (Jorgensen et al., 2009).

#### 1.2.5 The GTPase domain

The GTPase domain of LRRK2 belongs to the Ras-related superfamily of small GTPases and is termed ROC for Ras-of-Complex (Bosgraaf and Van Haastert, 2003). It has most homology to the Rab family. It can bind GTP and GDP *in vitro* although it displays poor GTP-hydrolysis activity (West et al., 2007). When the artificial mutations T1343G and R1398Q are engineered to resemble H-Ras, the hydrolase activity of the GTPase is increased (Ito et al., 2007). GTP $\square$ S, the non-hydrolyzable form of GTP, will

lock the protein in a kinase active state (Smith et al., 2006; Ito et al., 2007; Li et al., 2007; West et al., 2007). Thus, the current accepted model of LRRK2's enzymatic activity is that GTP binding is required for kinase activity and that the GTPase activity will return the kinase to basal levels. Next to the ROC domain is the COR domain (C-terminal of ROC) and together they define the key feature of the ROCO protein family which sometimes, but not always, also contain a kinase domain (Lewis, 2009). The COR domain is also implicated in the formation of the LRRK2 homodimer (Klein et al., 2009).

### 1.2.6 The kinase domain

The kinase domain of LRRK2 was the center of controversy due to resemblance to different subfamilies of human protein kinases. On the broader scale, the kinase domain belongs to the tyrosine kinase-like family which displays serine/threonine activity (Manning et al., 2002). Within this family, LRRK2 most closely resembles MAP Kinase Kinase Kinases (MAPKKK) and most specifically resembles receptor interacting protein kinases (RIPKs). Some reports considered LRRK2 to be of the Mixed Lineage Kinase group (MLKs) (Moore, 2008) but others view it as an offshoot of RIPKs similar to Interleukin 1 receptor activating kinase (IRAK) (Gandhi et al., 2009; Greggio and Cookson, 2009; Dauer and Ho, 2010). The kinase was shown to be an authentic kinase displaying autophosphorylation activity (West et al., 2005; Gloeckner et al., 2006; Greggio et al., 2006; MacLeod et al., 2006; Smith et al., 2006; Greggio et al., 2007; Ito et al., 2007; Jaleel et al., 2007; Li et al., 2007; West et al., 2007; Greggio et al., 2008; Imai

et al., 2008) and phosphorylating the synthetic myelin basic protein (MBP) (West et al., 2005; Luzon-Toro et al., 2007).

### 1.2.7 Biological Function

Some insights now give LRRK2 a broad role in synaptic vesicle endocytosis (Shin et al., 2008), sorting of vesicles between axons and dendrites (Sakaguchi-Nakashima et al., 2007), neurite morphology (MacLeod et al., 2006), and neuronal survival in oxidative stress (Imai et al., 2008). However, our limited knowledge of its phosphorylation substrates and interacting partners mostly comes from *in vitro* studies and much doubt can still be cast on the physiological relevance of their conclusions.

LRRK2 was shown to be an authentic kinase by autophosphorylation assays although the physiological relevance of this activity is not yet known. Several sites have however been found *in vitro* mostly in the ROC domain suggesting complex internal regulation (Gloeckner et al., 2010). Another assay method using MBP as substrate suggests that LRRK2 is a serine/threonine-directed kinase (West et al., 2005). The first substrate candidate to be described is moesin (Jaleel et al., 2007), which is part of a group of proteins termed ERM for ezrin/radixin/moesin. Moesin has an actin-binding site, and ERM proteins function to link the cytoskeleton to the plasma membrane thus influencing cell surface dynamics and process morphology and growth. LRRK2 was found to efficiently phosphorylate moesin in the test tube at Thr558, a previously-identified *in vivo* phosphorylation site that regulates actin binding. However in a later study, the same researchers were unable to induce ERM phosphorylation in HEK-293 cells casting doubt

on the physiological relevance of the finding (Nichols et al., 2009). Another substrate was however shown to be phosphorylated by LRRK2 *in vitro* and *in vivo*. 4E-BP, which stands for eIF4E-binding protein, was shown to be phosphorylated at two sites by LRRK2 and its *Drosophila* homolog dLRRK in *Drosophila* (Imai et al., 2008). 4E-BP is a repressor of protein translation implicated in mediating survival in the face of various stresses. Hyper-phosphorylation will suppress 4E-BP dissociating it from eIF4E and allowing for 5' Cap-dependent translation. This deregulated protein translation is a possible mechanism for the LRRK2-induced DA cell loss and hypersensitivity to oxidative stress in *Drosophila*. A more recent study however considered 4E-BP a weak substrate of LRRK2 phosphorylation as compared to autophosphorylation (Kumar et al.). LRRK2 overexpression was unable to significantly phosphorylate 4E-BP in HEK-293 cells, while another kinase, MAPK14/P38alpha was able to do so more efficiently (Kumar et al.). Thus to this date, uncertainty still looms and all LRRK2 phosphorylation substrates can only be considered provisional.

Some interacting partners have also been identified. LRRK2 was shown to interact with the RING2 domain of Parkin (Smith et al., 2005), with Rab5b from a yeast two-hybrid screen (Shin et al., 2008), and with alpha and beta tubulin (Gandhi et al., 2008). Inhibition of Hsp90 causes proteosomal degradation of LRRK2 mediated by CHIP (Wang et al., 2008b; Ding and Goldberg, 2009). Also, independent of GTP binding or kinase activity, LRRK2 was shown to promote ERK1/2 and ERK5 phosphorylation (West et al., 2007). While these studies are supportive of a general role of LRRK2 in neuronal maintenance, other studies found either no role for LRRK2 in neuronal survival (Wang et al., 2008a), or an unexpected protective function both *in vivo* (Saha et al., 2009)

and *in vitro* (Ohta et al., 2010). The following section will attempt to understand the discrepancy in the literature and to define the pathological mode of action for LRRK2 mutations, while examining the currently available *in vivo* models.

### 1.2.8 Pathological Function

The neuropathology of LRRK2 was considered more variable initially but later considered to confine to nigral degeneration, with LBD in most but not all cases. Four autopsies from members of the same family carrying the R1441C mutation (Zimprich et al., 2004b) revealed three different pathologies. These ranged from no distinct pathology, to LBD or diffuse LBD pathology, to phosphorylated tau with neurofibrillary tangles and an absence of LBD. G2019S conforms to typical PD pathology with LBs in most (Ross et al., 2006) but not all (Gaig et al., 2007) cases. Combined with the low penetrance of LRRK2 mutations, this highlights the importance of other contributing factors to disease pathogenesis and the commonality between the different neurodegenerative diseases.

In terms of molecular biology and starting with the GTPase domain, R1441C/G mutations are thought to lower GTPase activity as compared to wild type (Lewis et al., 2007; Li et al., 2007). Y1699C of the COR domain has not been directly tested, but an equivalent mutation in a prokaryotic homolog accomplished a similar result (Gotthardt et al., 2008). The controversy mostly lies with the kinase activity. All LRRK2 mutations were first thought to increase kinase activity however only G2019S is certain to do that (West et al., 2005; Greggio et al., 2006; MacLeod et al., 2006; Smith et al., 2006). For example, I2020T was shown to increase (West et al., 2007), slightly decrease (Jaleel et

al., 2007), or bear no effect (Luzon-Toro et al., 2007) on kinase activity. However I2020T was more prone to degradation *in vitro* which might account for the variability in measurements (Ohta et al., 2010). A similar controversy exists for the effects of the ROC/COR mutations on kinase activity (West et al., 2005; Greggio et al., 2006; MacLeod et al., 2006; Smith et al., 2006; Greggio et al., 2007; Jaleel et al., 2007; West et al., 2007). However, a genetically engineered mutation that blocks GTP and GDP binding lowered G2019S kinase activity and consequently cellular toxicity (Smith et al., 2006). Thus, the generally accepted idea is that G2019S increases kinase activity, and the ROC mutants decrease GTPase activity, with the effect on kinase activity being dependent on the presence of GTP/GDP in the assay system or the cellular context.

Interestingly, regardless of the apparent differences in kinase activity, all LRRK2 mutations lower cell viability equally and more than wild type (Greggio and Cookson, 2009). Kinase dead mutants can still cause toxicity but significantly less than kinase active ones (Greggio et al., 2006; Smith et al., 2006). This still suggests an important role for the kinase activity in LRRK2's proposed dominant gain-of-function mechanism. Mutations outside the kinase domain might alter protein stability, and interacting partners that are not phosphorylation substrates, and thus can have a different mechanism of action. The proposed toxic gain-of-function has been demonstrated in different experiments; cell death in primary cultured neurons or SH-SY5Y cells (Smith et al., 2005; Greggio et al., 2006; MacLeod et al., 2006; Smith et al., 2006; West et al., 2007), shorter neurite process morphology (MacLeod et al., 2006; Plowey et al., 2008), apoptosis by either the caspase 3 (Iaccarino et al., 2007), or caspase 8 pathway (Ho et al.,

2009), TUNEL-positive labelling suggesting either apoptosis or necrosis (Smith et al., 2005), and autophagy (MacLeod et al., 2006; Plowey et al., 2008).

Yet, a dominant-negative or haploinsufficiency mechanism is not excluded. For example, wild-type LRRK2 has been shown to protect cells under oxidative stress and this protection is abolished by the I2020T mutant. Prevention of I2020T degradation can restore this protection (Ohta et al., 2010). A *C. elegans* model has shown that even though G2019S expression reduced DA markers in the adult, overexpression of wild type, G2019S, R1441C, or kinase dead LRRK2 protected the nematode under oxidative stress (Saha et al., 2009). Knock down of the *C. elegans* homolog LRK-1 reduced survival which was associated with mitochondrial dysfunction (Saha et al., 2009). A more recent zebrafish model also demonstrated a role for LRRK2 in survival. Loss of zLRRK2 caused embryonic lethality, while loss of the WD40 domain caused a PD-like phenotype with loss of DA neurons and locomotor deficits (Sheng et al., 2010). This comes opposite to a previously proposed role for WD40 as being required for G2019S/R1441C toxicity (Jorgensen et al., 2009). In *Drosophila*, a dLRRK construct lacking the kinase and the WD40 domains was found to cause shrunken DA neurons and locomotor deficits in one study (Lee et al., 2007). Another two studies (Imai et al., 2008; Wang et al., 2008a) were not able to reproduce these results. And while one (Wang et al., 2008a) found preferential sensitivity to general oxidative stress by H<sub>2</sub>O<sub>2</sub>, and not DA-specific oxidative stress induced by rotenone or paraquat, the other found that LRRK-null flies were more resistant to oxidative stress. Rodent models thus far have not been able to provide a satisfactory recapitulation of cardinal PD signs. Two studies using two different ROC mutants reported no loss of DA neurons in mice, but only mild defects in

DA neurotransmission and locomotion (Li et al., 2010; Tong et al., 2010). A LRRK2 mouse model lacking the kinase domain also presented with a normal DA system and no sensitivity to MPTP (Andres-Mateos et al., 2009).

The above *in vivo* models suggest either a dispensable or mildly protective role for LRRK2 in DA neuronal survival which is opposite to expectations for a dominant disease gene. Fortunately, other invertebrate models provide a different perspective. A more recent *C. elegans* study found age-dependent DA neuronal loss with R1441C and G2019S mutants, with behavioral and locomotor deficits (Yao et al., 2010). These effects were rescued by a knock down of LRK-1 or the non-GTP binding mutant K1347A. In *Drosophila*, both wild type and G2019S LRRK2 were shown to cause DA neuronal loss and behavioral abnormalities in one study (Liu et al., 2008). Another study that used PD-pathogenic dLRRK mutants also demonstrated DA neuron loss (Imai et al., 2008). Finally, a new mouse model presented accelerated  $\alpha$ -synuclein-mediated neurodegeneration by LRRK2 overexpression independent of the kinase domain (Lin et al., 2009). This phenotype was ameliorated by LRRK2 deletion. LRRK2 overexpression on its own, mutant or not, did not cause neurodegeneration. However the fact that the cell loss was limited to cortical and striatal forebrain regions, limits the model's relevance to PD pathogenesis.

In light of the above review, it is evident that although LRRK2 seems to have a pathological function in DA neuron survival- especially under oxidative stress- much is still unknown about the physiological and pathological substrates and interacting partners of this large protein. Controversy still exists about the outcome and role of mutations

such as I2020T, and Y1699C. There is also no current work, as far as we know, that attempts to study the role of the leucine-rich repeat region via looking at its mutations. For this reason, we designed a *Drosophila* model of LRRK2 that attempts to fill some of the gaps in our knowledge regarding these LRRK2 mutations, namely I1122V of the LRR domain, Y1699C of the COR domain, and I2020T of the kinase domain. We have also successfully used this model to discover potential modifiers of our LRRK2-induced phenotype, and we hope that this would result in the discovery of relevant and authentic LRRK2 substrates or interacting proteins. But before we present our work, it is important to examine the rationale and methodology behind the use of *Drosophila* as a model organism for the study of neurodegenerative diseases, and particularly Parkinson's disease.

