Drosophila suppressor/enhancer screen to identify novel LRRK2 interactors

SAMEERA ABUAISH

This thesis is submitted as a partial fullfillment of the M.Sc. program in Cellular and Molecular Medicine

August 6th, 2013

Abstract

Parkinson's disease (PD) is a progressive neurodegenerative movement disorder characterized by the loss of dopaminergic (DA) neurons in the substantia nigra *pars compacta*. The mechanism by which these DA neurons die is still unclear and under investigation. Although mostly idiopathic, about 10% of PD cases have shown familial inheritance. Mutations in leucine-rich repeat kinase 2 (LRRK2), a large multi-domain protein with unknown physiological and pathological roles, have been linked to PD cases of autosomal dominant inheritance. A PD *Drosophilamelanogaster* model over expressing the human LRRK2(I2020T) kinase mutant using the GAL4/UAS system has shown a loss of DA neurons and locomotor deficiency. Additionally, ectopic overexpression of human LRRK2 in the eye caused a damaged eye phenotype characterized by roughness of the surface, loss of pigmentation and presence of black lesions (Venderova K*et. al*., 2009). The presence of this identifiable eye phenotype has allowed us to perform a suppressor/enhancer screen to identify possible genetic interactors of LRRK2. The LRRK2(I2020T) transgenic flies were crossed with genomic deficiency lines and the eye phenotype screened for either suppression or enhancement. Twenty-two genes, which are implicated in a variety of biological processes, have been identified thus far. Fourteen of these 22 interacting genes were assessed in the DA neurons of the *D.melanogaster* model. This functional screen is a rapid method to provide us with potential genetic interactions between LRRK2 and other genes, which will in turn, aid in elucidating the functional role of LRRK2 in PD pathology.

Table of Contents

List of Figures

List of Tables

List of Abbreviations

°C- degrees celsius
AD- Alzheimer's disease
Aly – Always early
AMPK- AMP-activated protein kinase
ANK- Ankryin like repeats
ArfGAP1 – ADP-ribosylation factor GTPase-activating protein 1
ATP – Adenosine-5'-triphosphate
ATPase - Adenosine-5'-triphosphatease
Bal – Balancer
Ca2+- Calcium
CaMKKβ - Ca2+/calmodulindependent protein kinase kinase β
CDC - Cell division cycle
CDK - Cyclin-dependent kinase
CLK - CDC-like kinase
CMA - Chaperon-mediated autophagy
COMT- Cathecolamine-O-methyltransferase
COR- C-terminal of ROC
Cyo – Curly
D.melanogaster- Drosophilamelanogaster
DA-Dopamin/Dopaminerigc
Dap160 - Dynamin associated protein 160
DAT - Dopamine active transporter
Ddc - *Dopa decarboxylase*
Def – Defeciency
dLRRK- Drosophila LRRK2 homolog
Drp1 - Dynamin-related protein
Elk-1 - ETS domain-containing protein
En – Enhancement
ERG - Electroretinogram
ERK- Extracellular-signal-regulated kinases
ERM-Ezrin/Radixin/Moesin
FADD - Fas-associated protein with death domain
gef64c - Guanine nucleotide exchange factor 64c
GMR – Glass multimer reporter
GTP – Guanine triphosphate
GWAS- Genome-wide association studies
hLRRK2 - human LRRK2
hsc70 - Heat shock congnat protein 70
Hz-Hertz
IFN-γ- Interferon-gamma
iPS - Induced pluripotent stem cells
JNK- c-Jun N-terminal kinases

K^+ - Potassium
KDa- kilo Dalton
KO - knock out
L-Dopa-Levodopa
LAMP-2A- lysosome-associated membrane protein type 2A
LBs- Lewy bodies
LRR- Leucine rich repeat
LRRK2- Leucine rich repeat kinase 2
MAO-B- Mono-amineoxidase-B
MAPKKK- Mitogen-activating kinase kinase kinase
MAPT - Microtubule-associated protein tau
MBP - Myelin basic protein
MKK4- Mitogen-activated protein kinase kinase 4
MLKs - Mixed lineage kninses
MPP+- 1-methyl-4-phenylpyridinium
MPTP- 1-methyl-4-phenyl-1,2,5,6-tetrahydropyr-idine
mRNA - Messenger RNA
MVBs - Multivesicular bodies
N - No interaction
Na^+- Sodium
NAS-NRC- The National Academy of Sciences/National Research Council
ND - Not determined
NF-κB - Nuclear factor kappa-light-chain-enhancer of activated B cells
PBMC - Peripheral blood mononuclear cells
PD – Parkinson's Disease
PET-Positron emission tomography
Pink1- PTEN-induced putative kinase 1
PPL1 - Protocerebral posteriolateral dopaminergic cluster neuron 1
PPL2 - Protocerebral posteriolateral dopaminergic cluster neuron 2
PPM1/2 - Protocerebral posteriomedial dopaminergic neuron 1/2
PPM3 - Protocerebral posteriomedial dopaminergic neuron 3
PRP4 - Precursor mRNA-processing factor 4
RIPK - Receptor-interacting protein kinases
RNA - Ribonucleic acid
RNAi – RNA interference
ROC- Ras of complex protein
ROS - Reactive oxygen species
SNc- Substantia nigra *pars compacta*
SNPs - Single nucleotide polymorphisms
Su – Suppression
TH – Tyrosine hydroxylase
Thr - Threonine
TUNEL - Terminal deoxynucleotidyl transferase dUTP nick end labeling
UAS-Upstream activation sequence
UPDRS- Unified Parkinson’s disease Rating Scale
WT - Wild type

Acknowledgements

I am heartily thankful to my supervisor, Dr. David Park, for giving me the opportunity to be part of his team of scientists and his immense insights and support. I wish to thank my committee members Dr. Akimenko and Dr. Colavita for their help and advice during the past two years.

I also extend my heartfelt gratitude to all of Paul Marcogliese, Elizabeth Abdel-Messih, and Dr. Yasmilde Rodriguez Gonzalez for their continuous encouragement, guidance and fruitful discussions that was of a great benefit for this project. I am deeply gratefulfor Sarah Seang's contribution to this project during her summer and honors training. I would like to extend my thanks to Dr. Katerina Vendorova andGhassan Kabbach for the initial training and help. I wish to express my appreciation to our lab manager,Steve Callaghan, for his endless support. I take this opportunity to convey my sincere thanks to all members of the Park lab who never hesitated to offer their insights and advice.

My sincere thanks to my fellow masters students, Alaa Fanous and Katie Don-Carolis, who have shared this journey with me through laughter and tears and whose friendship I will hold dearly to my heart and never forget.

Importantly, I would like to express my heartfelt gratitude to my country, Kingdom of Saudi Arabia, and The King Abdullah Scholarship Program for covering my study expenses for both undergraduate and graduate training and for investing and believing in the country's youth.

Lastly and most importantly, I offer my regards and blessings to my family, my parents Kawther and Yousuf, to whom I owe my life. They have always believed in me in times when I did notbelieve in myself.To my dear siblings, Rashad, Ahmed, Mohammed, and Yara, the source of my joy in life; I love you all. To my inspiration, my grandmother, Sameera, who has always encouraged me to pursue my dreams and whose prayers are always with me, I dedicate this work.

SAMEERA ABUAISH, 2013

Introduction

Parkinson's Disease

Background

Parkinson's disease (PD) was named after James Parkinson, an English physician, in honour of his pioneer description of the disease in 1817 in his monograph "Essay on the Shaking Palsy" (Parkinson, 1817). Fifty years later, a French neurologist Jean-Martin Charcot, who was the first to use the term PD to diagnose the syndrome, had a better characterization of the disease and established it as a distinct neurological disorder (Goetz, 1987).

PD is a neurodegenerative disorder predominantly affecting individuals over sixty years of age. Clinical symptoms primarily affect the motor system, yet as the disease progresses symptoms include non-motor dysfunction as well. The causes of PD are not completely known, however the datasuggest interplay between environmental and genetic factors acting on the background of an ageing brain.

Epidemiology

PD is the second most prevalent neurodegenerative disorder after Alzheimer's disease (AD). Statistics Canada reports that in 2010/2011 PD affected 1% of individuals of both sexes over 65 years of age (Statistics Canada, 2011). PD is known as an age-related disease where the prevalence increases up to 4% in populations over 80 years of age (De Lau and Breteler, 2006). In addition, Statistics Canada reports it as being the 13th leading cause of death in Canada causing 1901 deaths in 2009 (Statistics Canada, 2009).

This increase in number of cases leads to an increase of health care services creating a burden on the health care system. In a report by the Canadian Institute of Health Information, the economic burden associated with PD was estimated by the Public Health Agency of Canada to be $446.8 million between 2000-2001. They also reported a 10% increase in the number of emergency visits in 2006 with 40% of cases being admitted to the hospital for acute care (CIHI, 2007).

Clinical Features

PD presents with a broad spectrum of symptoms; patients typically exhibit four primary motor symptoms, which include bradykinesia combined with at least either resting tremor, rigidity, and/or balance impairment (Jankovic, 2008). Clinically, bradykinesia, referring to slowness of movement, is the most characteristic feature of PD (Berardelli, 2001). It manifests when planning, initiating and executing movement leading to features such as lack of facial expression, monotonic and hypotonic dysarthria, and reduced arm swing while walking (Berardelli, 2001; Jankovic, 2008). Resting tremor is the most recognizable feature of PD that manifests in a unilateral manner and is most prominent in the extremities like the hands and it is described as "Pill-rolling" tremors (Jankovic, 2008). "Cog-wheel" phenomenon is presented when tremors are associated with rigidity (Jankovic, 2008). Rigidity is characterized by increased resistance during passive movement and it is associated with musculoskeletal pain (Jankovic, 2008; Ha and Jankovic, 2012). As the disease progresses, postural imbalance becomes more common and a major issue causing fall injuries such as bone fractures (Williams *et. al.*, 2006).

In addition to the motor symptoms, non-motor symptoms are noted some of which are present in late stages of the disease while others appear pre-motor. The progression of PD leads to neuropsychiatric disorders like dementia, depression, anxiety and psychosis (Solla *et. al.*, 2011). Pre-motor symptoms might occur years ahead of manifestation of any motor symptoms and often fail to get noticed by the patients, yet as the disease progresses they become more severe having more effects on the quality of life of the patients compared to the effects of the motor symptoms. These symptoms include sleep disorder, pain, olfactory disturbance and autonomic dysfunction such as orthostatic hypotension, constipation, neurogenic bladder, and erectile dysfunction (Jankovic, 2008; Ruiz, 2011; Coelho and Ferreira, 2012).

Pathological Features

Underlying the motor symptoms is a progressive loss of dopaminergic (DA) neurons in the substantia nigra *pars compacta* (SNc) within the basal ganglia of the brain (Parent and Parent, 2009). The basal ganglia consists of multiple nuclei that communicate with the cerebral cortex through the thalamus and regulate movement and emotional behaviors(Clark *et. al.*, 2010). Simply described, the basal ganglia are thought to control movement through two pathways: a direct pathway that stimulates the motor cortex and causes movement, and an indirect pathway that inhibits the motor cortex and stops movement. The SNc projects to the striatum to coordinate these pathways using dopamine, which in turn coordinates movement (Clark *et. al.*, 2010). Consequently, the loss of these cells in this system will cause the movement impairment that is observed in PD patients. In fact, over 50% of the DA neurons in the SNc and 80% dopamine content in the striatum is believed to be lost years

before exhibition of any motor symptoms (Fearnley and Lees, 1991). PD is a multisystem disorder that affects other systems in the brain; in addition to DA neuronal death, about 30-50% cell loss is observed in non-DA neurons including the noradrenergic neurons of the locus coeruleus, and the serotoninergic neurons of the raphe nuclei (Zarow *et. al.*, 2003), which could explain some of the non-motor symptoms of the disease.

Another hallmark of PD is the presence of round hyaline neuronal cytoplasmic aggregates called Lewy bodies (LBs) found in the cell soma, as well as spindle-like inclusions called Lewy neurites in the axons and dendrites found in the affected regions (Ferrer *et. al.*, 2011). These inclusions are rich in ubiquitin and contain aggregates of several proteins, with α-synculein being the main component of these aggregates (Spillantini *et. al.*, 1998). LB pathology in PD is a subject of controversy as a staging system has been developed by Braak *et. al.* (2003) to predict the progression of PD from pre-clinical stages to clinical manifestation of motor symptoms and later exhibition of cognitive dysfunction based on the presence of LB lesions in the olfactory bulb and the medulla, expanding to the cortex. However, about 43% of the studied cases did not show the suggested pattern of pathology and 55% cases of wide spread LB pathology did not show any symptoms (Jellinger, 2009).

Treatment

Treatment of PD is always a challenge due to the disease complexity and its progressive nature. Based on the pathological DA cell loss, dopamine replacement therapy is the most common treatment to compensate for the loss of dopamine in the striatum. L-dopa (levodopa), a dopamine precursor, is the standard drug used to treat PD signs and symptoms (Yahr *et. al.*, 1969). The drug is co-administered with decarboxylase inhibitor to prevent the

formation of dopamine in the periphery (Toulouse and Sullivan, 2008). Long-term use of levodopa is associated with development of disabling motor fluctuation that affect up to 80% of patients, which includes the "wearing off" side effect observed when PD symptoms starts to reoccur, dyskinesias, and "on-off" movement patterns shifting from mobility to immobility within seconds (Jankovic and Stacy, 2007). Administration of cathecolamine-O-methyltransferase (COMT) inhibitors and monoamineoxidase-B (MAO-B) inhibitors are also used along with levodopa to reduce the rate of dopamine metabolism and increase levodopaefficiency as the disease progresses (Heikkinen *et. al.*, 2001;Waters *et. al.*, 2004). Dopamine agonists are also prescribed to alleviate PD symptoms, however they are contraindicated due to their hallucinogenic and psychotic side effects (Wood, 2010). Surgical deep brain stimulation of the subthalamic nucleus and the internal globus pallidus was found to improve motor fluctuation associated with levodopa treatment (Deep Brain Stimulation for PD Study Group, 2001). Rehabilitation and physical exercise are also recommended strategies to manage PD (Marchese *et. al.*, 2000; Hirsch *et. al.*, 2003).

Due to the progressive nature of the disease, managing PD becomes more challenging as it advances. Therefore, in addition to treating symptoms, treatments to stop or reverse the progression of the disease are needed. Understanding the causes and mechanisms of the underlying pathology of the disease and its causes offers promise for developing effective treatments.

Etiology

About 95% of PD cases are idiopathic with unknown cause, however aging appears to be the main risk factor for developing PD. More data are suggesting an interplay of environmental and genetic factors in the etiology of PD. The first evidence of the

involvement of environmental toxins in PD was back in 1979 by Davis *et. al.* when illicit drug users were diagnosed with PD when they presented with PD symptoms and later a pathology of degraded SNc. Further analysis of the drug showed contamination with 1-methyl-4-phenyl-1,2,5,6-tetrahydropyr-idine (MPTP) (Langston and Ballard, 1983). MPTP is converted into its active metabolite MPP+, which inhibits complex I of the mitochondrial respiratory chain of the DA neurons. Consequently, this compound was used to develop animal models of PD. This drew attention to other complex I inhibitors like rotenone, found to be a component of pesticides, which coincides with epidemiological studies reporting higher risk of developing PD in farmers and those exposed to pesticides (Wirdefeldt *et. al.*, 2011; Lai *et. al.*, 2002). Head trauma is also reported as a risk factor in developing PD (Wirdefeldt *et. al.*, 2011; Lai *et. al.*, 2002).

Genetics of PD

In addition to environmental factors, several genes have been discovered to cause familial PD and others have been considered as risk factors. Early twin studies have reported low concordance rate between monozygotic and dizygotic twins, indicating no role of genes in PD (Ward *et. al.*, 1983; Marttila *et. al.*, 1988). However, a study screening The National Academy of Sciences/National Research Council (NAS-NRC) World War II Veteran Twins Registry, reported 100% concordance rate in monozygotic twin who developed early onset PD (Tanner *et. al.*, 1999). In another study using [^{18}F] Dopa positron emission tomography (PET) scans to measure striatal dopaminergic function, reported a high concordance rate in monozygotic to dizygotic twins in reduction of [^{18}F] Dopa levels in the striatum after a 7 year follow up (Piccini *et. al.*, 1999). Furthermore, family aggregation studies have reported

a higher relative risk of developing PD when having a first-degree relative with PD, and that relative risk was higher in the early onset cases (Wirdefeldt *et. al.*, 2011).

Autosomal dominant forms

From the aforementionedfamily aggregation studies, the first PD gene was discovered: α-Synuclein (SNCA). Point mutations were found in rare kindred (Kruger et al., 1998; Polymeropoulos et al., 1997; Zarranz et al., 2004). Duplication and triplication of the SNCA containing locus were also found in other families causing an increased dosage of α-synuclein (Singleton *et. al.*, 2003; Ibanez *et. al.*, 2004). These families showed a dominant form of inheritance of PD with high penetrance. Subjects developed PD at an early age, had a rapid disease progression, and most of them developed severe dementia. Neuropathology showed loss of DA neurons in the SNc and wide spread LBs in both the midbrain and cerebrum (Puschmann, 2013). Studies suggest that overexpression of α-synuclein, such as in the multiplication events and the toxic oligomer formations due to the missense mutations, contribute to the pathology of PD (Farrer *et. al.*, 2004; Narhi *et. al.*, 1999).

Mutations in leucine-rich repeat kinase 2 (LRRK2), the focus of this study, were first identified in 2004 (Zimprich *et. al.*, 2004; Paisan-Ruiz *et. al.*, 2004) after the identification of the PARK8 locus in a Japanese family (Funayama *et. al.*, 2002). Later, more studies reported several missense mutations in other families and in sporadic cases as well (Healy *et. al.*, 2008). LRRK2 accounts for up to 13% of familial cases and are found in about 4% of sporadic cases, making it the most common genetic cause of PD (Berg *et. al.*, 2005; Healy *et. al.*, 2008). LRRK2 is further discussed below.

Autosomal recessive forms

Parkin, another PD gene, was discovered in a Japanese family causing autosomal recessive early onset PD (Kitada *et. al.*, 1998). Several mutations and deletion of the Parkin gene have been reported in different families with different ethnic origins (Lucking *et. al.*, 2000; Klein *et. al.*, 2000), and is the major cause of early onset PD (≤20 years of age) and even childhood PD (Lucking *et. al.*, 2000; Kitada *et. al.*, 1998). These cases are characterized by slow progression of the disease and frequent dystonia (Lucking *et. al.*, 2000). Post-mortem investigation showed selective loss of SNc and absence of LBs in most cases (Mori *et. al.*, 1998; Hayashi *et. al.*, 2000). Parkin is an E3 ligase that participates in the ubiquitination and protein degradation process in the cell (Imai *et. al.*, 2000). Mutations cause loss of function and several reports suggest a role for Parkin in mitochondrial function or quality control (Palacino*et. al.*, 2004; Grunewald *et. al.*, 2010).

Several mutations and large deletions in the PTEN-Induced Putative Kinase 1 (PINK1) gene cause autosomal recessive PD with early onset (Valente *et. al.*, 2004; Myhre 2008). They also contribute to early onset sporadic cases (Valente *et. al.*, 2004). Patients present similar characteristics to Parkin patients, but with increased risk of psychological dysfunction (Ibáñez *et. al.*, 2006; Steinlechner *et. al.*, 2007;Samaranch *et. al.*, 2010). Similar to Parkin, Pink1 is shown to be involved in mitochondrial functioning (Kumar *et. al.*, 2011). In fact, Parkin was shown to genetically interact with Pink1 by rescuing the effect of Pink1 deletion in a *Drosophila* model (Clark *et. al.*, 2006).

DJ-1, originally identified as an oncogene, was discovered to have mutations and deletions that cause a rare autosomal recessive PD with an early onset in several families (Bonifati *et. al.*, 2003). DJ-1 is known as an oxidative stress sensor and is suggested to act as

an antioxidant (Kahle *et. al.*, 2009). Recent reports suggest its role in mitochondrial functioning (Irrcher *et. al.*, 2010; Wang *et. al.*, 2012).

In addition to familial mutations, genome-wide association studies (GWAS) have been performed to examine the contribution of genes as potential risk factors in PD. In the past few years, several loci implicated in PD have been identified (Bekris *et. al.*, 2010). These studies promise to further our understanding of the disease.

Leucine-Rich Repeat Kinase 2

Background

LRRK2 is a complex protein encoded by a gene that spans 51 exons and is found on chromosome 12 (Zimprich *et. al.*, 2004). It is a multi-domain protein of 2527 amino acids that includes a GTPase, kinase, and protein-protein interaction domains (Mata *et. al.*, 2006). The GTPase domain is made up of a Ras of complex protein (ROC) domain, and followed by a C-terminal of ROC (COR) domain, which is a characteristic of the ROCO superfamily of proteins (Bosgraaf *et. al.*, 2003). The activity of the GTPase domain is believed to regulate the kinase domain of the protein (Ito *et. al.*, 2007; Taymans *et. al.*, 2011). The remaining domains, leucine rich repeat (LRR), Ankryin like repeats (ANK), and WD40 sequence, are all domains that serve as protein interaction platforms (Figure 1) (Mata *et. al.*, 2006).