## 1.3 *Drosophila melanogaster*

### 1.3.1 Introduction

Thomas Hunt Morgan is credited as the father of the field of *Drosophila* genetics. In 1910 he started the first lab at Columbia University and made the first genetic discovery; discovering that *white* gene resulted in loss of *Drosophila*'s red eye color and that it's located on the X chromosome (Morgan, 1910). Ironically and on the other side of the Atlantic, Friederich Lewy was publishing his work on the pathologic anatomy of Parkinson's disease (Holdorff, 2002). Would these men have predicted the convergence of their seemingly distant discoveries some hundred years later? The answer is left for us to ponder. But since then, *Drosophila*, also named the fruit fly and the vinegar fly, has become the most commonly used organism in genetic studies, and an invaluable tool for the study for neurodegenerative disease.

Multiple factors amount to the fruit fly's value. Its small size and simple habitat result in ease of use and storage and lower costs. It has a short life cycle resulting in large numbers of progeny within two weeks. The *Drosophila* has only 3 pairs of autosomal chromosomes (named 2, 3 and 4) and a pair of sex chromosomes. Effectively however, only chromosomes 2 and 3 and the X chromosome are of importance since the fourth chromosome and the Y chromosome are extremely small. This, along with the fact that female virgins are very easy to identify, make genetic crossing and manipulation much easier. Currently, the availability of the genome since the year 2000 (Adams et al., 2000),

and an array of molecular biology tools that facilitate the creation of transgenic mutants, all epitomize the value of *Drosophila* as a model organism.

### 1.3.2 P-Element Transposons and *Drosophila* transformation

P-Elements (Bingham et al., 1982) are highly mobile small pieces of DNA that are thought to have been originally foreign to *D. melanogaster*. Their primary transcript is a transposase enzyme surrounded by small inverted repeats. When a male of a fruit fly strain carrying a P element is crossed with a female lacking P elements, this P element will become highly mobile in the germline cells of the F1 causing mutations and chromosomal breakage and rearrangements. This will reduce the fertility of the F1 progeny and the few surviving progeny will carry many mutations and exhibit many phenotypes.

Scientists realized the potential benefit of P elements and created a system in which the transposase gene was isolated in a separate vector termed the Helper plasmid, and the inverted repeats surround cDNA for a gene of interest in another plasmid that is the Transformation plasmid. The Helper plasmid cannot continue to multiply in the genome as it lacks the inverted repeats, and thus only the foreign gene of interest will be incorporated.

On the other hand, perhaps one of the most useful features for *Drosophila* geneticists and particularly in the field of neurodegeneration is the large red compound

eye. Morgan's discovery of the *white* gene provided a very useful genetic tool that parallels the use of Ampicillin resistance in *E. Coli*. This mutation causes the flies to have white eyes. A wild type copy of the *white* gene is usually cloned into the Transformation plasmid along with the transgene, and the construct, along with the Helper plasmid, are injected into the germline of  $w^-$  (white eyed) embryos (Rubin and Spradling, 1982). By reintroducing the red eye color, an easily visible marker, geneticists can identify lines in which incorporation of the transgene has been theoretically successful.

### 1.3.3 Ectopic gene expression

The GAL4/UAS bipartite expression system (Phelps and Brand, 1998) is also based on P elements and allows for spatial/temporal ectopic expression of the gene of interest. A "Driver" fly line will contain the GAL4 gene which is a yeast-only transcription factor under the control of tissue-specific promoters. These promoters are under endogenous control in the fly thus permitting tissue-specific expression of GAL4. The "Reporter" fly will contain an Upstream Activation Sequence (UAS) controlling the expression of the transgene of interest. When the two fly lines are crossed, GAL4 will drive the expression of the target gene by activating UAS in specific tissue or cell subtypes. This means that a potentially toxic human gene will be dormant and harmless in the fly until the researcher chooses to drive its expression using the large arsenal of Driver fly lines available. These UAS constructs will also contain a copy of the *white* gene for the purpose explained above.

This however does not guarantee successful expression of the target gene as faulty placement into the genome can happen. This is where the eyes also come in handy. Instead of painstakingly analyzing tens of different fly lines by means of Western blotting for protein expression, or performing brain dissections for DA neuron loss for example, the target gene is initially driven in the eyes. If a transgene is indeed working in a dominant toxic gain-of-function manner, then an initial hypothesis would be that its overexpression will cause retinal degeneration, or stated more broadly, eye defects. These eye defects could be easily observed under the optical microscope and can manifest as different phenotypes; pigmentation loss, surface roughness, size reduction, loss of interommatidial bristles, surface holes, black surface lesions, or even lethality. They can also manifest as loss of rhabdomeres within the ommatidia which might only be detectable upon sectioning of the eye. Thus, flies that manifest an eye phenotype upon expression of a potentially toxic gene are considered candidates for further analysis such as DA neuron loss, mitochondrial defects, locomotor deficits, etc.

#### 1.3.4 Chromosomal rearrangements and gene knockout

Before the discovery of P elements, geneticists used X ray radiation and chemicals to cause chromosomal breakage and rearrangements. These sometimes resulted in useful chromosomes termed Balancer Chromosomes. These chromosomes have multiple overlapping inversions that prevent crossovers between themselves and a normal chromosome. They also frequently display a dominant marker that could be visually

traced, and importantly a recessive lethal allele. Thus, these become invaluable stock keeping tools. When a fly has incorporated a recessive lethal gene either via transformation or P-element disruptions, geneticists can keep a healthy stock by introducing a Balancer chromosome that carries a different recessive lethal gene. Thus, the stock will be kept in the viable heterozygous state. Some useful body markers for Balancer chromosomes are used in our study such as *Cyo* which results in Curly wings.

Other than creating Balancer chromosomes, X ray radiation can also result in deletions of large segments of the genome. As long as deleted regions did not include haploinsufficient genes, i.e. genes that are lethal in single allele dosage, the flies could be kept in stock with Balancer chromosomes. One particular *Drosophila* feature made this phenomenon even more useful. Larval salivary glands contain gigantic chromosomes that have undergone 10-11 replications without cell division. These Polytene chromatids are perfectly aligned with each other and display patterns of dark and light bands. These bands provided scientists with a readily available detailed map of the genome! Thus, when deletions or chromosomal arrangements were made, it was possible to map their location precisely. Libraries of these deletions, termed Deficiencies, are thus kept and cataloged.

Using X ray radiation was useful before the discovery of P elements. However since then, new approaches have been taken to produce more precise large deletions or single gene silencing and disruptions. Three methods could be used: Transposon-mediated mutagenesis and single gene disruptions (Thibault et al., 2004), transgenic RNA interference (RNAi) (Kondo et al., 2006), and gene knockout by homologous

recombination (Parks et al., 2004). These all share the same basic technique with small important differences. For example, RNAi silencing employs two inverted cDNA sequences surrounded by the inverted P element repeats and separated by a small linker region. When this DNA is expressed under the control of a UAS promoter, the resulting mRNA will snap back at the linker region resulting in double stranded RNA which will degrade the endogenous mRNA signal of the targeted gene. Recombination-based mutagenesis or knockout uses FRT flanking repeats that are recognized by FLP recombinase, and the cDNA carrying the mutation will be excised, linearized by a different enzyme, and then recombine with and replace the endogenous gene. Similar to Deficiency libraries then, libraries of RNAi lines and single gene knockouts are available for use and when combined with ectopic overexpression techniques and *Drosophila's* short life span, will provide to our disposal very valuable and efficient tools for the study of human disease.

### 1.3.5 Modifier screens

The final role that the *Drosophila* eye can play in disease modeling is the modifier screen. After establishing a disease model, researchers can use the eye phenotype to quickly discover *in vivo* modifiers. A modifier is a drug or a gene that can either suppress or enhance the phenotype established in the model. An ideal phenotype is thus one that is both easy to score, and moderate enough to allow both suppression and enhancement. As mentioned above, multiple libraries are available to perform these modifier screens.

Deficiency libraries are the most common first step because they encompass larger quantities of deleted genes. If a modification arises, the researcher can then look into individual genes within the region by means of lines of smaller deficiency regions, single gene disruptions, or targeted gene silencing by RNAi. This method can detect only dominant alleles as only one allele is deleted in the deficiency. Also, only genes that are not haploinsufficient can be included in such deficiency or deletion libraries. Thus, if a deleted allele of gene *A* is found to cause enhancement of a phenotype, it could be hypothesized that overexpression or reintroduction of gene *A* can downregulate the toxicity of the original transgene. Researchers can then study the effects of this interaction in other cells that are otherwise harder to employ in a first-step modifier screen. For example, the DA system in the case of PD, and mammalian systems in later stages. This provides a quick method to help discover new pathways of neurodegeneration or refine already known ones in the hope of developing future disease therapeutics.

### 1.3.6 Modeling Neurodegenerative diseases

Using *Drosophila*'s array of tools, geneticists have been able to study many human diseases and in particular neurodegenerative diseases such as Alzheimer's disease (AD), Polyglutamine diseases, Prion diseases, Amyotrophic lateral sclerosis, and Parkinson's disease. Amongst many things, *Drosophila* has contributed to our knowledge of Tau phosphorylating kinases (Shulman and Feany, 2003; Nishimura et al., 2004),

mechanisms of A $\beta$  peptide-induced amyloid plaque formation in AD (Iijima et al., 2004), and new genetic factors involved in polyglutamine disease (Fernandez-Funez et al., 2000). The eye phenotype, for example, has been used successfully in large modifier screens in polyglutamine disease models.

Four general strategies are employed to design models of neurodegeneration. The first one is forward genetic screens, in which flies with a phenotype are identified first, and then the gene responsible is isolated. The second strategy concerns mutant neurodegenerative disease genes working in a dominant fashion such as  $\alpha$ -synuclein and LRRK2. These are modeled by transgenic overexpression of the mutant protein, for example by using the GAL4/UAS system. Recessive disease genes such as Parkin and Pink1 are assumed to work in a loss-of-function fashion and thus they are modeled by disruption of the endogenous protein by the methods outlined earlier. The fourth strategy is using pharmacological agents which are readily delivered to the brain due to the lack of a stringent blood-brain barrier and could be easily mixed with the fly food (Lu and Vogel, 2009).

*Drosophila* is an ideal model organism for the study of Parkinson's disease. It contains well defined DA clusters (Nassel and Elekes, 1992; Mao and Davis, 2009) that could be visualized by immunostaining the Tyrosine hydroxylase (TH) enzyme, the rate limiting enzyme behind the production of Dopamine. Pharmacological intervention by toxic Parkinsonian drugs such as rotenone, H<sub>2</sub>O<sub>2</sub>, and paraquat, or by L-Dopa can be utilized to test exacerbation or rescue. Locomotor activity could also be assessed in *Drosophila* by means of its climbing ability since the flies will always aspire to climb to

the top of their housing vial, a phenomena termed startle-induced negative geotaxis (Rhodenizer et al., 2008).

Excellent reviews have been written about the modeling of PD in *Drosophila* (Muqit and Feany, 2002; Botella et al., 2009; Lu, 2009; Lu and Vogel, 2009). Fly models of all PD disease genes have demonstrated at least some if not simultaneously all hallmark PD features including DA cell loss, locomotor deficits, cellular inclusions, and a response to L-Dopa. Other phenotypes have also been studied such as defective mitochondria, eye defects, and sensitivity to oxidative stress. Novel interactions between different PD genes have been demonstrated in *Drosophila*, as well as new mechanistic pathways. A more thorough discussion of the different *Drosophila* PD models and how they compare with our model will be presented in Chapter 4.

## 1.4 Rationale and study limitations

Parkinson's disease has been identified almost two hundred years ago with still no cure in sight. The discovery of a genetic cause of such a magnitude as that of *LRRK2* has revived our hope of finding a solution by attempting to learn the pathways of pathogenesis. With this in mind, our work provides a viable model of uncovering *LRRK2*'s complex biological and pathological functions by examining novel mutations and interactions. The study aims to discover targets for therapeutic intervention for PD. It is for this reason that we believe our work is novel and is worthy of pursuit.

Other than the reasons outlined above for our choice of *Drosophila* as a PD animal model, it's important to point out that no rodent animal model has thus far been successful at recapitulating key Parkinsonian features as important and central as dopamine neuron loss (Betarbet et al., 2002; Melrose et al., 2006; Betarbet and Greenamyre, 2007; Harvey et al., 2008). However there are clear and understandable limitations for the use of invertebrates as opposed to vertebrates for the modeling of human disease. Obvious reasons include the inability to model more complex symptoms such as drug-induced dyskinesias, complex aspects of the motor symptoms, or non-motor and cognitive symptoms. 70% of human disease loci are estimated to have clear *Drosophila* orthologs (Reiter et al., 2001), and while this is an encouraging figure, it does present its limitation such as the lack of an obvious  $\alpha$ -synuclein fly ortholog. Finally, drugs tested in *Drosophila* might be unable to cross the human blood brain barrier. Yet,

the fact that such a simple organism can capture the most intricate details of a complex human disorder should warrant it our study and consideration.