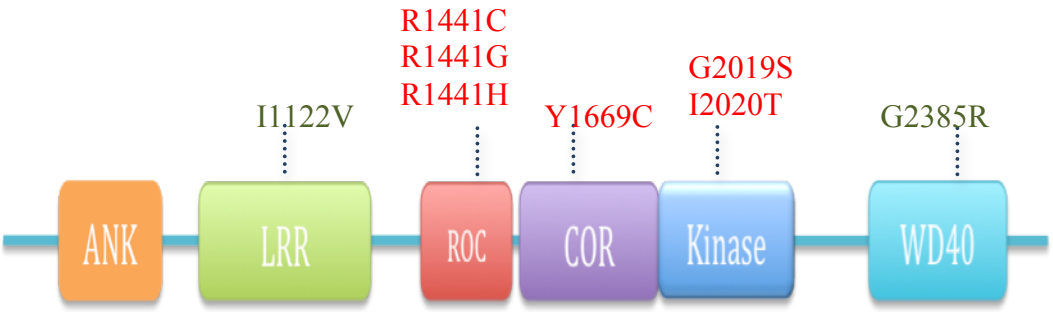

Figure 1 LRRK2 domains and mutations. Two enzymatic domains, kinase and ROC-COR GTPase domain, harboring pathogenic mutations indicated in red. Protein- protein interaction domains: WD40, LRR, and ankryin domains, harboring PD associated mutations indicated in green (adapted from Lu and Tan, 2008)

LRRK2 Mutations

Several missense mutations have been identified in the various domains of LRRK2. Six mutations in the ROC, COR, and kinase domains are considered to be pathogenic (Biskup and West, 2009) (Figure 1). Mutations in the ROC domain (R1441C, R1441G, and R1441H) and COR (Y1699C) are suggested to decrease GTPase activity (Li *et. al.*, 2007; Lewis *et. al.*, 2007). G2019S and I2020T are adjacent mutations found in the kinase domain and lie at its activation loop (Mata *et. al.*, 2006). G2019S is a suggested gain of function mutation and causes an increase in kinase activity (West *et. al.*, 2005), and is the most common pathological mutation with a prevalence of up to 36% in North African Arab and 28% in Ashkenazi Jewish patients (Healy *et. al.*, 2008). Despite the high prevalence of the G2019S mutation, it shows a low, incomplete, and age-dependent penetrance of 28% at the age of 59 and increasing to 74% at 79 years (Healy *et. al.*, 2008). In addition to the pathogenic mutations, few risk factor mutations were identified in genetic association studies including I1122V mutation in the LRR domain and G2385R in the WD40 domain (Figure 1) (Ruiz, 2009). Patients with LRRK2 mutations present with typical clinical PD even indistinguishable from idiopathic PD in the case of G2019S carriers (Ruiz, 2009). However, the neuropathology is heterogeneous with some showing eitherLB inclusions, tau positive neurofibrillary tangles, or pure nigral neurodegeneration with no additional features (Zimprich *et. al.*, 2004; Poulopoulos*et. al.*, 2012).

LRRK2 Structure and Localization

The kinase domain is the most controversial domain of LRRK2 particularly due to its resemblance to different kinase subfamilies. The kinase domain falls under the tyrosine-kinase like family. Different reports have reported resemblance ofLRRK2 to either MLKs (mixed lineage kinases) or RIPK (receptor-interacting protein kinases) subfamilies (Tsika and Moore, 2012). Kinase activity has been measured in several studies by autophosphorylation assays and phosphorylation of the generic kinase substrate myelin basic protein (MBP) (Jaleel *et. al.*, 2007; West *et. al.*, 2005).

The second catalytic domain is the Roc GTPase domain followed by the COR domain which arecharacteristic of the ROCO family and resemble the Ras superfamily (Bosgraaf *et. al.*, 2003). *In vitro*, the GTPase domain binds GTP, however, hydrolysis activity is very weak or undetectable (West *et. al.*, 2007; Li *et. al.*, 2007; Lewis *et. al.*, 2007). The weak GTPase activity could be due to the absence of GTPase-activating proteins (GAPs) in these *in vitro* studies as addition of ArfGAP1 was found to increase GTPase activity and interact with LRRK2 (Stafa *et. al.*, 2012; Xiong *et. al.*, 2012). Several groups have documented the regulation of the kinase activity through GTP binding and hydrolysis, and even regulation of dimerization of LRRK2 (Smith *et. al.*, 2006; Ito *et. al.*, 2007). In addition to the enzymatic domains, several repeat sequences are found and are suggested to act as protein-protein interaction domains, indicating that LRRK2 may act as a scaffold protein (Mata *et. al.*, 2006).

LRRK2 is a cytosolic protein expressed ubiquitously in many tissues and cells. Examining the expression patterns of LRRK2 in human and rat tissues revealed its high expression in the kidney, lung, and lymph nodes (Westerlund *et. al.*, 2008). In the brain it is

highly expressed in DA innervated regions including striatum and cerebral cortex as well as in the cerebellum and hippocampus; however, it is detected at low levels in DA regions such as the SNc and the ventral tegmental area (VTA) (Higashi *et. al.*, 2007; Higashi *et. al.*, 2007). LRRK2 is expressed primarily in neurons and several studies reported its association with several membrane structures such as endosomes, lysosomes, mitochondrial outer membrane, Golgi complex and the endoplasmic reticulum (Biskup *et. al.*, 2006; Dodson *et. al.*, 2011; Hatano *et. al.*, 2007). Specifically, it was found to associate with lipid rafts in these membranous organelles through its WD40 domain suggesting the involvement of protein-protein interaction for membrane targeting (Hatano *et. al.*, 2007). Recent reports show its expression in immune cells, which includesastrocytes and microglia in the brain and macrophages and monocytes in the periphery (Miklossy *et. al.*, 2006 ;Hakimi *et. al.*, 2011).

LRRK2 Function

The function of this complex protein is still unknown and numerous studies have been conducted to try to understand the mechanism by which LRRK2 functions. To date, studies of LRRK2 and its mutations report its involvement in several cellular processes and pathways some of which are cell death, cytoskeletal and vesicular trafficking, autophagy and lysosomal pathways, mitochondrial function, and inflammation.

Cell Death

Several cell culture studies have reported increased cellular toxicity when overexpressing wild type (WT) and pathogenic mutants of LRRK2, the latter expressing

relatively higher toxicity (Smith *et. al.*, 2006; Greggio *et. al.*, 2006; West *et. al.*, 2007; Guo *et. al.*, 2007). This cell toxicity was induced by kinase and/or GTPase activity, as using kinase or GTPase dead constructs attenuated the LRRK2-induced cell death (Smith *et. al.*, 2006; Greggio *et. al.*, 2006; West *et. al.*, 2007; Guo *et. al.*, 2007). In addition, protein-protein interaction domains could play a role in cytotoxicity of LRRK2, as deletion of LRR and WD40 domains was found to rescue G2019S and R1441C mutant cell death (Iaccarino *et. al.*, 2007; Jorgensen *et. al.*, 2009). This suggests that LRRK2 could be mediating its toxicity not only through its enzymatic domains but also its protein-protein interaction domains and their candidate interactors. This induced cytotoxicity is reported to be mediated by apoptosis as measured by TUNEL staining and caspase activation (Smith *et. al.*, 2006; MacLeod *et. al.*, 2006). The apoptosis induction could be through intrinsic pathways dependent on Apaf1 or extrinsic through interaction with Fas-associated protein with death domain (FADD), which is enhanced with the mutant constructs of LRRK2 (Iaccarino *et. al.*, 2007; Ho *et. al.*, 2009). A recent study illustrated that the MKK4-JNK-c-Jun pathway mediated the DA cell loss in LRRK2 (G2019S) transgenic mice (Chen *et. al.*, 2012).

Cytoskeleton and Vesicular Dynamics

Certain human LRRK2 autopsy cases displayed phospho-tau-positive inclusions in the brain (Poulopoulos*et. al.*, 2012). Both *in vitro* and *in vivo* studies reported altered phosphorylation of tau by LRRK2 G2019S (Kawakami *et. al.*, 2012; Melrose *et. al.*, 2010; Lin *et. al.*, 2010). Tau is a microtubule interacting protein that stabilizes the microtubules (Gandhi *et. al.*, 2008). LRRK2 was also shown to interact with α/β tubulin and phosphorylate β-tubulin to increase microtubule stability (Gillardon, 2009). In addition, LRRK2 was

reported to interact with and phosphorylate ERM proteins, which act as anchors for actin filaments to connect to the plasma membrane, causing rearrangement of the cytoskeleton (Parisiaduo *et. al.*, 2009). To further support LRRK2's involvement in cytoskeletal organization it was shown to interact with Rac1, a Rho GTPase that plays a role in actin remodeling (Chan *et. al.*, 2011). This interaction increased Rac1 activity which increased its binding to p21 activated kinase affecting cytoskeletal dynamics. The G2019S and R1441C mutations attenuated this interaction, while I2020T and Y1699C mutations increased binding (Chan *et. al.*, 2011). These reports were suggested to explain the neurite-shortening morphology reported by several studies subsequent to mutant LRRK2 overexpression both *in vivo* and *in vitro* (Chan *et. al.*, 2011; Ramonet *et. al.*, 2011; Kawakami *et. al.*, 2012; Plowey *et. al.*, 2008). However, this reported morphology is controversial, as it is not known if this is a transient or a sustained effect, if it affects dendrites and/or axons, or if it is a delayed outgrowth or retraction of processes.

Cellular localization of LRRK2 indicates its association with lipid rafts, which play a role in signal transduction, vesicular trafficking and endo/exo-cytosis (Hatano *et. al.*, 2007). In fact, a number of LRKK2 mouse models have impaired neurotransmitter release (Melrose *et. al.*, 2010; Li *et. al.*, 2009; Li *et. al.*, 2010). A closer look at presynaptic and postsynaptic properties of cortical neurons in absence of LRRK2 revealed altered synaptic transmission by disturbing vesicular dynamics and distribution within the recycling pool (Piccoli *et. al.*, 2011). Several reports explored the interaction of LRRK2 and endo/exo-cytosis related proteins. For instance, LRRK2 was found to interact with Rab5b in a yeast two hybrid screen.Further,coexpressing active Rab5b rescued the impaired vesicular endocytosis observed by LRRK2 overexpression (Shin *et. al.*, 2008). Furthermore, in a *Drosophila* model

overexpressing LRRK2(G2019S), endocytosis was regulated through endophilinA, which was phosphorylated by LRRK2 (Matta *et. al.*, 2012).

Autophagy and Lysosomal Pathway

Several reports have implicated a role for LRRK2 in autophagy regulation. Cells overexpressing G2019S or R1441C constructs showed autophagic stress characterized by accumulation of autophagic vacuoles and multivesicular bodies (MVBs) which LRRK2 was found to localize with (Alegre-Abarrategui*et. al.*, 2009). As well, *in vivo*, LRRK2 knock out (KO) mice presented impairments in the autophagy-lysosomal pathway in their kidneys, which showed accumulation of lipofuscin granules and altered levels of LC3-II and p62, which are autophagy markers (Tong *et. al.*, 2010). Further analysis indicated that these animals had presented with age-dependent bi-phasic alterations in the autophagic activity in the kidneys indicated by changes in the level of LC3. This was also accompanied by increased levels of lysosomal protein proteases, and progressive accumulation of autolysosomes and lipofuscin granules (Tong *et. al.*, 2012). Deregulation of autophagy is also reported in old mice overexpressing G2019S LRRK2 (Ramonet *et. al.*, 2011) and in induced pluripotent stem cells (iPS) generated from PD patients with the LRRK2 G2019S mutation (Sánchez-Danés *et. al.*, 2012). Some studies have investigated the mechanism by which LRRK2 regulates the autophagy-lysosomal pathway and have reported a Ca^2+ dependent manner of regulation through the Ca^2+/calmodulin dependent protein kinase kinase β/AMP-activated protein kinase (CaMKKβ/AMPK) pathway (Gómez-Suaga et, al,. 2012), while another reported induction of autophagy in G2019S mutant fibroblasts via the MEK/ERK pathway (Bravo-San Pedro *et. al.*, 2013). A recent study explored the role of

LRRK2 in chaperone-mediated autophagy (CMA), where cytosolic soluble proteins are recruited to lysosomes through a translocation complex comprising of the heat shock congnat protein 70 (hsc70) and lysosome-associated membrane protein type 2A (LAMP-2A). They have found that LRRK2 is degraded via CMA. However high levels of WT LRRK2 or G2019S mutant hindered this process and caused accumulation of other CMA substrates like α-synuclein (Orenstein *et. al.*, 2013).

Mitochondrial Function

Reports of LRRK2's involvement in mitochondrial dysfunction were investigated in both PD patients and animal models. Skin autopsies from PD patients with LRRK2 G2019S mutation revealed increased interconnectivity and elongation of mitochondrial morphology as well as decreased membrane potential and ATP levels (Mortiboys *et. al.*, 2010). iPS-derived neuronal cells from fibroblasts of LRRK2 G2019S patients had decreased mitochondrial oxygen consumption rate and increased mobility (Cooper *et. al.*, 2012). Moreover, fibroblasts obtained from LRRK2 G2019S patients in another study have shown increased proton leaking that was mediated by an increase in uncoupling protein expression (Papkovskaia *et. al.*, 2012). Mitochondrial abnormalities were also observed in LRRK2 G2019S transgenic mice featured by presence of condensed mitochondrial aggregates (Ramonet *et. al.*, 2011). Similarly, mitochondrial defects including enlarged mitochondria with dilated cristae were reported in DA neurons, indirect flight muscles (Ng *et. al.*, 2012) and in ommatidia of LRRK2 G2019S transgenic flies (Hindle *et. al.*, 2013). Two studies reported that LRRK2 regulates mitochondrial dynamics causing mitochondrial fragmentation that was mediated through its interaction with dynamin-related protein (Drp1), a

mitochondrial fission factor (Niu *et. al.*, 2012; Wang *et. al.*, 2012).

Inflammation

In addition to being a PD-linked gene, GWAS reported that single nucleotide polymorphisms (SNPs) in LRRK2 were accounted as risk factors in both inflammatory bowle disease (Umeno *et. al.*, 2011) and leprosy (Zhang *et. al.*, 2009), indicating a possible function of LRRK2 in the immune system. Several studies have investigated expression of LRRK2 in the immune cells; consistent reports indicated LRRK2's expression found in human peripheral blood mononuclear cells (PBMC) including B cells, macrophages, and lower levels in T cells. LRRK2 was increased upon induction of monocyte activation induced through IFN-γ stimulation and after exposure to microbial agents (Hakimi *et. al.*, 2011, Thévenet *et. al.*, 2011). Another study identified LRRK2 as an IFN-γ target gene by investigating immune cells *in vitro* and *in vivo*. They also showed LRRK2 induction of NF-κB pathways and its recruitment near pathogens and contribution to the antibacterial activity of the macrophages by regulating reactive oxygen species (ROS) (Gardet *et. al.*, 2010). Exploring LRRK2's role in the brain's immune system revealed LRRK2 expression in microglia upon their activation both *in vitro* and *in vivo*. LRRK2 knockdown or inhibition attenuated the activation of microglia and its pro-inflammatory signaling (Moehle *et. al.*, 2012; Kim *et. al.*, 2012).

Drosophila Model of LRRK2

The aim of this study is to identify genetic interactors of LRRK2 to gain a better understanding of LRRK2's physiological function. To perform this study we employed an *in vivo Drosophila melanogaster* model of LRRK2-linked PD to identify specific, functional genetic/cellular pathways that LRRK2 may be involved in. *Drosophila melanogaster* is a model organism that offers advantages for examining the cellular and molecular pathology of human diseases. Compared to mammalian murine models, where genetic manipulations are costly and time consuming, *Drosophila* models offer advantages by providing a simpler system with a wider array of genetic manipulation, convenience in housing, and relatively short life-span.

Drosophila offers a wide array of genetic tools through the manipulation of P-elements. These are higly mobile pieces of DNA that could inster themseleve randomly into the genome creating mutations (Hummel and Klambt, 2008). In addition, P-elements vectors are used for transgene expression along with reinteroducing a *Drosophila* reporter gene, such as the white gene causing a red eye phenotype (Hummel and Klambt, 2008). Further, P-elements are used along with GAL4/UAS system allowing for ectopic overexpression of a transgene in a tissue specific manner (Brand and Perrimon, 1993). Transgenic flies containing a tissue specific driver fused to the yeast transcription factor GAL4, which can be obtained from the Bloomington *Drosophila* stock centre, are crossed with flies containing the transgene fused to the yeast upstream activator sequence (UAS) (Brand and Perrimon, 1993). The progeny of this cross expresses GAL4, which will activate the expression of the transgene after it binds to the UAS sequence in a GAL4 specific tissue (Figure 2) (Liu *et. al.*, 2008). In addition to the genetic tools that *Drosophila* offer with a fully sequenced genome,

homologous genes associated with human PD can be found in the *Drosophila* genome with high levels of conservation (Reiter *et. al.*, 2001). The adult fly has a characterized dopaminergic system found in 6 clusters in the brain (Friggi-Grelin *et. al.*, 2003) and locomotion could be assesed offering a good model in which to study PD. This makes *Drosophila* a great model to help us study LRRK2's function in an *in vivo* system.

Drosophila have a LRRK2 homolog (dLRRK) that is composed of a LRR, ROC, and a kinase domain, which are 38%, 46%, and 44% identical with those of human LRRK2, respectively (Lee *et. al.*, 2007). Characterization of dLRRK was reported to have different effects in different studies. For instance, while overexpressing WT dLRRK in DA neurons did not show any alteration in numbers, overexpressing pathogenic dLRRK mutations similar to that of PD mutations have shown conflicting results (Imai *et. al.*, 2008; Lee *et. al.*, 2007). On the one hand, overexpressing the COR or kinase equivalent mutations of LRRK2 have lead to significant reduction in some DA clusters and decrease of DA content in the brain (Imai *et. al.*, 2008), while overexpressing WT or the ROC equivalent mutation have not caused any alteration of DA numbers (Lee *et. al.*, 2007). Deletion of dLRRK has also shown different effects. Two studies reported no detectable change in DA neurons in dLRRK deletion lines (Wang *et. al.*, 2008; Imai *et. al.*, 2008), while increased DA content in the brain was observed in one study (Imai *et. al.*, 2008). In another study, although they did not observe decrease in tyrosine hydroxylase (TH) positive cell numbers, they reported a decrease in the TH staining intensity and a shrunken cell morphology which correlated with the locomotor defects that they observed (Lee *et. al.*, 2007).

Further, other studies examined the overexpression of either WT human LRRK2 (hLRRK2) or mutant hLRRK2 in *Drosophila*. A previous study conducted in the Park lab by

Venderova *et. al.* (2009) was able to generate a transgenic fly overexpressing wild type or mutant (I1122V, Y1699C, and I2020T) hLRRK2. The flies were generated using the GAL4/UAS system, which allows for ectopic overexpression of hLRRK2 in a tissue specific manner (Brand and Perrimon, 1993). The results showed a loss of DA neurons and locomotor deficiency in the flies overexpressing wild type or mutants, with the I2020T mutation having the most prominent effect (Venderova *et. al.,* 2009). Also, they were able to examine the effect of hLRRK2 overexpression in the eyes of the transgenic flies using an eye-specific Glass Multimer Reporter (GMR) driver. At room temperature, no eye defects were observed upon hLRRK2 overexpression. However, due to the sensitivity of the GAL4/UAS system to change in tempreture, flies raised at 29°C, showed an abnormal eye phenotype that showed a loss of pigmentation, surface roughness and presence of black lesions. These black lesions are a marker of ommatidial degeneration, which has been used to assay neurodegeneration in several other studies (Wang *et. al.*, 2006; Kanao *et. al.*, 2010).

Study Rational

As discussed earlier, LRRK2 is implicated in many different cellular processes, and mutations in LRRK2 cause several abnormalities in different cellular systems. However, the function and the pathways that LRRK2 participate in are poorly understood. Therefore, identifying *in vivo* interactors of LRRK2 will help understand its biological and pathological function and the cellular pathways that it is involved in. Using the fly model generated by Venderova *et. al.*, (2009), which presents with a readily identifiable phenotype in the eye, will allow for a convenient and quick screen for potential genetic interactions between LRRK2 and other genes using a suppressor/enhancer screen.

The ectopic overexpression of hLRRK2 in the eye of the flies grown and housed at 29°C provides baseline phenotype that will either be enhanced or suppressed in the absence of potential hLRRK2 interactors. In order to perform this analysis, a set of commercially available well-defined deficiency lines from the Bloomington *Drosophila* Deficiency Kit are used. These deficiency lines are flies with hemizygous deletions. Deficiency lines are crossed with the transgenic flies that are overexpressing hLRRK2(I2020T) which have been generated in the Venderova *et. al.* (2009) paper. If hLRRK2(I2020T) potentially interacts with one of the deleted genes in a specific deficiency line, we expect to observe an enhancement or a suppression of the baseline eye phenotype (Figure 3) (see methods for eye scoring criteria). In order to narrow down the candidate genes, multiple subregions of the interacting deficiency region are ordered and screened for modification. Finally, genes of the interacting subregions are screened using gene disruption, RNAi, and overexpression lines; this way the interacting genes were screened in an unbiased fashion (Figure 4).

The Bloomington *Drosophila* centre third chromosome's deficiency kit contains 179 fly lines. These lines were crossed with GMR>hLRRK2(I2020T) transgenic flies and GMR>+ control flies at 25°C and 29°C. The screen has revealed several interacting genes. These interactors were examined with other hLRRK2 transgenic lines. In addition, a number of these interactions has been investigated in the DA system of the fly for modification of the TH cell loss obsereved in the hLRRK2(I2020T) transgenic flies.