The second chapter will guide you through the design and characteristics of our fly model. Here, we used to the GAL4-UAS system to drive the expression of 4 different LRRK2 constructs (Wild type, I1122V, Y1699C, and I2020T) in different tissues. We demonstrate eye defects, DA neuron loss, complex locomotor alteration, unexpected increase of basal life span, sensitivity to oxidative stress, and complex interaction with the recessive PD genes. In the third chapter we demonstrate the suitability of this model by performing a modifier screening and uncovering regions of reactivity in the eye. These regions of reactivity present an exciting opportunity to learn about interactors and substrates of LRRK2, so that perhaps we can develop a clearer and more thorough understanding of the mechanism of Parkinson's disease.

## Chapter 2 – Manuscript

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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## 2.1 Author Contributions

Ghassan Kabbach's contributions: Helped design the experiments, mapped and balanced LRRK2 stocks, and created double transgenic lines by genetic crossing. Performed experiments for Figure 2, Figure 9, Table 3, Figure 10, Figure 11, Figure 12, Figure 13, Table 4, Figure 14, and Took pictures of Figure 3. Performed I2020T screen at 25°C and 29°C (Table 5), took pictures for Figure 15 (Chapter 3), and designed Figure 1 (Intro)

Katerina Venderova's contributions: Helped design the experiments, wrote the manuscript, performed all statistical analysis and prepared all figures in the manuscript. Performed experiments for Figure 4, Figure 6, Figure 7, Figure 8, Table 2. Analyzed images for Figure 11, Figure 12, Figure 14.

Elizabeth Abdel-Messih's contributions: Assisted in maintenance of all stocks, and in preparation of almost all experiments. Performed initial I1122V screen and identified Deficiency X (Figure 15, Chapter 3).

M. Emdadul Haque's contributions: Prepared pUAST LRRK2 constructs for microinjection. Helped map and balance stocks, and design experiments.

Yi Zhang's contributions: Performed experiments for Figure 5.

Randy Ringuette's contributions (Acknowledgments): Assisted in preparation of some experiments. Performed experiments for Figure 3.

Douglas Holmyard's contributions (Acknowledgments): Performed Scanning Electron Microscopy on fly samples for Figure 9, Figure 11, Figure 12, and Figure 14.

Louise Pelletier's contributions (Acknowledgments): Performed eye sections for Figure 9

## 2.2 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.

## 2.3 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 Parkinson' disease, 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* (Smith et al., 2005; MacLeod et al., 2006; 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 fulllength 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) (Lee et al., 2007; Imai et al., 2008) or human LRRK2 (hLRRK2) (Liu et al.,

2008). However, the reported neurochemical and behavioral 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 others 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.

## 2.4 Results

### 2.4.1 Generation of LRRK2 transgenic lines

We first generated WT and mutant LRRK2 transgenic flies by microinjecting a UAS-hLRRK2-containing vector into w<sup>1118</sup> 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. 2A). 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/ϕ fly (Fig. 2B). We thus concluded that all of our lines express the LRRK2 transgenes.

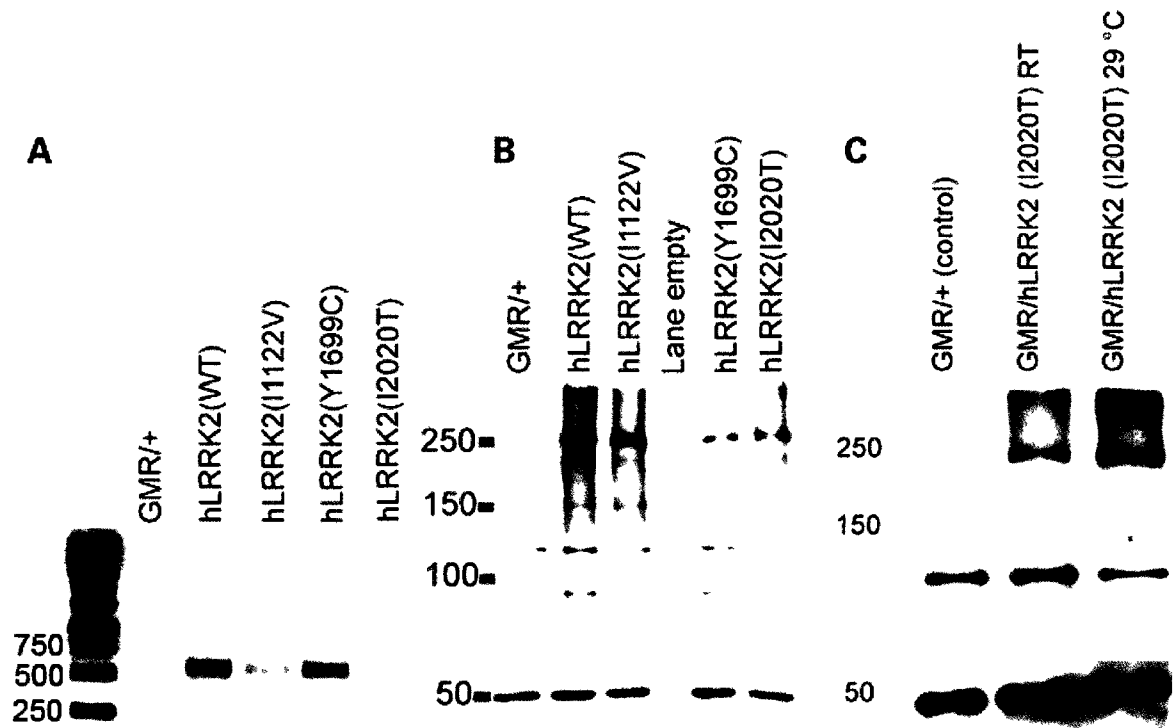


Figure 2. Expression of hLRRK2 in *Drosophila*. (A) RT-PCR of all lines expressing hLRRK2 under Da promoter; (B) western blot from heads expressing hLRRK2 in GMR fashion. The band above the 250 kDa marker corresponds to hLRRK2.  $\beta$ -tubulin is a loading control. (C) Effect of temperature on LRRK2 expression levels under the UAS-Gal4 expression system. Western blot from heads expressing hLRRK2 in GMR fashion.  $\beta$ -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 with controls (Fig. 3A and B). Accordingly, gross development appeared normal with flies expressing hLRRK2(I2020T).

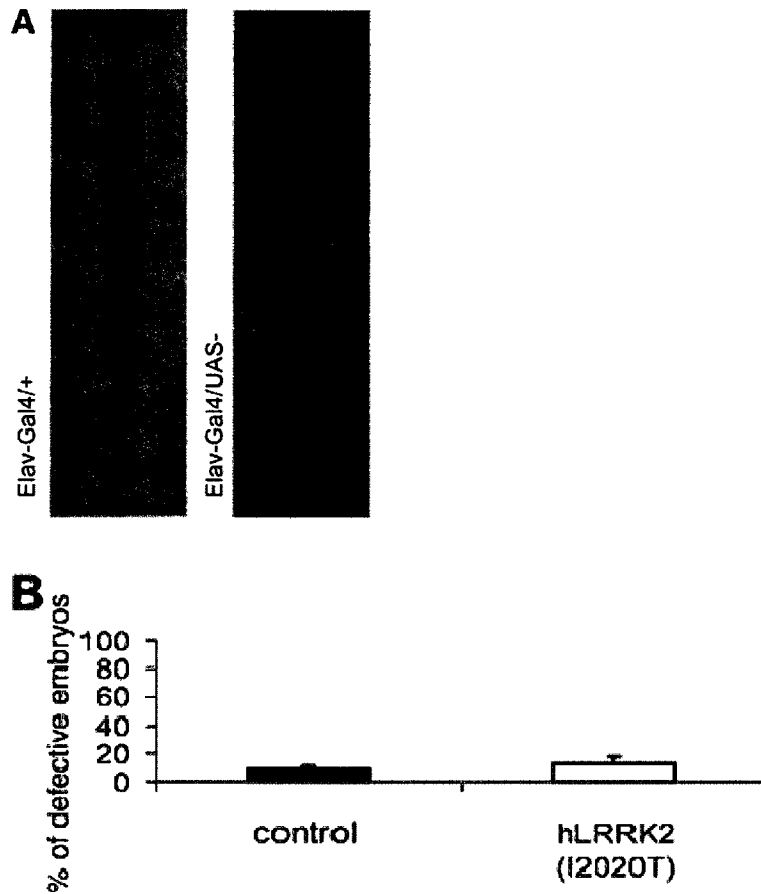


Figure 3. hLRRK2 expression has no effect on axonal growth in embryonic development.

(A) hLRRK2 (I2020T) expression does not affect axonal growth in embryonic development. Representative images of control (Elav/p) 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.

## 2.4.2 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. 4E). 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. 4B 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. 4A 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. 4C). 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. 2C). 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. 4D).

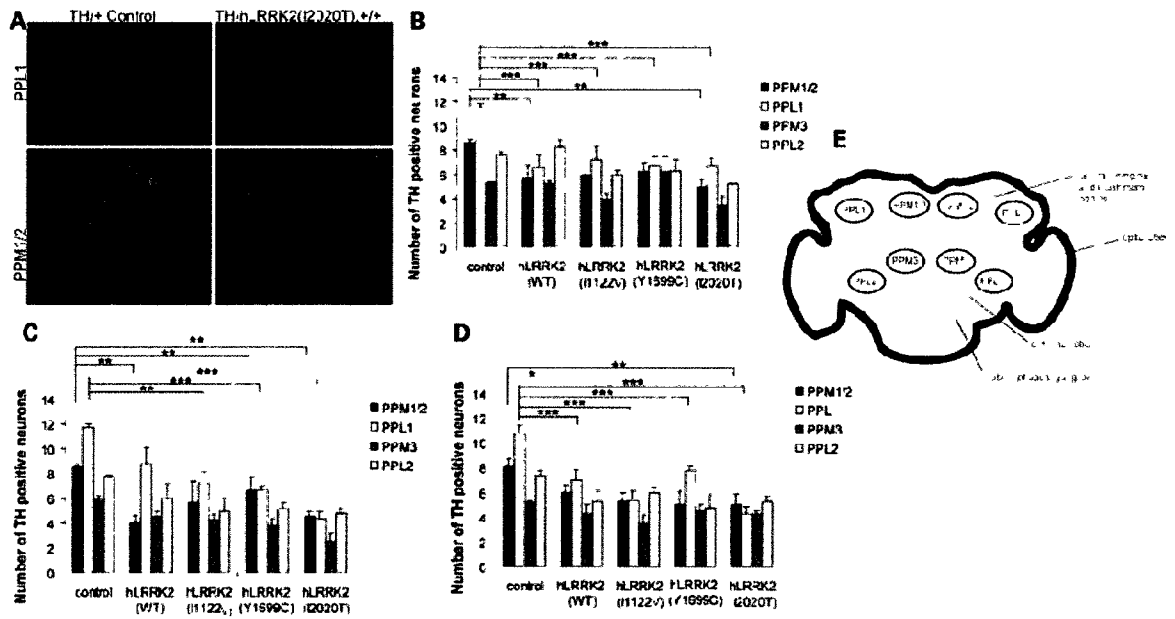


Figure 4. hLRRK2 expression causes loss of dopaminergic neurons. (A) Representative images of TH staining in PPL1 and PPM1/2 DA clusters in 50-day-old control and TH-Gal4/UAS-hLRRK2(I2020T) flies maintained at room temperature. (B) Graph summarizing the findings above. (C) The effect of hLRRK2 expression 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.

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(R144C) 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 with 4.90±1.17% in lacZ control) (Fig. 5).

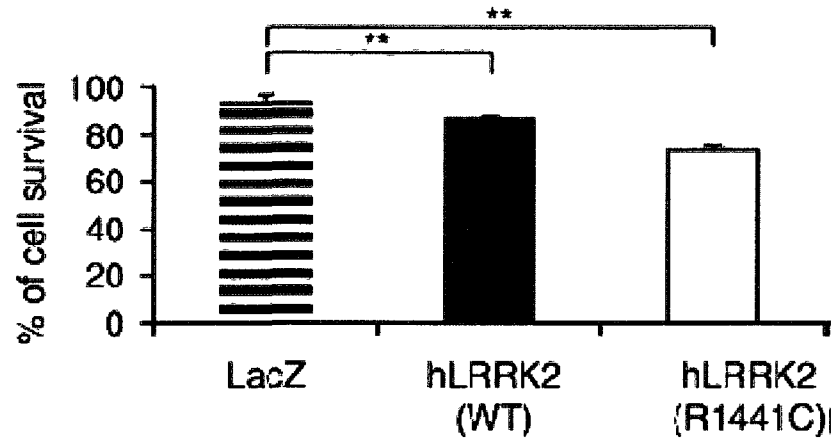


Figure 5. Expression of WT or mutant hLRRK2 kills primary cortical neurons.  $n = 3$ , the experiments were performed in triplicates. One-way ANOVA followed by Dunnett's post-test.