The wide spectrum of LRRK2 functions explored in the literature and its effects on the different processes in the cells, calls for the identification of respective interactors to explain the nature of the role that LRRK2 plays in these different pathways. Therefore, this study is an excellent opportunity to identify these candidates in a well-characterized*in vivo*

model of PD. The genetic interactions identified in this screen could be further explored within the same model or other *in vivo* (i.e. mammalian model) or *in vitro* (i.e. cell culture) models to get a mechanistic explanation of these interactions.

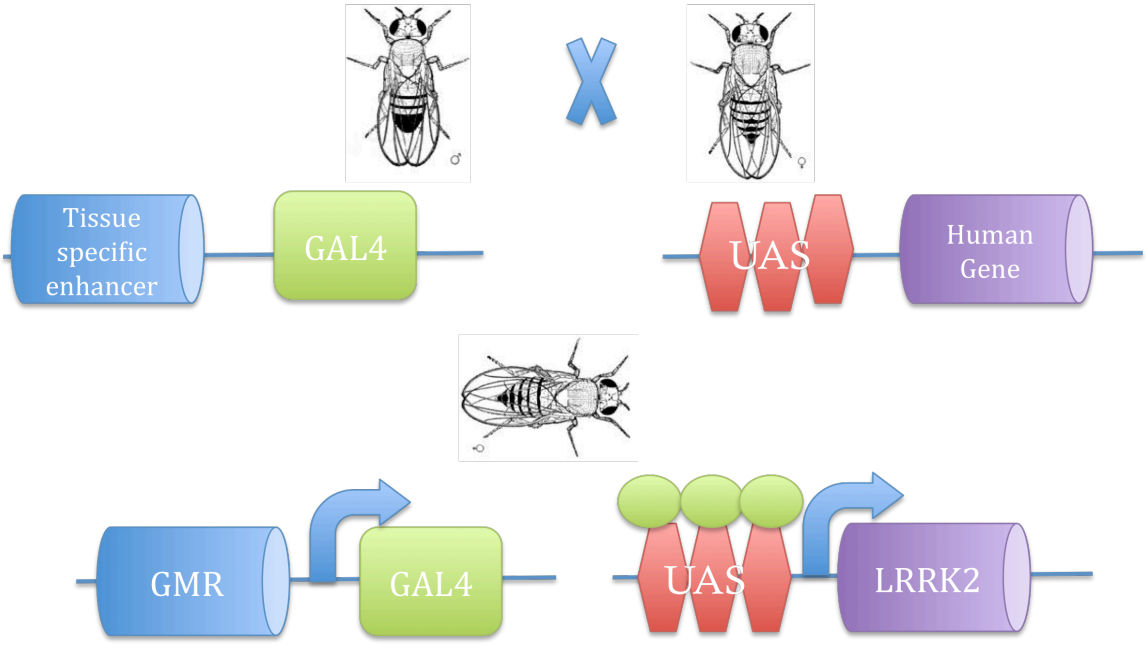

Figure 2 GAL4/UAS system used to create transgenic flies overexpressing hLRRK2 in a tissue specific manner. GMR is an eye specific enhancer that drives the expression of GAL4, which binds to the UAS leading to the ectopic expression of hLRRK2 in the eye.

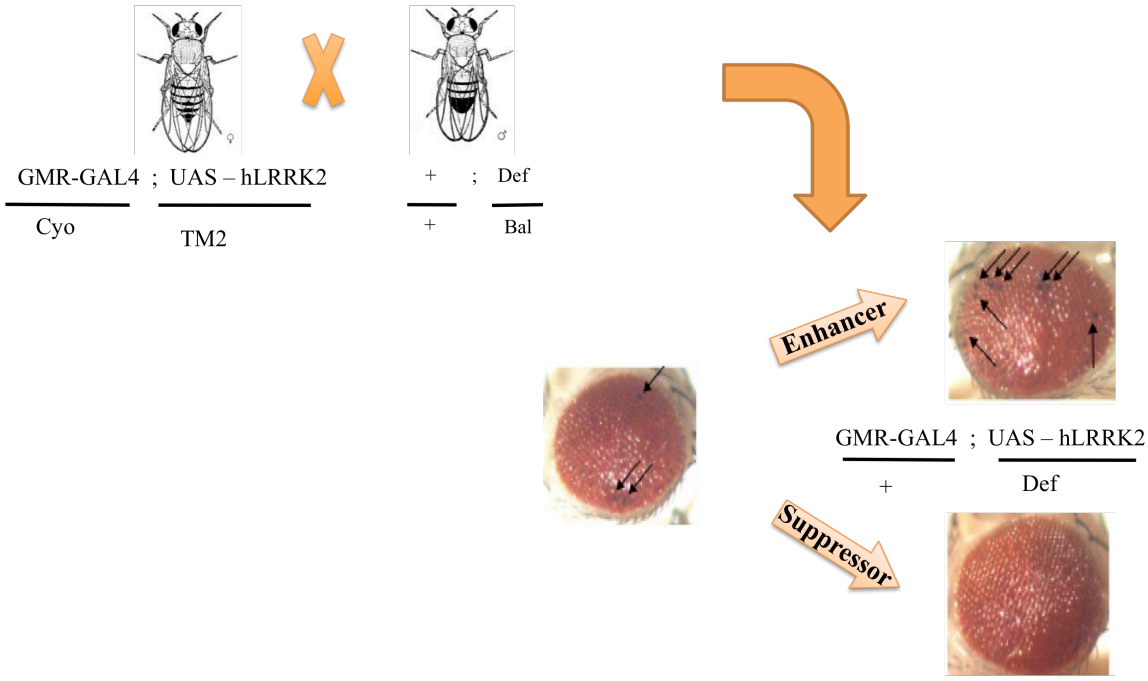

Figure 3 Suppressor/enhancer screen used to identify LRRK2 interactors. hLRRK2(I2020T) transgenic flies are crossed with deficiency lines and the progeny of this cross is screened for modification of the of the baseline phenotype seen in hLRRK2(I2020T) transgenic flies (Modified from Venderova *et. al.* (2009).

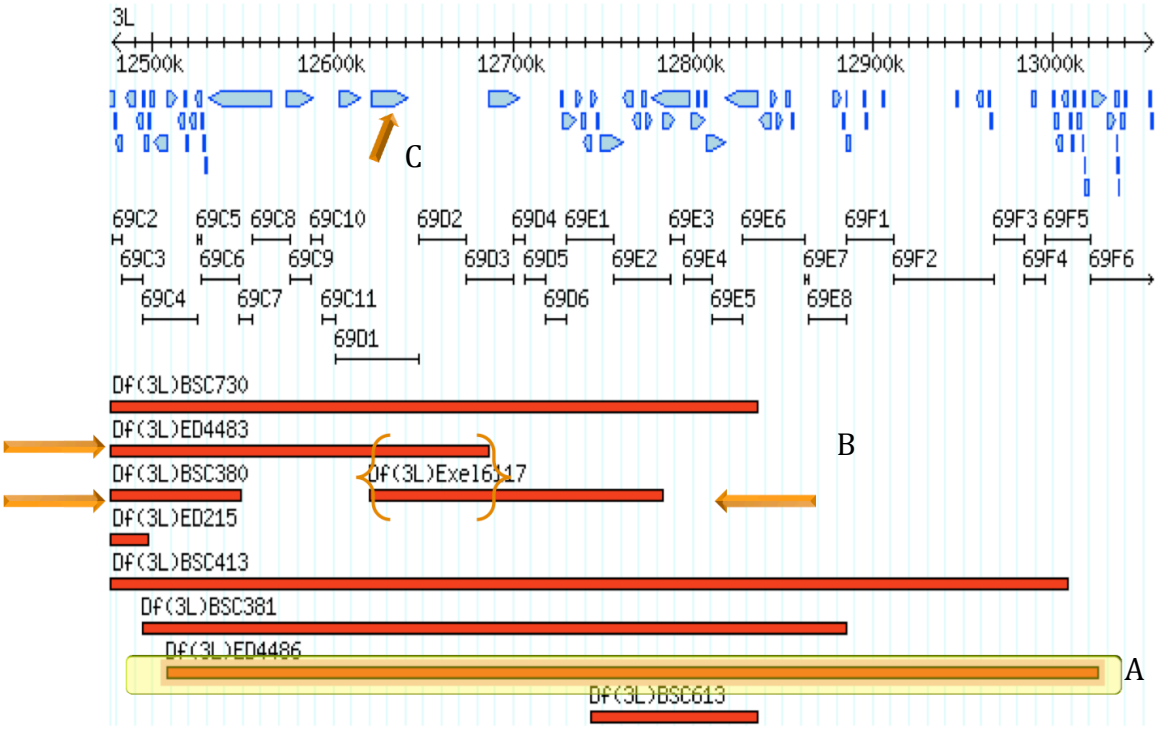

Figure 4 Methodology used to identify interacting genes. (A) Hemizygous defiecncy line showing modification. (B) Subregions with hemizygous deletion are further screened for modification. (C) Genes from interacting subregions are later screened using gene disruption, RNAi, and overexpression lines.

Results

A suppressor/enhancer screenwas performed to identify possible genetic interactors of LRRK2. Transgenic flies overexpressing hLRRK2(I2020T) in the compound eye under the GMR driver (GMR>hLRRK2-I2020T) presented with eye defects when raised at 29°C. These defects were characterized by presence of black lesions, loss of pigmentation and roughness of the eye surface (Figure 4). Modification of this phenotype by enhancement or suppression was indicative of a genetic interaction. In order to measure this modification, a scoring system was developed for assessment of eye damage (Figure 5). The hLRRK2 transgenic flies show no phenotype at 25°C, therefore only an enhancement is expected in case of an interaction increasing from 1- survival. On the other hand, the transgenic flies show a baseline phenotype at 29°C characterized by rough eye, loss of pigmentation and presence of black lesions at level 2.5. An enhancement of this phenotype is scored from 3 – survival, while suppression is scored by 2 – 0.

GMR>hLRRK2(I2020T) flies were crossed with the 3rd chromosome deficiency (Def) kit lines obtained commercially from the Bloomington *Drosophila* stock centre. The kit has 179 genomic deletion lines covering about 97% of the 3rd chromosome. At 29°C, the GMR>hLRRK2(I2020T) fly is crossed with each deficiency line along with three other control crosses per deficiency line (Table 1). GMR>hLRRK2(I2020T) control has an eye damage phenotype at a level 2.5, which is termed the baseline phenotype to compare to the experimental crosses. The deficiency control (GMR>Def) was generated to examine if there is any interaction between the GMR driver and the deficiency line. The experimental cross (GMR>hLRRK2/Def) was screened to examine for any modification of the baseline phenotype. Six interacting regions that caused suppression were discovered from the original large deletion screen. Sub-regions and genes within respective deleted regions of the

discovered interactions from the different deficiency lines are addressed using gene specific RNAi and gene deletion lines. Finally, five interacting genes were identified causing suppression of the eye phenotype (Table 2).