### 2.4.3 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 with 62.0–88.0% at 10 days of age (Fig. 6A). 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. 6A). 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. 6B). In comparison, the 10-day-old hLRRK2 flies were less able to climb up the vial within 10 s compared with controls (Fig. 6A); this difference was smaller when observing the same flies for 20 s (Fig. 6B). 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. 6A and C). A very similar pattern of initial depression of movement 30 and 50 day 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. 6C). The implications of this finding, particularly in relation to loss of dopaminergic neurons, are discussed further below.

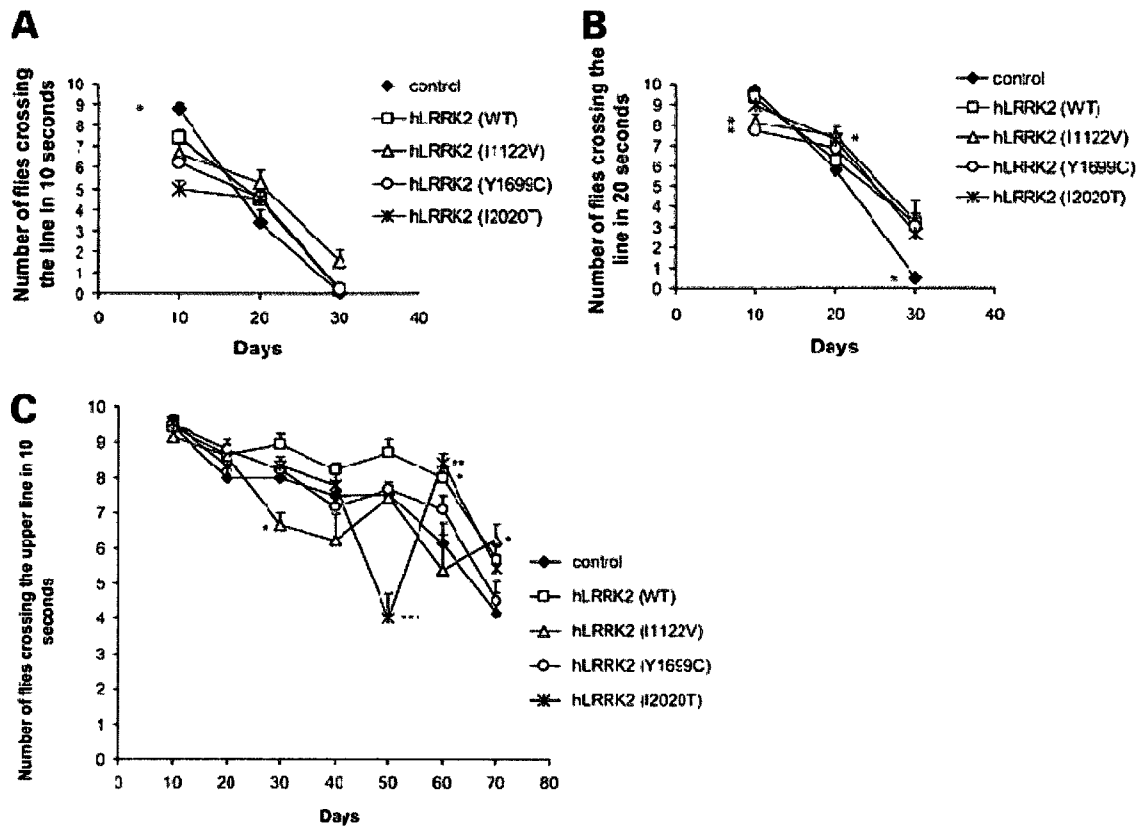


Figure 6. hLRRK2 expression causes alterations in locomotor activity. Climbing behavior of TH-Gal4/UAS-hLRRK2(WT), TH-Gal4/UAS-hLRRK2(I1122V), TH-Gal4/p;UAS-hLRRK2(Y1699C)/p and TH-Gal4/UAS-hLRRK2(I2020T) flies raised and maintained at 29°C and recorded for 10 s (A) r 20 s (B). n = 4–7 sets of 10 for 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 s post-test. Asterisks next to control signify that all genotypes were significantly different from control.

#### 2.4.4 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. 7A; Table 1). The hLRRK2(I1122V) mutant was not significantly different from control.

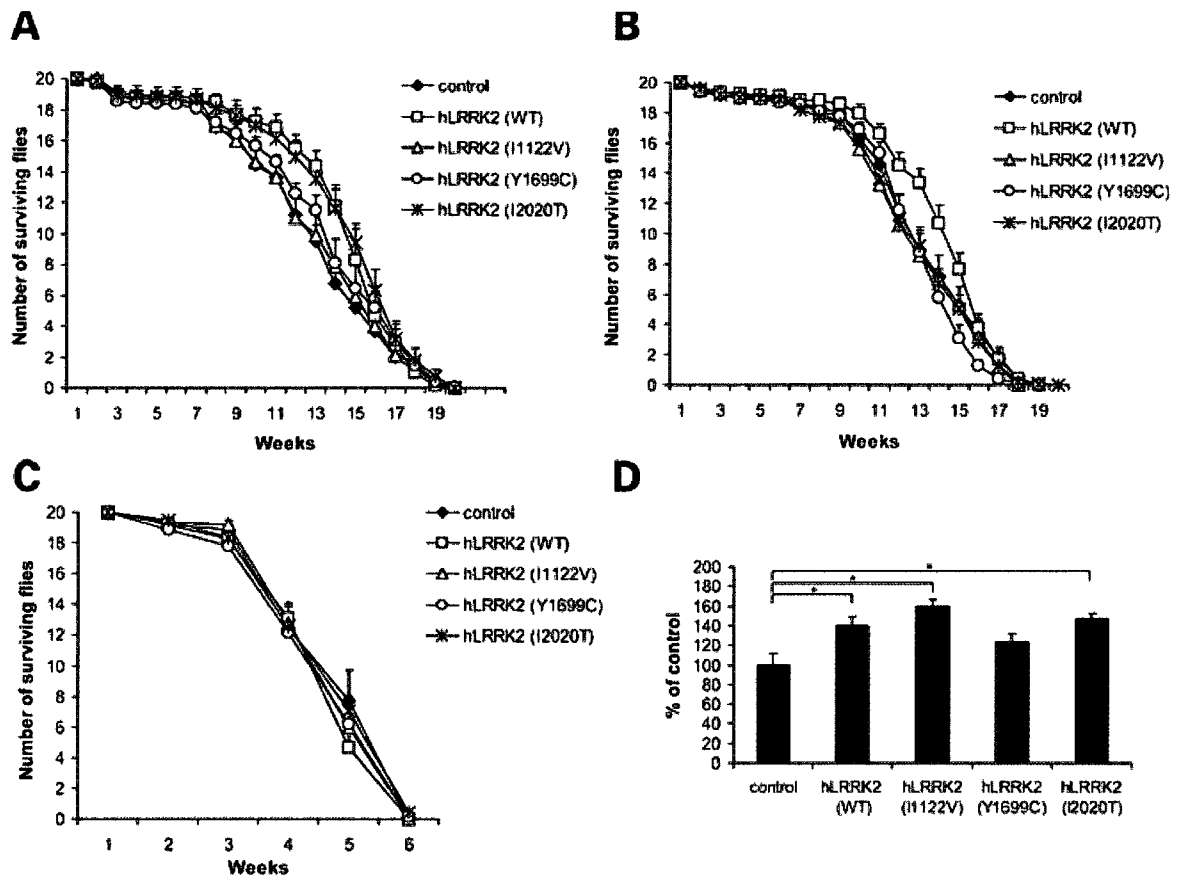


Figure 7. hLRRK2 expression affects the lifespan and the number of progeny. (A) Effect of ubiquitously expressed hLRRK2 on the lifespan at room temperature.  $n = 10\text{--}17$  sets of 20 per genotype. (B) Effect of pan-neuronally expressed hLRRK2 on lifespan at room temperature ( $n = 20\text{--}32$  sets of 20 per genotype). (C) Effect of pan-neuronally expressed hLRRK2 on lifespan at  $29^{\circ}\text{C}$  ( $n = 10$  sets of 20 per genotype). (D) hLRRK2 expression increases the number of progeny.  $n = 5\text{--}8$  sets of parents ubiquitously expressing hLRRK2. One-way ANOVA, Bonferoni's post-test.

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 panneuronal hLRRK2(WT) expression significantly increased lifespan at room temperature (Fig. 7B; Table 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. 7C). 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. 7D). 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 with control (data not shown). The experiment was carefully standardized and controlled, as crowding may have a profound effect in this type of experiment.

Table 1. hLRRK2 expression increases basal fly lifespan at RT

Genotype	50% survival (weeks)	95% CI	<i>P</i> -value
Da-Gal4/+	13	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	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	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	12.6–13.4	0.7223 (n.s.)

Summary of the effects of hLRRK2 expression on basal fly lifespan at room temperature.

The data were analyzed by nonlinear regression analysis.

Many of the identified PD genes modulate sensitivity to reactive oxygen species. In this regard, LRRK2 has been shown to increase sensitivity to H<sub>2</sub>O<sub>2</sub> 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 nM) at room temperature rendered all flies ubiquitously expressing hLRRK2 significantly more susceptible to this toxin compared with control (Fig. 8A; Table 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. 8B).

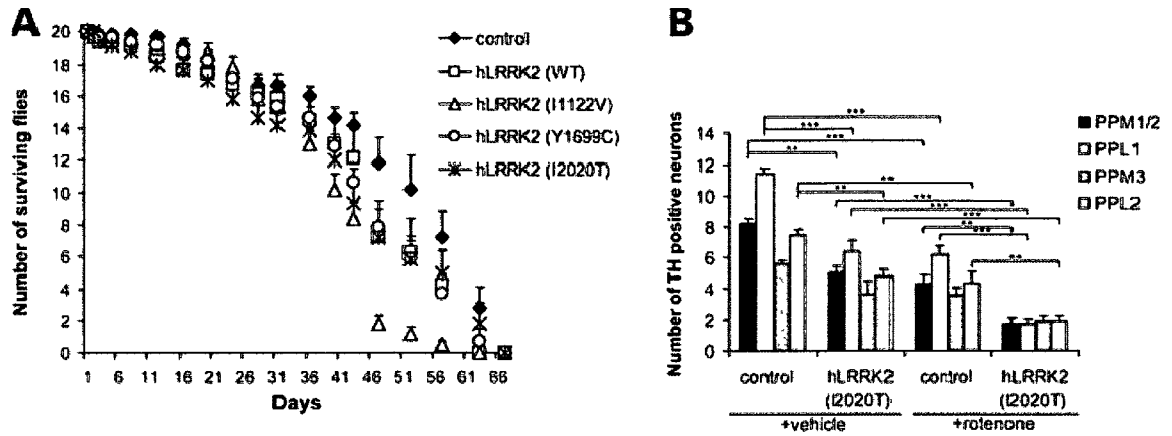


Figure 8. hLRRK2 expression increases sensitivity to oxidative stress. (A) Effect of rotenone at room temperature on lifespan of flies ubiquitously expressing hLRRK2.  $n = 6-8$  sets of 20 per genotype. (B) Effect of rotenone on survival of dopaminergic neurons in flies expressing hLRRK2 specifically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

Table 2. hLRRK2 expression increases sensitivity of the flies to rotenone

Genotype	50% survival (days)	95% CI	<i>P</i> -value
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	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	40.7–41.5	0.0003

Summary of the effects of hLRRK2 expression on sensitivity to rotenone at room

temperature. The data were analyzed by nonlinear regression analysis

#### 2.4.5 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. 9). 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 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 8 and 9A).

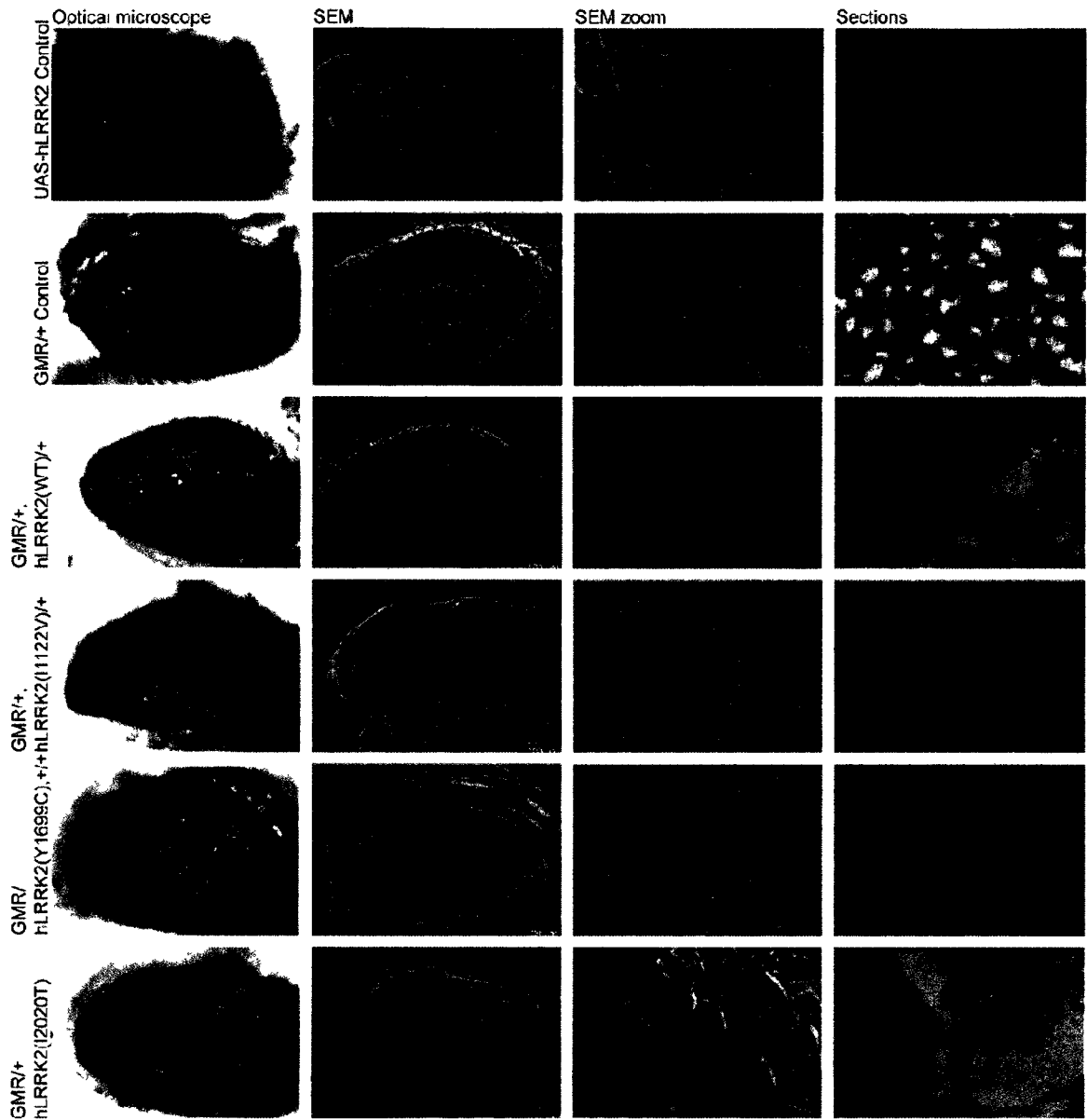


Figure 9. hLRRK2 expression causes structural and pigmentation abnormalities at 29°C. Representative images from optical microscope, SEM and tangential eye sections. The arrows point to a black lesion.

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 with non-GMR controls (Fig. 9). 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. 9). 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 lines, 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. 9). The regular trapezoidal arrangement of the photoreceptor cells was very severely disrupted. The cell lattice between photoreceptor cell arrays of different ommatidia 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).

#### 2.4.6 Interactions of LRRK2 with other PD causing genes

The presence of eye defects allowed us to screen for genetic interactions with other known PD 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.

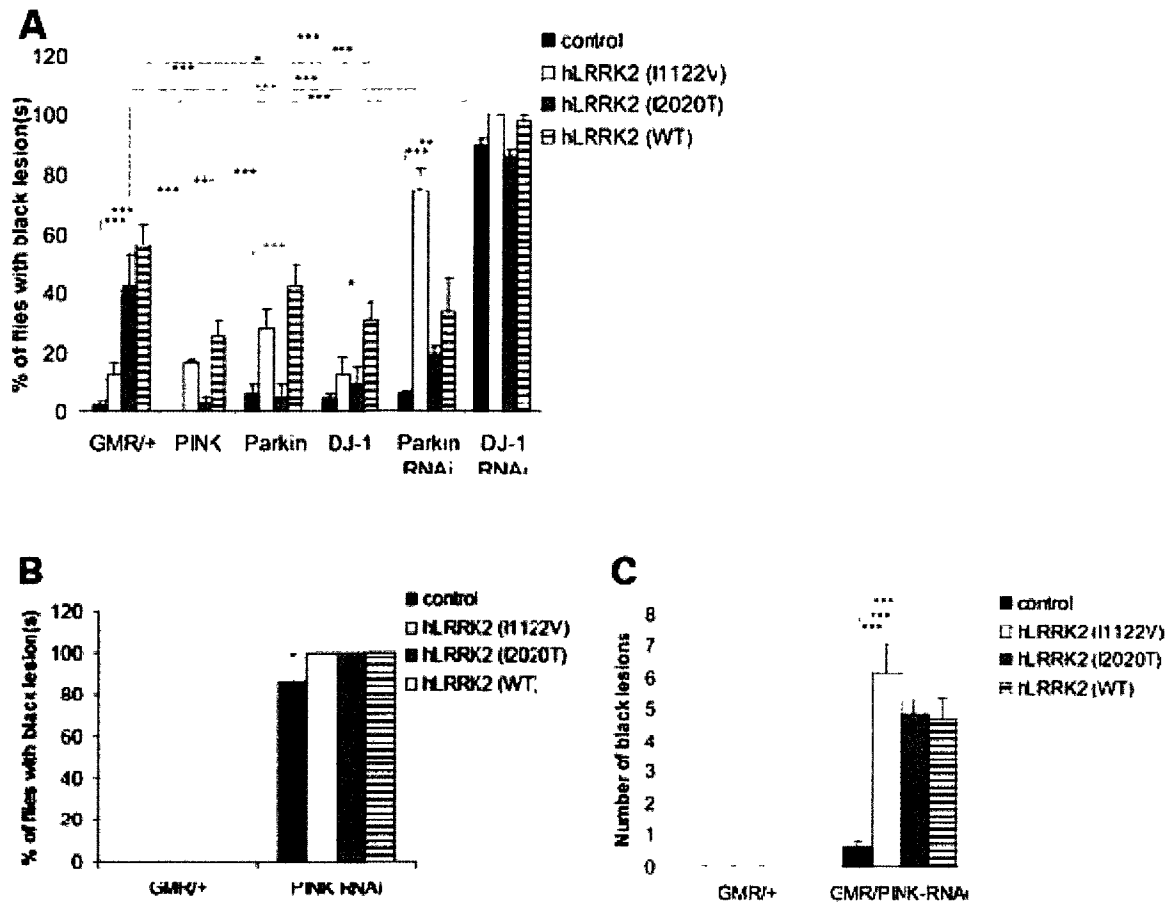


Figure 10. hLRRK2 interacts with hParkin, hPINK1 and hDJ-1 in the eye. Summary of hLRRK2 effects and of interaction with PINK1, DJ-1 and Parkin on the formation of black lesions (88–412 flies per genotype). (A) The effects at room temperature; (B and C) the effect of LRRK2 overexpression in PINK1 deficient flies at 29°C. One-way ANOVA followed by Bonferonni's post hoc test.

hPINK1 overexpression alone did not cause a significant formation of black lesions (Fig. 10A), holes or loss of pigmentation (Table 3), but the eyes had some loss and disorganization of interommatidial bristles (Fig. 11). 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 3), the roughness of the eye surface (Fig. 11) or the formation of holes (Fig. 11) 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. 11). 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. 10A). 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.

Table 3. Summary of the effects of hLRRK2 expression on eye pigmentation at 29°C.

Interaction with hPINK1, hParkin and hDJ-1

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

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.

In order to better analyze these interactions, we further performed loss-of-function experiments. However, because the double transgenic GMR/PINK1-RNAi; hLRRK2/þ 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 (Figs 11 and 12; Table 4). In addition, nearly all PINK1-RNAi flies displayed black lesions (Figs 9B and C and 12). Co-expression of hLRRK2 did not significantly elevate the number of animals with black lesions (because PINK1-RNAi alone caused a dramatic effect) (Fig. 10B). However, the number of black lesions per animal was dramatically elevated with hLRRK2 (WT and mutants) expression (Figs 9C and 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.