In parallel with the 29°C screen, a 25°C screen was carried out. At 25°C GMR>hLRRK2(I2020T) flies lack eye defects, hence identification of subtle enhancements possibly missed in the 29°C is more feasible. The screen at 25°C is performed by crossing GMR>hLRRK2(I2020T) transgenic flies with the different deficiency lines and raised in a 25°C incubator. The experimental crosses (GMR>hLRRK2/Def) were compared with two control crosses (Table 1). From this screen 24 regions were identified as modifiers from which later were narrowed to 17 specific interacting genes that showed modification (enhancement) (Table 2).

In total, 22 interacting genes were identified by crossing different gene disruption lines (GD) such as RNAi, gene deletion, or over-expression lines with the GMR>hLRRK2(I2020T) fly. An example of an enhancement at 25°C and a suppression at 29°C are represented in figure 6 and figure 7, respectively. The amino acid sequences of these genes are then BLASTED via NCBI online to identify their human homolog (Table 2). In addition, weaker interactions of 16 genes have been identified from the screen (Table 3). These interactions showed mainly loss of pigmentation and no presence of black lesions at 25°C scored at a level 2, while a mild suppression scored between level 1-2 was observed by the genes screened at 29°C.

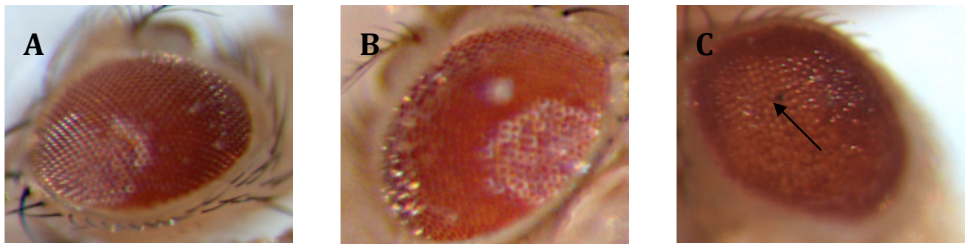

Figure 5 Over-expression of hLRRK2(I2020T) in the compound eyes causes eye defects at 29°C. (A) UAS-hLRRK2(I2020T) flies show no eye phenotype. (B) GMR-GAL4 fly shows mild roughness of eye surface while GMR>hLRRK2(I2020T) presents black lesion (arrow), loss of pigmentation, and roughness of the eye (C).

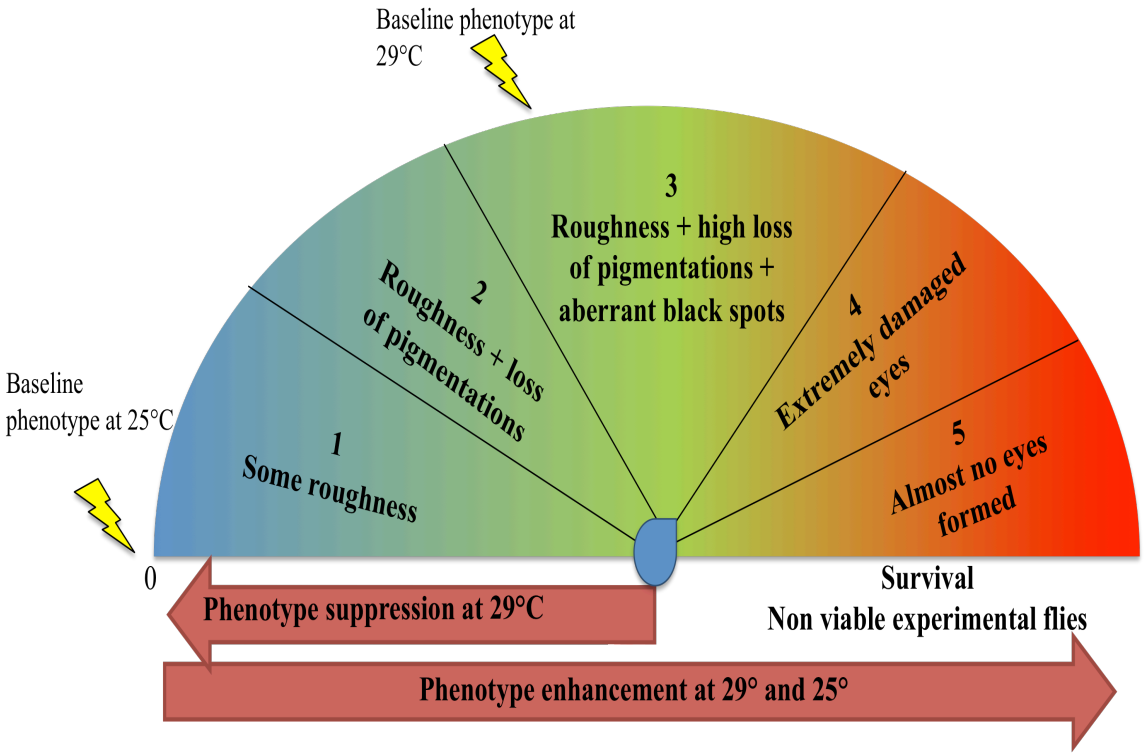

Figure 6 A schematic presentation of the scoring criteria used to analyse the eye phenotype in the screen. The modifier levels are presented along with their expected eye phenotype. The arrows show the increase of interaction with the increase of either enhancement or suppression**.**

Table 1 Genetic crosses summarized, showing the experimental cross in red along with three control crosses.

X	Def ; + Bal. +	+ ; UAS-LRRK2 (I2020T + UAS-LRRK2(I2020T)	W1118 ; + ; + + +
GMR-GAL4 ; UAS-hLRRK2 (I2020T) CYO TM6B	GMR-GAL4 ; UAS-LRRK2(I2020T) + Def	N/A	N/A
GMR-GAL4 ; + BC +	GMR-GAL4 ; Def + +	GMR-GAL4 ; UAS-hLRRK2 (I2020T) + +	GMR-GAL4 ; + + +

Def= deficiency, Bal= balancer.

Figure 7 A 25°C screen cross showing an enhancement of the eye phenotype. (A) GMR>hLRRK2(I2020T) control cross with a normal eye phenotype. (B) GMR>GD control cross that shows a normal eye phenotype. (C) Experimental cross (GMR>hLRRK2(I2020T)/GD) showing an enhancement at level 3 with loss of pigmentation, roughness of the eye, and presence of black spot indicated.

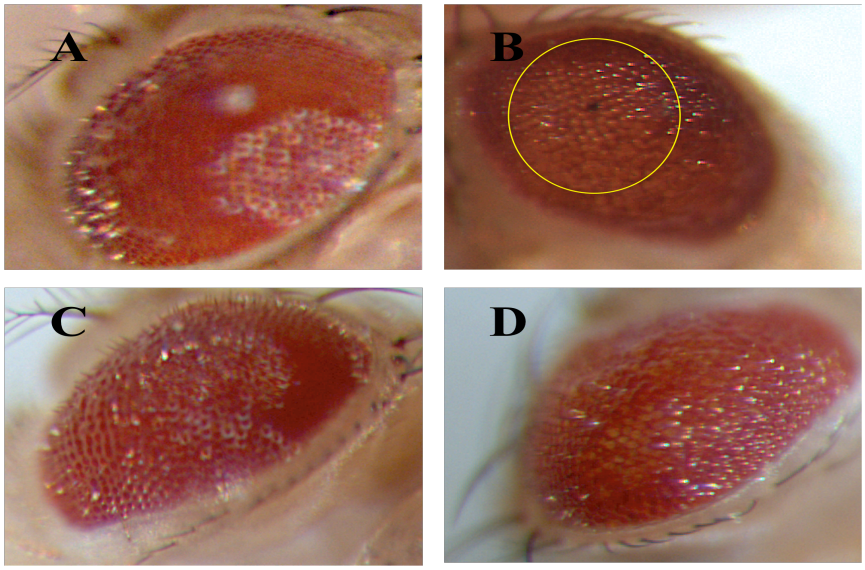

Figure 8 A 29°C screen cross showing a suppression of the eye phenotype. (A) GMR>+ control cross showing some roughness in the eye. (B) GMR>hLRRK2(I2020T) control cross with a baseline eye phenotype at level 3. (C) GMR>Def control cross that shows some roughness. (D) Experimental cross (GMR>hLRRK2(I2020T)/GD)showing a suppression at a level 2 with mild loss of pigmentation and some roughness of the eye.

Table 2 Summary of the *Drosophila* hit genes revealed from the screen on the third chromosome and their BLAST results from NCBI for their closest human homolog.

Gene	Human Homolog[a]	Modification
CG2003	Vesicular glutamate transporter 1	En
Modulo	RNA binding Proteins	En
CG9614	HEPARAN SULFATE 2-O-SULFOTRANSFERASE 1	En
mlp84b	Cysteine and glycine-rich protein 2	En
CG1091	Terminal uridylyltransferase 7	En
vtd	RAD21	En
rpd3	HDAC2	En
Cyp1	Cyclophilin A	En
gef64c	Intersectin 1	En
CG32266	Sorbin and SH3 domain-containing protein 2	En
αTub85E	Tubulin alpha- 1C	En
Rasp	HHAT	En
aly	Lin-9	En
CG5191	FAAH	En
tx	Protein atonal homolog/ neurogenic differentiation factor 2	En
CG6154	Dipeptidase 1	En
beat-VII	MUC18[b]	En
CG10809	Ankyrin repeat domain-containing protein 54[b]	Su
rdl	Glycine receptor	Su
Atpα	Na+/K+ ATPase	Su
CG7028	Serine/threonine-protein kinase PRP4 homolog	Su
cdk8	Cyclin-dependent kinase 8	Su

a- All lines were loss of function transgenic lines unless indicated
b- Over-expression lines
En=Enhancement, Su=Suppression

Table 3 Summary of the *Drosophila* weak interactors revealed from the screen on the third chromosome and their BLAST searched human homolog genes.