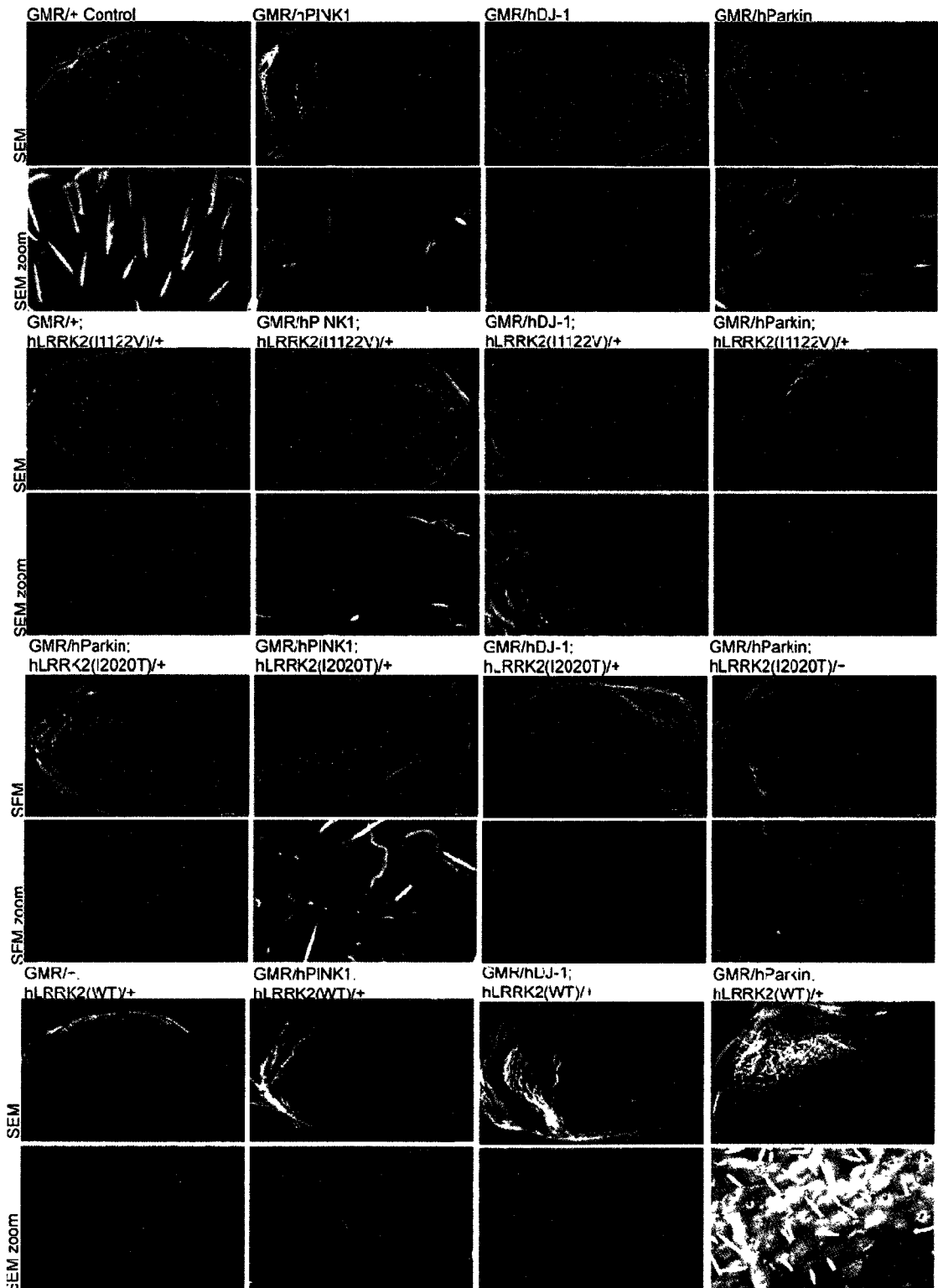


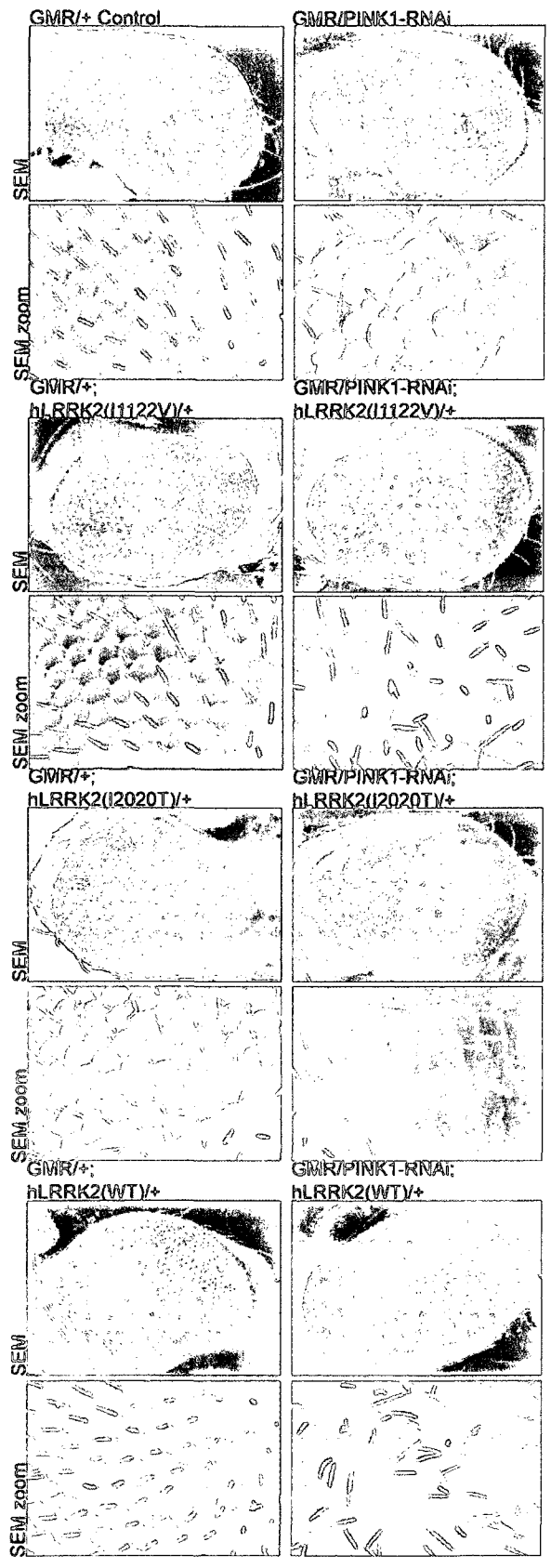
Figure 11. hLRRK2 interacts with hParkin, hPINK1 and hDJ-1 in the eye at 29°C.

Representative images from SEM.

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. 11), as well as counts of pigment loss (Table 3). This phenotype was not rescued by expression of WT or mutant hLRRK2 (Fig. 11). However, hParkin expression, similar to hPink1 expression, diminished the formation of black lesions in hLRRK2(I2020T) lines (Fig. 10A). 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. 10A). 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. 14). This again indicates a complex interaction between Parkin and LRRK2.

Figure 12. 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. 11) and pigmentation loss (Table 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. 11). In common with hPINK1 and hParkin, hDJ-1 expression significantly ameliorated black lesions formation in hLRRK2(I2020T) or hLRRK2(WT) (Fig. 10A). Under SEM, loss of DJ-1 led to a phenotype that was qualitatively and quantitatively similar to hDJ-1 overexpression (Fig. 14). Moreover, this phenotype was potentiated by hLRRK2 expression in a similar fashion (Fig. 14). 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. 10A).

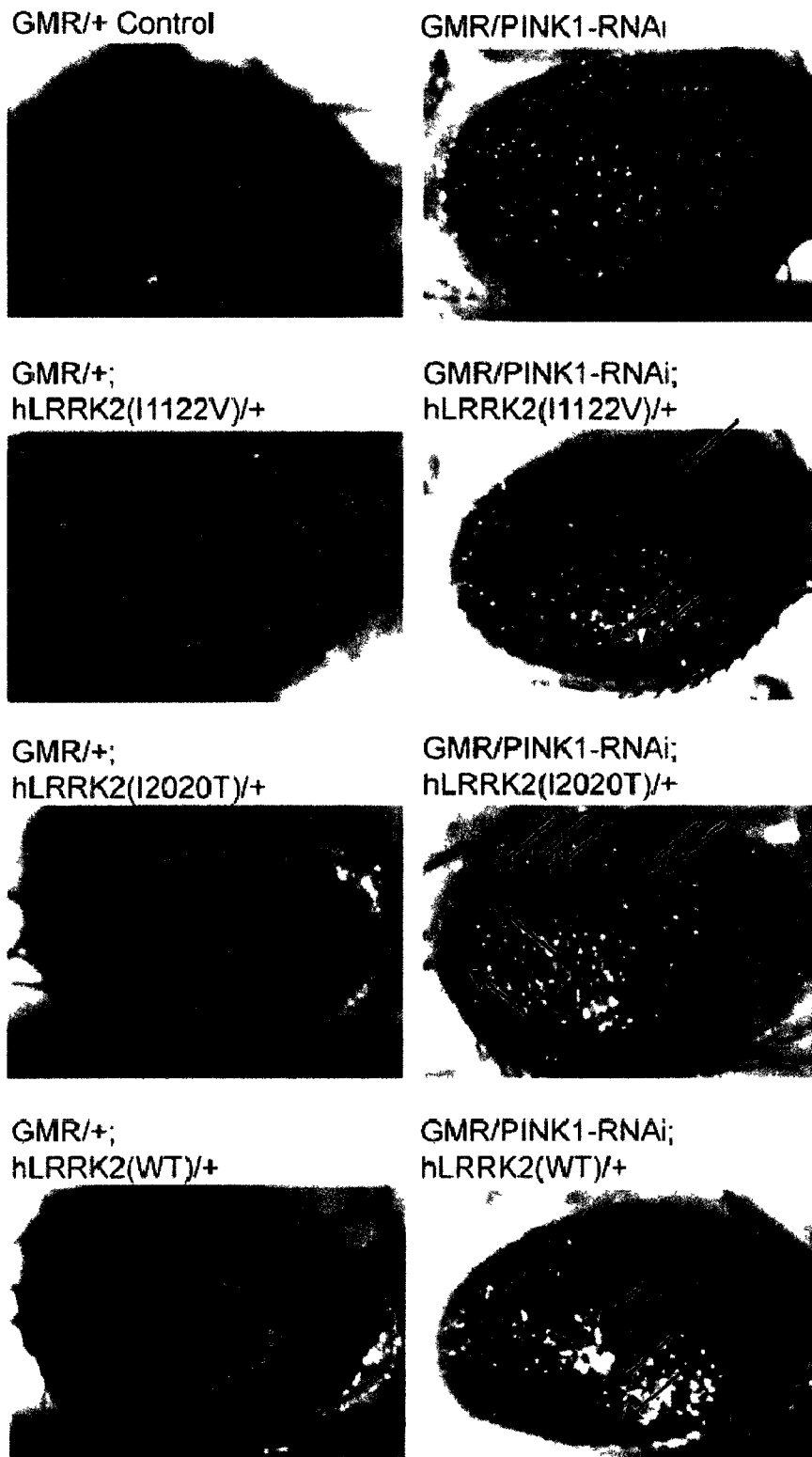


Figure 13. Interactions of hLRRK2 with PINK1-RNAi in the eye at room temperature.

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

Table 4. Summary of the effects of hLRRK2 expression on eye pigmentation at RT.

Interaction with PINK1

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

Loss of pigmentation in the eye as seen under optical microscope. Interactions of

hLRRK2 with PINK1-RNAi at room temperature.

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.

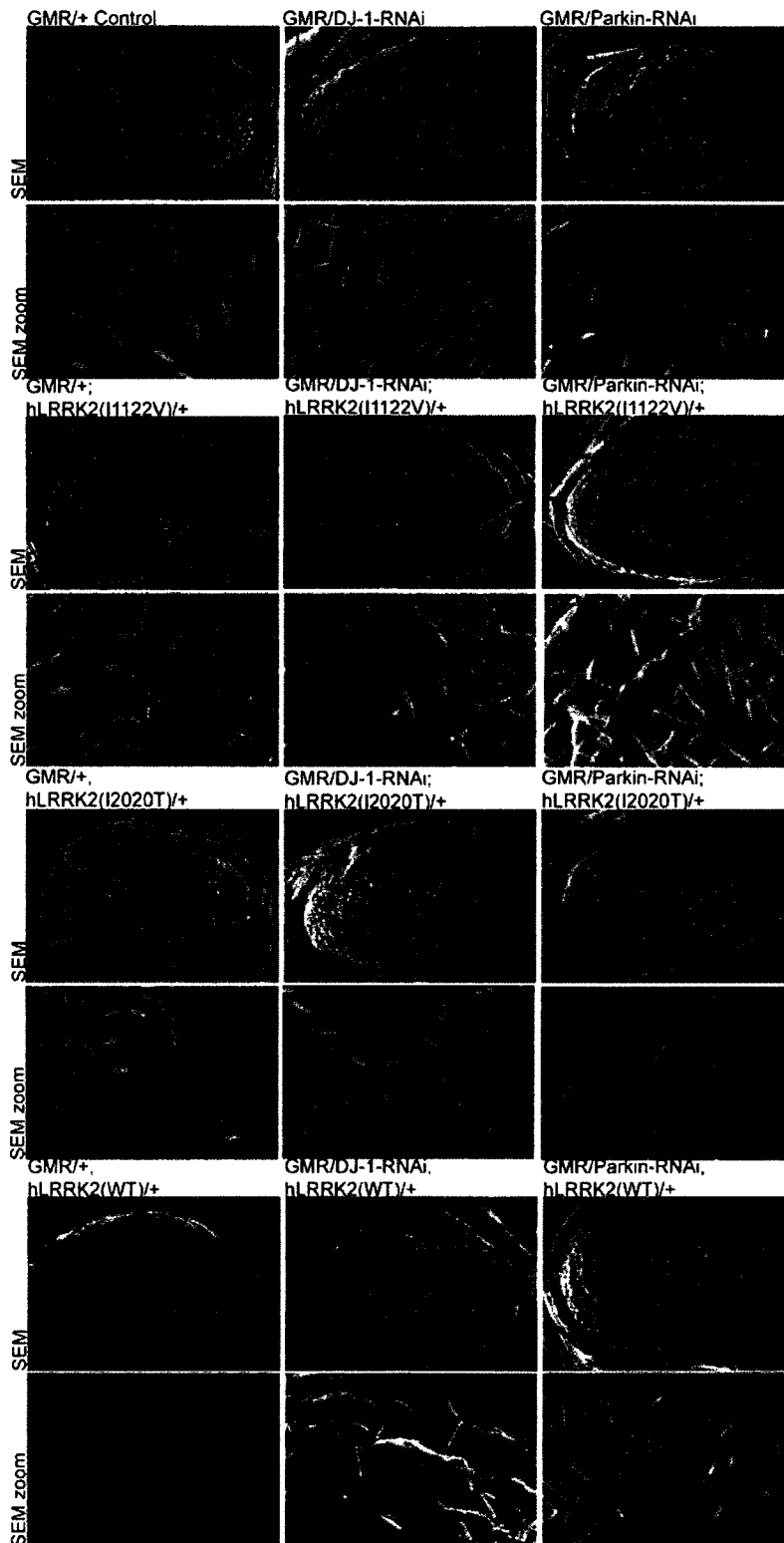


Figure 14. Interactions of hLRRK2 with DJ-RNAi and Parkin-RNAi in the eye at 29°C.

Representative images from SEM.

## 2.5 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 LRRK2 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. (Lee et al., 2007) shows no loss of dopaminergic neurons or deficits in climbing ability. In contrast, Imai et al. (Imai et al., 2008) shows loss of dopamine and of dopaminergic neurons accompanied by behavioral deficits. 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 (Marin, 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. (Liu et al., 2008) showed a similar degree of loss of dopaminergic neurons in all clusters for both WT and kinase domain mutant of hLRRK2. 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 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., 2008a). This suggests that LRRK2, 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 behavioural 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. LRRK2 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 do not 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 versus 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.

## 2.6 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 *Bam*H1/*Xho*I double digests and cloned to pUAST vector at *Bgl*III/*Xho*I site. The plasmids were microinjected to w1118 fly embryo (Genetic Services, Cambridge, MA, USA). 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), respectively. 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: 50-CGATCCATGGCTAGTGGCAGCTGT-30 (forward) and 50-CCTCTGAGACTCTCTCAAACAGC-30 (reverse). For the western blot, we used an anti-LRRK2 rabbit polyclonal antibody (Novus Biologicals), and E7 mouse monoclonal anti- $\beta$ -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/ $\beta$  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 20 s was recorded, as indicated. All behavioural 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 48 h 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 mM). The food containing rotenone was made fresh and changed every 2–4 days. LRRK2 or w<sup>1118</sup> 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 24 h 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 2 mm, 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 10 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 R144C 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 h on standard agar-apple juice plates at 48°C, fixed and incubated with the primary mouse anti-CNS axons BP-102 antibody (Developmental Studies Hybridoma Bank) followed by the goat antimouse HRP-conjugated secondary antibody (Promega, Madison). HRP activity was detected by precipitation of 3-30 diaminobenzidine (DAB) in the presence of H<sub>2</sub>O<sub>2</sub>. 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  $P \leq 0.001$ .