Gene	Human Homolog[a]	Modification
heph	Polypyrimidine track-binding protein	En
CG4335	Trimethyllysine dioxygenase	En
Ash1	Histone-lysine N-methyltransferase ASH1L	En
CG4159	Pseudouridine synthase 1	En
eIF2Balpha	Translation initiation factor eIF-2B	En
sprouty	Protein sprouty homolog 2[b]	En
axo	Contactin-associated protein-like 2[b]	En
CG6821	LRRFIP2	En
kurtz	Beta-arrestin-1	En
lds	Transcription termination factor 2	En
CG43318	ATR-interacting protein isoform 1	En
wah	KAT8 regulatory NSL complex	En
CG6420	WD repeat-containing protein 20	En
CG5484	YIF1B	En
orco	Syntaxin-11	Su
CG12951	Plasma Kallikrein	Su

a- All lines were loss of function transgenic lines unless indicated
b- Over-expression lines
En=Enhancement, Su=Suppression

In order to confirm the interactions identified above and to further explore whether the interaction was dependent upon specific LRRK2 mutations, the identified hits were additionally screened with WT and I1122V (LRR domain mutation) transgenic flies (Table 4). Five of the hits (CG2003, vtd, aly, tx, Atpα) screened with WT LRRK2 transgenic flies showed comparable modification to the I2020T screen, twelve (Modulo, CG9614, CG1091, rpd3, Cyp1, gef64c, αTub85E, CG5191, CG6154, CG10809, rdl, CG7028) showed no modification, one (cdk8) did not survive, and four (mlp94b, CG32266, Rasp, beat-VII) have yet to be determined. Furthermore, seven interactors (CG9614, vtd, Cyp1, CG5191, tx, CG6154, beat-VII) showed comparable modification to I2020T when screened with the I1122V transgenic fly, while ten (CG2003, Modulo, CG1091, rpd3, gef64c, aly, rdl, Atpα, CG7028, cdk8) showed no modification, and four (mlp84b, CG32266, αTub85E, Rasp) have not been determined yet. Interestingly, one (CG10809) showed an opposite modification to I2020T. Moreover, weak interactors were also screened with WT and I1122V transgenic flies (Table 5). Out of the 16 weak interactors, three genes (heph, sprouty,lds) when screened with the WT transgenic fly showed comparable results to the I2020T screen, one (CG6420) had a stronger interaction, nine (CG4335, Ash1, CG4159, eIF2Balpha, CG6821, CG43318, wah, CG5484, orco) had no interaction, and three (axo, kurtz, CG12951) have not been determined yet. In addition, four (sprouty, CG6821, wah, orco) genes displayed similar results to the I2020T screen when crossed with I1122V transgenic flies, three (heph, CG6420, CG5484) presented a stronger interaction, six (CG4335, Ash1, CG4159, eIF2Balpha, lds, CG43318) had no interaction, and three (axo, kurtz, CG12951) have not been screened yet.

Table 4 Comparison of the interactions of the hit genes with WT, I2020T, and I1122V hLRRK2 transgenic flies.

Gene	Human Homolog[a]	I2020T	WT	I1122V
CG2003	Vesicular glutamate transporter 1	En	En	N
Modulo	RNA binding Proteins	En	N	N
CG9614	HEPARAN SULFATE 2-O-SULFOTRANSFERASE 1	En	N	En
mlp84b	Cysteine and glycine-rich protein 2	En	ND	ND
CG1091	Terminal uridylyltransferase 7	En	N	N
vtd	RAD21	En	En	En
rpd3	HDAC2	En	N	N
Cyp1	Cyclophilin A	En	N	En
gef64c	Intersectin 1	En	N	N
CG32266	Sorbin and SH3 domain-containing protein 2	En	ND	ND
αTub85E	Tubulin alpha- 1C	En	N	ND
Rasp	HHAT	En	ND	ND
aly	Lin-9	En	En	N
CG5191	FAAH	En	N	En
tx	Protein atonal homolog/ neurogenic differentiation factor 2	En	En	En
CG6154	Dipeptidase 1	En	N	En
beat-VII	MUC18[b]	En	ND	En
CG10809	Ankyrin repeat domain-containing protein 54[b]	Su	N	En
rdl	Glycine receptor	Su	N	N
Atpα	Na+/K+ ATPase	Su	Su	N
CG7028	Serine/threonine-protein kinase PRP4 homolog	Su	N	N
cdk8	Cyclin-dependent kinase 8	Su	Survival	N

a. All lines were loss of function transgenic lines unless indicated
b. Over-expression lines

En=Enhancement, Su=Suppression, N=No interaction, ND=Not determined

Table 5 Comparison of the interactions of the weak interactors with WT, I2020T, and I1122V hLRRK2 transgenic flies.

Gene	Human Homolog[a]	I2020T	WT	I1122V
heph	Polypyrimidine track-binding protein	En	En	En+
CG4335	Trimethyllysine dioxygenase	En	N	N
Ash1	Histone-lysine N-methyltransferase ASH1L	En	N	N
CG4159	Pseudouridine synthase 1	En	N	N
eIF2Balpha	Translation initiation factor eIF-2B	En	N	N
sprouty	Protein sprouty homolog 2[b]	En	En	En
axo	Contactin-associated protein-like 2[b]	En	ND	ND
CG6821	LRRFIP2	En	N	En
kurtz	Beta-arrestin-1	En	ND	ND
lds	Transcription termination factor 2	En	En	N
CG43318	ATR-interacting protein isoform 1	En	N	N
wah	KAT8 regulatory NSL complex	En	N	En
CG6420	WD repeat-containing protein 20	En	En+	En+
CG5484	YIF1B	En	N	En+
orco	Syntaxin-11	Su	N	Su
CG12951	Plasma Kallikrein	Su	ND	ND

a- All lines were loss of function transgenic lines unless indicated
b- Over-expression lines
En=Enhancement, En+= Stronger enhancement Su=Suppression, N=No interaction, ND=Not determined

In order to study these interactions in a PD context, we explored the effects of the interactors identified in the original screen in the DA system of the fly. First, we generated transgenic flies that overexpress hLRRK2(I2020T) in the DA system using the *Dopa decarboxylase* (Ddc) driver, and then assessed the effect of this model on the DA system by staining the brains for TH positive DA clusters. This DA driver was utilized instead of the TH driver originally published in Venderova *et. al.* (2009) in our laboratory because of the position of the TH driver on the 3rd chromosome along with hLRRK2. The Ddc>hLRRK2(I2020T) were raised for ten days at 25°C and later whole brains were dissected and stained for TH and cells were quantified under confocal microscopy. The Ddc>hLRRK2(I2020T) whole brain mounts showed less TH staining compared to control Ddc>+ brains, this was parallel to the loss observed in TH>hLRRK2(I2020T) (Venderova et. a., 2009) (Figure 8).

After confirming the effects of hLRRK2(I2020T) on the DA system of the *Drosophila*, Ddc>hLRRK2(I2020T) transgenic flies were crossed with the different interacting genes and the brains were analyzed for modification of the cell loss phenotype observed in the Ddc>hLRRK2(I2020T) transgenic flies. The brains of ten-day-old flies were dissected, stained for TH and cells were quantified under confocal microscopy. The experimental cross (Ddc>hLRRK2(I2020T)/GD) was compared to all of Ddc>hLRRK2(I2020T), Ddc>+, and Ddc>DG control flies. To date, 14 of the interacting genes were crossed and analyzed for modification (Table 6). Eleven interacting genes showed modifications by rescuing the TH cell loss observed in the Ddc>hLRRK2(I2020T) transgenic flies (Figure 9-19), while three interacting genes showed no modifications (Figure 20-22)

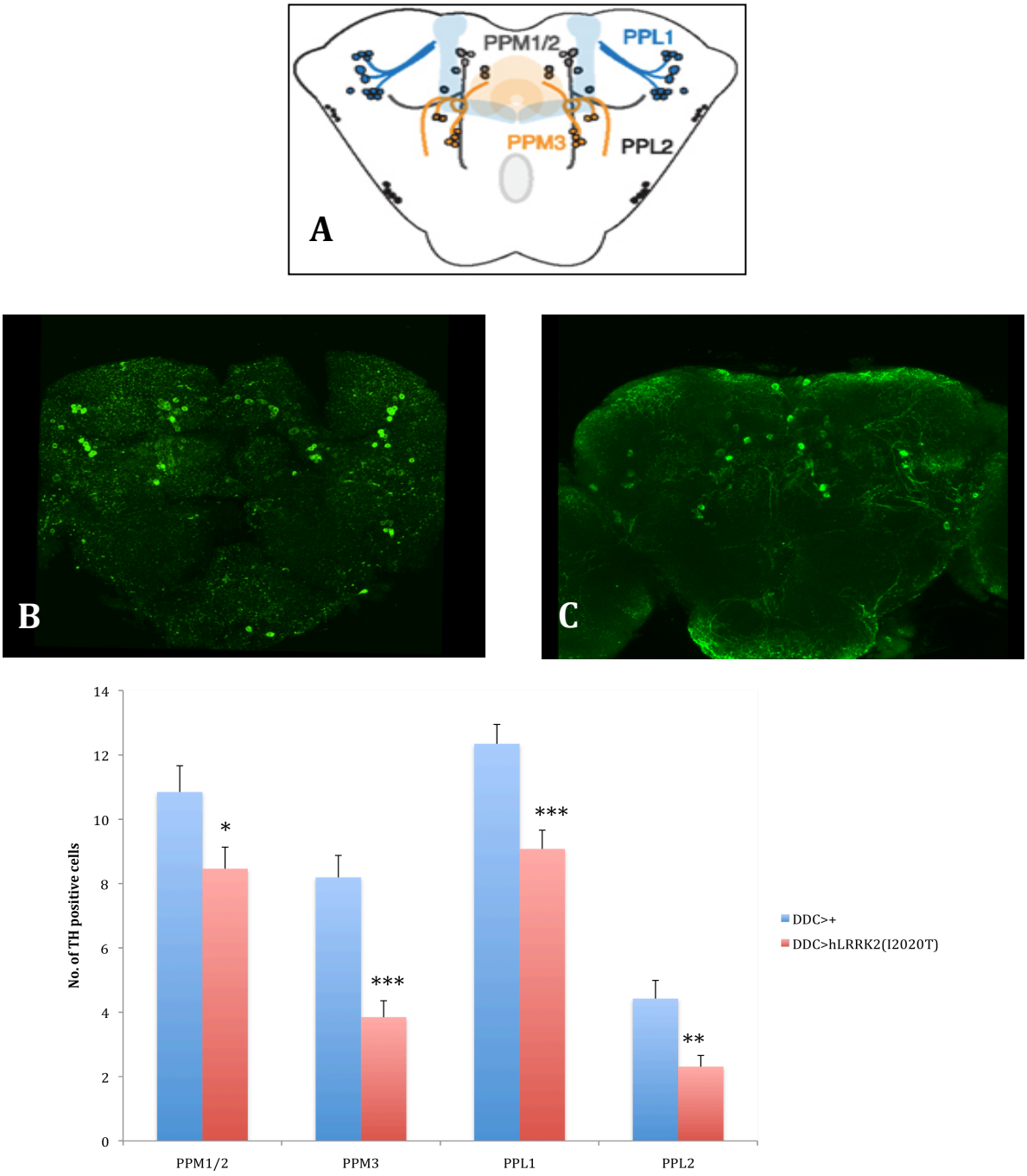

Figure 9 Loss of DA neurons in hLRRK2(I2020T) transgenic flies using Ddc-*GAL4* driver.

(A) Schematic representation of the posterior DA clusters in *Drosophila* protocerebral posteriolateral dopaminergic cluster neuron 1/2 (PPL1/2) and protocerebral posteriomedial dopaminergic neuron 1/2/3 (PPM1/2/3) (White *et. al.*, 2010). Representative images of Tyrosine hydroxylase (TH) staining in DA clusters in Ddc control flies (B) and Ddc>hLRRK2(I2020T) (C). (D) Quantification of the DA clusters in (n=13 Ddc>+ and n=13 Ddc>hLRRK2(I2020T)). Unpaired two-tail Student's t-test.