## 2.7 Acknowledgements

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*Conflict of Interest statement.* None declared.

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## Chapter 3 – Suppressor/Enhancer Screen

### 3.1 General Introduction

A suppressor/enhancer screen is a screen for dominant phenotype modifiers. After establishing a phenotype that is easy to score, a second important characteristic is the potential of the phenotype to be modifiable in both, the negative and the positive direction. A negative modification (suppressor) is one that reduces the phenotype towards the wild type state, and a positive modification (an enhancer), is one that exacerbates the phenotype.

Two components make up the screen. The first is the gene of interest which displays a modifiable phenotype, and the second is a library of flies that can potentially interact with and modify the phenotype. The screen was performed at two different temperatures. At 25°C, *LRRK2* has no basal phenotype, and thus the screen will only permit the identification of positive modifiers. At 29°C, *LRRK2* flies have an eye phenotype which will permit the identification of negative modifiers as well as positive modifiers. At times, a second *LRRK2* construct was also used to verify results obtained from the first *LRRK2* construct. The screen was performed against a library of Deficiency flies that lack small regions of their genome. Modifiers resulting from the screen were categorized into 5 arbitrary levels according to their strength with Level 1 being the weakest and level 5 being the strongest (Table 5).

Table 5. Outline of the characteristics of the different positive modifier levels.

Positive modifier Level	25°C	29°C
Level 1	5-10% pigmentation loss	10-20% pigmentation loss
Level 2	25%+ pigmentation loss	30%+ pigmentation loss
Level 3	50%+ pigmentation loss or increase of black lesions	60%+ pigmentation loss or increase of black lesions
Level 4	40%+ pigmentation loss AND increase of black lesions	50%+ pigmentation loss AND increase of black lesions
Level 5	Similar to level 4 AND loss of eye size/structures	Similar to level 4 AND loss of eye size/structures

Eye phenotypes of the different positive modifier levels at 25°C and 29°C. Levels at 29°C require a slightly higher threshold of pigmentation loss due to the background pigmentation loss of the GAL4 control.

## 3.2 Results

Using the hLRRK2 (I1122V) line, we screened a small portion of the second chromosome at 29°C and found one Level 3 positive modifier which we termed Deficiency X. This modifier greatly enhanced I1122V's black lesion phenotype (Fig. 15). After this proof-of-concept experiment, we turned to I2020T to confirm the findings and to continue screening the second chromosome. Indeed, Deficiency X also enhanced I2020T's black lesion phenotype (Data not shown). A total of 11 positive modifiers and 2 negative modifiers were found using I2020T at this temperature (Table 6).

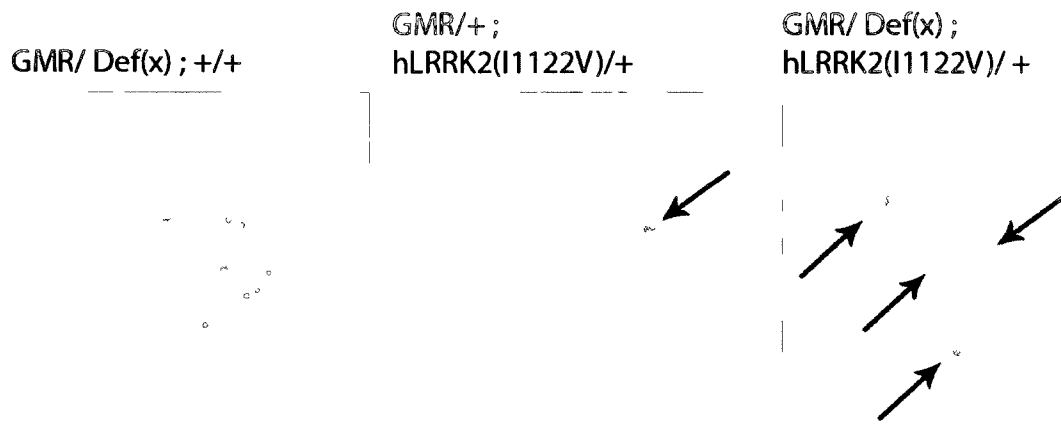


Figure 15. Interaction of hLRRK2(I1122V) with Deficiency X in the eye at 29°C.

Representative optical microscope images. Arrows point to black lesions.

However, since LRRK2 will have a strong basal phenotype at 29°C, the 25°C cross will permit a finer identification of minor enhancements that could be missed at the higher temperature. A higher percentage of hits were obtained at 25°C (46.5%) than at 29°C (14.4%). Thus far, only two hits were shared between the two temperature groups. One Level 1 positive at 29°C was detected as a Level 2 positive at 25°C, and one Level 3 positive at 29°C (Deficiency X), was detected as a Level 2 positive at 25°C. The rest of the modifiers were found either strictly at the temperature given and were not detectable at the other temperature, or have not yet been examined at the other temperature. The results are summarized in Table 6.

Table 6. Multiple modifiers uncovered from screening hLRRK2 (I2020T) against second chromosome Deficiencies.

Modifier Level	Number of fly lines at 25°C	Number of fly lines at 29°C
Positive modifier Level 1	11	9
Positive modifier Level 2	7	0
Positive modifier Level 3	2	2
Positive modifier Level 4	0	0
Positive modifier Level 5	0	0
No modification	23	77
Negative modifier Level 1	0	0
Negative modifier Level 2	0	1
Negative modifier Level 3	0	0
Negative modifier Level 4	0	1
Negative modifier Level 5	0	0
Total Deficiencies screened	43	90

Summary of results obtained by screening at two different temperatures. Eyes were examined under optical microscope and ranked from weakest (Level 1) to strongest (Level 5).

### 3.3 Materials and Methods

#### *Drosophila* genetics

The flies were maintained on a standard cornmeal/agar medium at RT or at 29°C, just as outlined in section 2.6. GMR-Gal4/Cyo ; UAS-LRRK2(I2020T)/TM6B was designed by crossing each of GMR/BC; +/+ and +/+; LRRK2(I2020T) with the doubly balanced line Sp/Cyo; TM2/TM6B. Progeny such as GMR/Cyo ; +/TM6B and Sp/Cyo ; LRRK2/TM2 were crossed to obtain the final stock. Deficiency flies were obtained from Bloomington *Drosophila* Stock Centre.

## Chapter 4 – General Discussion

We have generated four *Drosophila* lines harboring four different human LRRK2 constructs; wild-type, I1122V mutant of the leucine-rich repeat domain, Y1699C mutant of the COR domain, and I2020T mutant of the kinase domain. By means of ectopic expression, we demonstrated DA cell loss, complex biphasic locomotor activity, increased life span, increased sensitivity to oxidative stress, a rough eye phenotype, and potential genetic interaction with recessive PD genes. Overall, our results are significant because they recapitulate key Parkinsonian features, constitute an *in vivo* model for previously unreported mutations, and demonstrate persisting controversies as they support some aspects of already-published reports, and contradict others.

### 4.1 Results of the study

#### 4.1.1 Effects on the DA system

Work by my colleague Dr. Katerina Venderova demonstrated DA cell loss by overexpression of all LRRK2 constructs including wild type. This comes in agreement with a report in 2008 by Liu et al. Also in agreement, we demonstrate that this cell loss is most prominent with the kinase mutant I2020T, which is adjacent to their kinase mutant G2019S that also displayed a higher degree of cell loss. A recent report (Ng et al., 2009) also demonstrate DA cell loss by overexpression of human LRRK2 mutants G2019S,

Y1699C, and the association-mutant G2385R. However, they report no loss using wild type. Another group (Lee et al., 2007) overexpressed dLRRK and dLRRK R1069C, which is equivalent to the human R1441C mutant, and reported no cell. On the other hand, Imai reported DA cell loss using other dLRRK mutants: Y1383C (equiv. Y1699C), and I1915T (equiv. I2020T), but not wild type (Imai et al., 2008). And while Lee et al demonstrate shrunken DA neurons in a dLRRK knockdown background, Wang et al (2008) report no effect, and Imai report increased DA levels in the brains.

*Drosophila* has 15 DA clusters in its brain (Nassel and Elekes, 1992) that are arranged roughly in parallel bilateral pairs. We report the cell loss in clusters PPM1/2, PPL1 and PPL2. Similarly, Imai et al using the dLRRK mutants report the loss in PPL1 and PPM1/2, and Ng et al report the loss in PPM1 and PPM3. However, Liu et al report the loss in all DA clusters.  $\alpha$ -synuclein models that detected specific DA neuron loss reported it either in the PPM1/2 (Feany and Bender, 2000; Auluck et al., 2002), or the PPL1 (Trinh et al., 2008). Parkin models report PPL1-specific loss (Whitworth et al., 2005). Thus our results come in line with most indices of the DA loss phenotype, albeit raising a few more questions regarding specificity to certain DA clusters and effect of the wild type LRRK2.

When the original paper on *Drosophila*'s dopamine clusters was published (Nassel and Elekes, 1992), no attempt was made to understand the function or the innervations of the clusters. A more recent study sheds some light about some of those innervation pathways and highlights the underscored complexity of the system (Mao and Davis, 2009). According to this study, PPL1 neurons are made up of at least five different subtypes that mainly innervate the mushroom body, which is the proposed center for

learning. One type of PPL1 neurons innervate the Central Complex, which controls locomotion, but also possibly learning. The PPM3 was found to be the major innervator of the Central Complex.

With the lack of enough studies on the functions of the different neuronal populations with the clusters, it's thus difficult to speculate on the discrepancies seen in the different fly models. However, there does seem to be more specificity towards the PPL1 cluster, and it would be interesting to know whether or not the subpopulation that innervates the Central Complex is the most affected if it indeed plays a specific role in locomotion.

#### 4.1.2 Effects on lifespan and sensitivity to oxidative stress

While some overexpression reports (Liu et al., 2008; Ng et al., 2009) demonstrate increased mortality, our model surprisingly showed increased life span for all constructs except I1122V. This increased life span was not due to reduced crowding resulting from reduced fertility and progeny. On the contrary and as mentioned in the manuscript, our flies also demonstrate increased egg laying. This later result comes in agreement with Lee's report of reduced egg laying with the dLRRK knockdown model. It's important to keep in mind that this increased life span occurred to a limited extent with ubiquitous expression of LRRK2 and not pan-neuronal at two different temperatures.

Dietary restriction is an important factor that extends life span in *Drosophila*. Several reports and discussions have been devoted to this topic (Piper et al.; Bass et al.,

2007) and that recommend Brewer's yeast for a healthy extended life span. Rarely do reports mention details about the type of food medium used but usually the word "standard" is used to describe the medium. The Bloomington *Drosophila* Stock Center at Indiana University, an authority on *Drosophila* protocols, considers Dry inactive yeast as part of their standard medium. In our study, we have used Brewer's yeast, and while it's unknown what other studies employed as their yeast source, differences in the food medium might have contributed to the general lengthening of the life span seen in our study. However, this does not explain the extension of life span induced by LRRK2 over control flies in Figure 7A, or at least the lack of the expected effect of life span reduction in Figures 7 B-C.

A recent publication in the journal Cell (Zid et al., 2009) found 4E-BP to be upregulated following dietary restriction in *Drosophila*. Interestingly, 4E-BP was found to be hyperphosphorylated and inhibited by LRRK2 in *Drosophila* resulting in loss of DA neurons (Imai et al., 2008). Thus, yeast dietary restriction in our study might have directly contributed to an attenuation of LRRK2's toxicity in a 4E-BP dependent manner, causing either an unexplained increase in life span, or no difference from controls.

Most results agree on a toxic role for LRRK2 under oxidative stress. Wang et al demonstrate increased general sensitivity to H<sub>2</sub>O<sub>2</sub>, but not to rotenone or paraquat, with the dLRRK lacking the C-terminal region after the ROC domain, i.e. lacking the COR, kinase, and WD40 domains. However, a complete loss of dLRRK caused increased resistance to H<sub>2</sub>O<sub>2</sub> and paraquat in Imai's study, while overexpression of dLRRK point mutations resulted in sensitivity. Imai however used half the amounts of paraquat and H<sub>2</sub>O<sub>2</sub> that Wang used. Ng et al showed that their mutant flies, except Y1699C, were more

sensitive to rotenone which aggravated their DA cell loss (Ng et al., 2009). This is in agreement with our results using rotenone. Thus, lower levels of oxidative stress could have increased longevity in a LRRK knockdown background, while increasing those levels would result in the opposite effect. A recent *C. elegans* report supports this idea (Schulz et al., 2007).

#### 4.1.3 Locomotor function

While Imai et al do not assess locomotor activity in their dLRRK overexpression model, both Liu and Ng report clear locomotor deficits by overexpression of their mutants. G2385R was the only exception in Ng's paper. We also report an initial regression of locomotor function, however towards the end of the experiment, our mutant flies surpass the control in a recurring biphasic fashion that we saw at different temperatures and different time measurements. As discussed in Chapter 2, this might be due to compensatory mechanisms upregulating DA receptors in response to DA neuron loss.

Startle-induced negative geotaxis that is employed in this behavioral test is not a completely understood phenomenon. Reduced climbing speed is thought to be the main mechanism involved in aging *Drosophila* (Rhodenizer et al., 2008), but this does not rule out other effects in disease paradigms. When tetanus toxin, a potent neurotransmitter release inhibitor, was expressed in *Drosophila* using TH-Gal4, flies were hyperexcited and this prevented them from climbing efficiently to the top of the vial (Friggi-Grelin et

al., 2003). Thus although the expression of tetanus caused perceived locomotor deficits, the underlying mechanism seemed to be hyperactivity rather than reduced motor function resulting from shutting down of the DA system. It's not clear that the initial locomotor deficits seen in our model is due to any hyperexcitment because flies still attempted to climb slowly and eventually reached the top line. But the differential effects on DA clusters might have played a role in the differences in results. Both Liu and Ng report loss in the PPM3 cluster which is the major innervator of the Central Complex (Mao and Davis, 2009), while our study reports loss in different clusters. This, or other compensatory mechanisms that seem to be at work in the Dopamine system (Jones et al., 1981; Tirelli and Terry, 1993), can play a role in the biphasic locomotor response seen with our flies.

#### 4.1.4 Eye Defects

Liu et al is the only group to demonstrate a mild eye phenotype. They demonstrate loss of rhabdomere counts per ommatidium that is higher in G2019S than in wild type, but report no surface phenotype. At the same temperature, we also cannot detect any surface eye roughness, and did not see rhabdomere degeneration (data not shown). However by raising the temperature to 29°C, we detect surface roughness and pigmentation loss, surface holes, ommatidial disorganization suggestive of retinal degeneration, and surface black lesions. I1122V is the only construct not displaying significant formation of black lesions, and Y1699C is the only construct displaying loss

of mechanosensory bristles. Interestingly, wild type LRRK2 showed the highest black lesion penetrance, but not intensity per animal which was rather uniform. We speculate that the rise in temperature causing a rise in LRRK2 expression levels is what is causing the occurrence of the phenotype. It is important to note that at this temperature, the GMR-GAL4/+ control also displays some limited surface roughness and pigmentation loss (Kramer and Staveley, 2003).

The glass multiple reporter (GMR) promoter region belongs to a gene called glass in *Drosophila*. This gene encodes a DNA-binding Zinc-finger transcription factor and is expressed in all cell types in the eye during eye development (Ellis et al., 1993). The eye is made up of ~800 ommatidia which in turn is made up of 8 photoreceptor cells, plus cone cells and pigment cells. Glass does not induce eye cells to become neurons but is rather required for the differentiation of neurons into photoreceptors (Ellis et al., 1993). When UAS-dPinkRNAi was driven in the eye using GMR-Gal4 (Wang et al., 2006), the phenotype that was obtained was greatly similar to ours. This group reported similar phenotypes of black surface lesions, disorganized interommatidial bristles, ommatidial degeneration, surface roughness, and some pigment loss. They found that ommatidial degeneration was in fact due to photoreceptor loss, and that antioxidants, and not anti-apoptotic factors, rescued these phenotypes. We similarly saw a high degree of disruption in the ommatidial structure and loss or disorganization of photoreceptor cells that was difficult to quantify due to the high degree of disorganization (Figure 9). However, the high degree of similarity between our phenotypes, and the fact that LRRK2 further sensitizes the black lesion phenotype of dPink1-RNAi flies (Figure 13) suggests that a similar mechanism might underlie these defects.

#### 4.1.5 Interaction with recessive PD genes

The multiple parameters displayed in our eye phenotype allow for both qualitative and quantitative assessment of defects. Black lesions are small spots that are visible on the eye surface under optical microscope. Typically they present as 1-2 spots per animal, and thus an increase in their penetrance or number can serve as a quantifiable measurement of exacerbation of phenotype. On the other hand, a glossy eye surface that is due to surface roughness from fusing ommatidia, and loss of pigmentation are two phenotypes that are more readily recognized as a qualitative measure of defects. On the ultramicroscopic level, Scanning Electron Microscopy can reveal bristle loss and hole formation. In summary, we found that ectopic overexpression of all three recessive PD genes rescued the black lesion formation phenotype of wild type and I2020T LRRK2. On the other hand, knockdown of Pink1 by means of RNAi greatly increased black lesion formation (Figure 13). This increase was evident in LRRK2-expressing flies at room temperature that do not have any basal phenotype. The effect on other indices of the phenotype was complex but yet suggestive of a selective interaction. Both Parkin RNAi and DJ1 RNAi flies show bristle loss, but I2020T suppressed the former's phenotype while it exacerbated the latter's. However I1122V's effect was opposite in direction as it caused a dramatic increase in Parkin RNAi's black lesion formation. Parkin was recently found to suppress DA neuron loss caused by G2019S in *Drosophila* (Ng et al., 2009). This comes in line with I1122V's exacerbation of lesion formation. Unfortunately, it was

not possible to test the effect of Parkin overexpression on I1122V since this line did not present with significant black lesions to begin with.

## 4.2 Suppressor/ Enhancer screen

The I1122V construct displays prominent pigmentation loss and surface roughness even though it presents with less black lesion penetrance. And since it's also an overall healthier stock than I2020T in the GMR double transgenic construct (GMR/Cyo;LRRK2(I1122V)/TM6b), we decided to pre-screen some lines using this construct first. Screening with this mutant did indeed result in the identification of Deficiency X which increased black lesion number and penetrance. Taking this into consideration, we decided to continue further by using I2020T which has three advantages. First, it presents with the most prominent DA neuron loss and thus can be more physiologically relevant if we decide to test some candidate genes in the DA neurons. Second, it displays a significant black lesion phenotype that can be assessed for rescue. Finally, it's clinically confirmed as a causative PD gene while I1122V is associative.

When performing the screen at 25°C, a higher number of positive modifiers were discovered. This is not readily appreciated because LRRK2 has a stronger phenotype at 29°C and should then present with a higher number of positives. However, at this temperature the control also has an eye phenotype making differentiating low differences more difficult. Thus, the 25°C cross can pick up small differences in exacerbation

provided that enough dosage of LRRK2 is present, while the 29°C cross is most useful to discover negative modifiers. Indeed, the strongest modifier discovered thus far is a negative modifier at 29°C. This modifier, and Deficiency X, both contain less than 20 identified and putative genes making the isolation of the gene responsible for the phenotype easier. We are currently scanning these regions by using smaller deletions, P-element gene disruptions, and RNAi lines. Also, better defined deletions continue to be added to the Bloomington Stock Center further enhancing and refining the coverage of the euchromatin and reducing redundant deletions.

### 4.3 Common pathways

One *Drosophila* ortholog of human LRRK1 and LRRK2 has been found and is termed *dLRRK*. It shares 24% identity and 38% similarity at the amino acid level to LRRK2 (Wang et al., 2008a). Additionally, it shares all the key domains and most of the pathogenic PD mutation sites. Analysis by Marín in 2008 considered LRRK2 not to be a true ortholog of *dLRRK*, but  $\alpha$ -synuclein models have been very successful thus far even though *Drosophila* does not have any clear  $\alpha$ -synuclein gene. Several LRRK2 *Drosophila* models have already been described in the above discussions. Generally, overexpression of wild type, Y1699C, G2019S, and G2385R LRRK2 mutants and *dLRRK* mutants have been shown to be toxic to DA neurons, while knockdown of *dLRRK* was shown to have no bearing on DA neuron viability. Most of these studies reported no eye phenotype for LRRK2 overexpression, or an eye phenotype that was

unpractical to use for a large modifier screen (Liu et al., 2008). No studies exist for I2020T and I1122V. In our work, we present the first model that demonstrated DA neuron loss in these novel mutations, and is also suitable for a modifier screen via the eye phenotype.

$\alpha$ -synuclein was the first PD gene to be successfully modeled in *Drosophila* in 2000 (Feany and Bender, 2000). Expression of wild type, A30P, or A53T  $\alpha$ -synuclein caused a mild eye phenotype, loss of DA neurons, cytoplasmic inclusions, and locomotor deficits. A later study by the same group was the first to demonstrate the idea that protein aggregation in PD can in fact be a protective cellular response (Chen and Feany, 2005). Phosphorylated  $\alpha$ -synuclein at Ser129 was shown to be soluble and toxic, while non-phosphorylated  $\alpha$ -synuclein formed aggregates and was less toxic. Visualizing DA neurons in *Drosophila* is indirect and relies on either immunostaining of the TH enzyme, or TH-GAL4-driven expression of Green Fluorescent Protein (GFP). Different reports using different analysis methods- brain sections or ideally whole-brain confocal microscopy- resulted in a controversy on whether or not the  $\alpha$ -synuclein models result in true loss of DA neurons (Pesah et al., 2005).

Parkin models of PD were next in line to be developed. These flies presented with reduced life span, locomotor deficits, and male sterility. Mitochondrial pathology is seen as the possible underlying cause (Greene et al., 2003). DA neuron loss was also demonstrated in one Parkin fly model in the PPL1 cluster (Greene et al., 2005). Parkin overexpression in *Drosophila* rescued neuronal loss in  $\alpha$ -synuclein-overexpressing flies (Haywood and Staveley, 2006).

Pink1 models were less conclusive. Loss-of-function/deletion mutants resulted in no DA neuron loss (Clark et al., 2006) while RNAi gene silencing of Pink1 did (Wang et al., 2006; Yang et al., 2006). This could be due to off-target effects, but the flies did indeed present with a phenotype resembling of Parkin's phenotype. Most DA clusters were affected in the Pink1 model, but opposite to Parkin's phenotype, PPL1 was less affected. Swollen mitochondria were found in Pink1 mutant lines suggesting increased mitochondrial fusion (Park et al., 2006). Indeed, it was shown that enhancing fission, through Drp1 or other modes, rescued the Pink1 phenotype (Lutz et al., 2009). Overexpression of Parkin, but not vice versa, was able to rescue Pink1's phenotype which was the first evidence of interaction between the two recessive PD genes (Clark et al., 2006; Park et al., 2006). Pink1 was shown to recruit Parkin to the mitochondria in *Drosophila*, and evidence of direct phosphorylation has been demonstrated in some (Kim et al., 2008), but not all reports (Vives-Bauza et al.).

DJ-1 models were harder to dissect due to the presence of two *Drosophila* orthologs of DJ-1 named DJ-1A and DJ-1B, with the former being dubbed the closer homolog. Similar to the case of Pink1, RNAi silencing and gene deletions yielded different results. Different methods of DJ-1B knockdown had no bearing on DA neuron viability (Menzies et al., 2005; Meulener et al., 2005; Park et al., 2005) but demonstrated a possible compensatory mechanism of DJ-1A upregulation in the brain (Menzies et al., 2005). RNAi silencing of DJ-1A reported loss of DA neurons (Yang et al., 2005), but DJ-1A/B double deletion mutants did not (Meulener et al., 2005). All studies however agree on an important role for DJ-1 in ROS management in the fly. H<sub>2</sub>O<sub>2</sub>, rotenone, or paraquat treatments render mutant or RNAi flies of DJ1A/B more sensitive. DJ-1 was

unable to rescue Pink1's muscle and degeneration phenotypes (Yang et al., 2006) although it has been shown to interact with Parkin under oxidative stress in mammals (Moore et al., 2005).

It is thus evident that a recurring theme in all of these recessive PD models is the involvement of the mitochondria. There is strong evidence to link Pink1 and Parkin to the same pathway, but this is less obvious for DJ-1. Dominant PD genes appear to work more closely together, evident from the latest  $\alpha$ -synuclein mouse model (Lin et al., 2009), although yet, few *Drosophila* studies including our own, have linked them pathologically to Pink1 or Parkin. In order to advance our knowledge in this direction, it is imperative to first define the enzymatic activity and physiological substrates of these proteins. One way that we believe this is feasible is by the creation of relevant animal models, and performing large-scale blind modifier screens.

#### 4.4. Conclusion

Few *Drosophila* models of LRRK2 have emerged thus far but we believe that our model, due to its readily recognizable eye phenotype, has the most potential to be used for suppressor/enhancer screening. We have presented a model that is novel in the quality of both the mutations presented and the parameters of toxicity. By presenting DA neuron loss, oxidative stress, and a curious case of locomotor disregulation, we have recapitulated some of the main cardinal features of Parkinson's disease. We hope that we can use this model to aid our understanding of the biological and pathological role(s) of LRRK2, which might one day provide a new avenue for Parkinson's disease therapeutics.

## Chapter 5 – References

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