Table 6 Summary of the *Drosophila* interacting genes revealed from the screen on the third chromosome and their BLAST searched human homolog genes along with the type of modification in the eye and the DA system.

Gene[a]	Human Homolog	Eye Modification	DA Modification
CG2003	Vesicular glutamate transporter 1	En	ND
Modulo	RNA binding Proteins	En	N
CG9614	HEPARAN SULFATE 2-O-SULFOTRANSFERASE 1	En	ND
mlp84b	Cysteine and glycine-rich protein 2	En	ND
CG1091	Terminal uridylyltransferase 7	En	Su
vtd	RAD21	En	Su
rpd3	HDAC2	En	ND
CG32236	Cyclophilin A	En	Su
gef64c	Intersectin 1	En	Su
CG32266	Sorbin and SH3 domain-containing protein 2	En	ND
αTub85E	Tubulin alpha- 1C	En	Su
Rasp	HHAT	En	ND
aly	Lin-9	En	Su
CG5191	FAAH	En	ND
tx	Protein atonal homolog/ neurogenic differentiation factor 2	En	Su
CG6154	Dipeptidase 1	En	Su
beat-VII	MUC18[b]	En	ND
CG10809	Ankyrin repeat domain-containing protein 54[b]	Su	Su
rdl	Glycine receptor	Su	Su
Atpα	Na+/K+ ATPase	Su	Su
CG7028	Serine/threonine-protein kinase PRP4 homolog	Su	N
cdk8	Cyclin-dependent kinase 8	Su	N

a- All lines were gene disruption lines unless indicated
b- Over-expression lines
En=Enhancement, Su=Suppression, N=No interaction, ND=Not determined

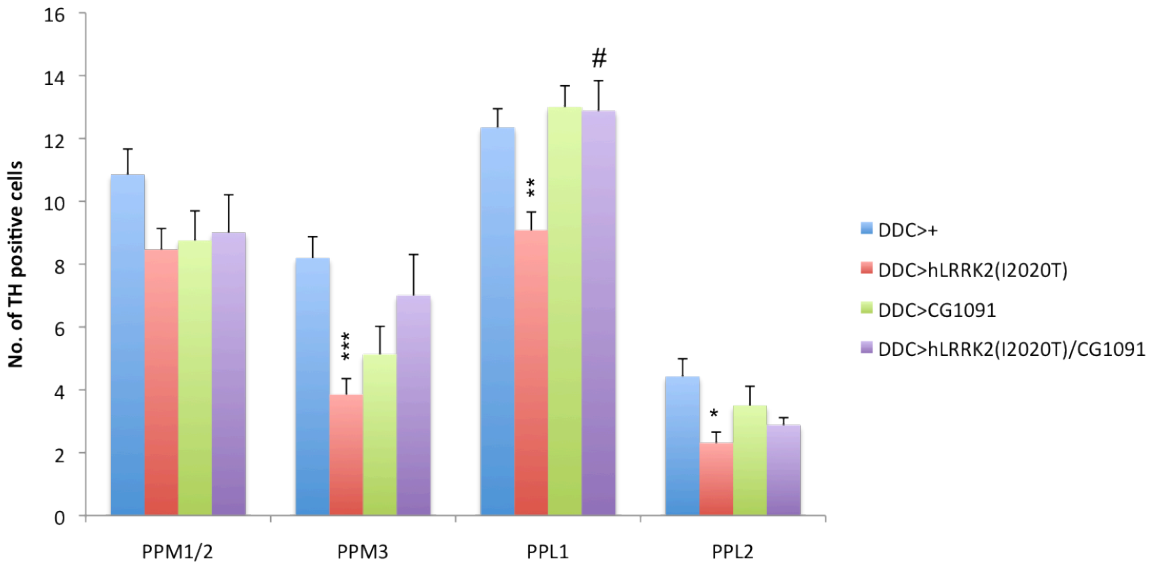

Figure 10 Rescue of DA cell loss mediated by CG1091. Quantification of TH-positive neurons per DA cluster in 10-day-old flies of the indicated genotypes. Rescue of DA loss in the PPL1 DA cluster of Ddc>hLRRK2(I2020T)/CG1091 (n=4). The same Ddc>+ (n=13) and Ddc>hLRRK2(I2020T) (n=13) sample have been used among the several experiments. Statistically significant differences between the control Ddc>+ and Ddc>hLRRK2(I2020T) transgenic lines are indicated: *, $P \leq 0.05$, **if $P \leq 0.01$, *** if $P \leq 0.001$. Statistically significant differences between Ddc>hLRRK2(I2020T) and Ddc>hLRRK2(I2020T)/ CG1091 flies are indicated: #, $P \leq 0.05$. All data were analyzed by one-way ANOVA followed by Bonferonni's post hoc test.

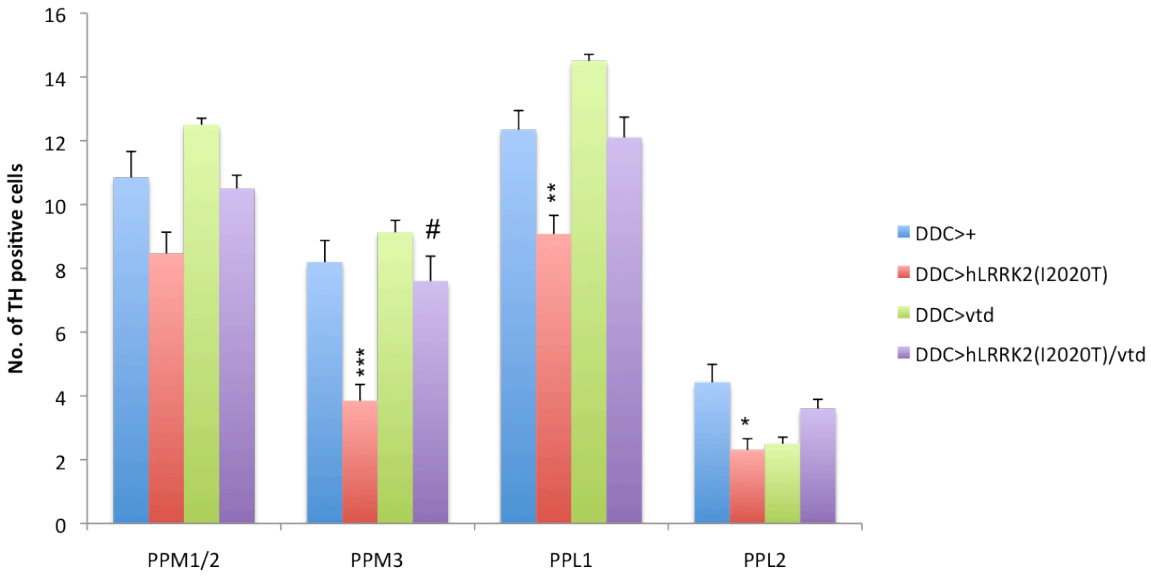

Figure 11 Rescue of DA cell loss mediated by vtd. Quantification of TH-positive neurons per DA cluster in 10-day-old flies of the indicated genotypes. Rescue of DA loss in the PPM3 DA cluster of Ddc>hLRRK2(I2020T)/vtd (n=5). The same Ddc>+ (n=13) and Ddc>hLRRK2(I2020T) (n=5) sample have been used among the several experiments. Statistically significant differences between the control Ddc>+ and Ddc>hLRRK2(I2020T) transgenic lines are indicated: *, $P \leq 0.05$, **if $P \leq 0.01$, *** if $P \leq 0.001$. Statistically significant differences between Ddc>hLRRK2(I2020T) and Ddc>hLRRK2(I2020T)/vtd flies are indicated: #, $P \leq 0.05$. All data were analyzed by one-way ANOVA followed by Bonferonni's post hoc test.

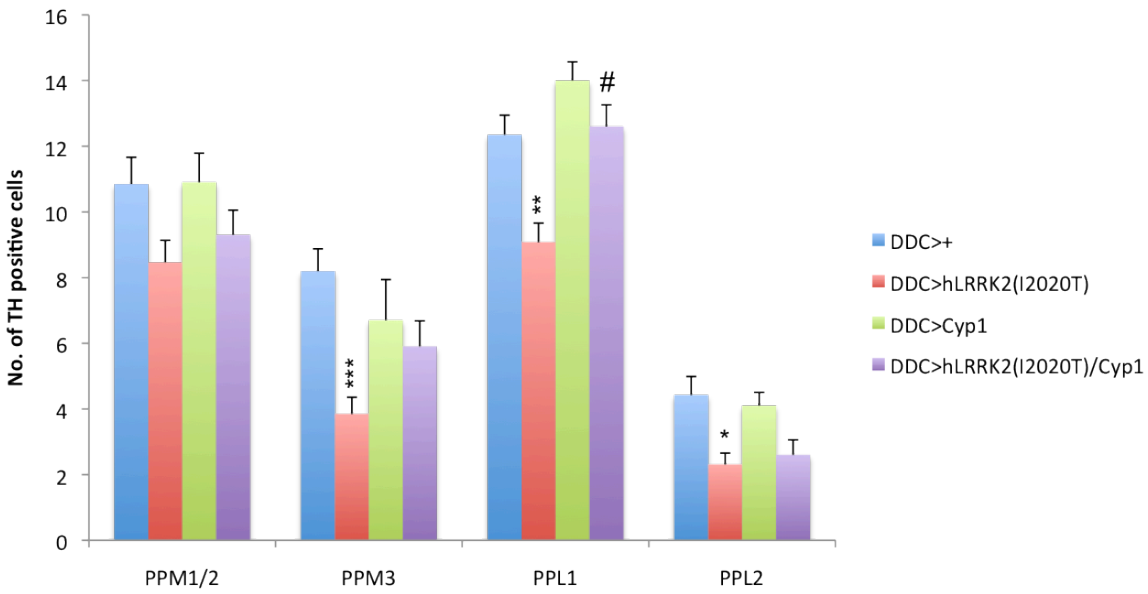

Figure 12 Rescue of DA cell loss mediated by Cyp1. Quantification of TH-positive neurons per DA cluster in 10-day-old flies of the indicated genotypes. Rescue of DA loss in the PPL1 DA cluster of Ddc>hLRRK2(I2020T)/Cyp1 (n=5). The same Ddc>+ (n=13) and Ddc>hLRRK2(I2020T) (n=13) sample have been used among the several experiments. Statistically significant differences between the control Ddc>+ and Ddc>hLRRK2(I2020T) transgenic lines are indicated: *, $P \leq 0.05$, ** if $P \leq 0.01$, *** if $P \leq 0.001$. Statistically significant differences between Ddc>hLRRK2(I2020T) and Ddc>hLRRK2(I2020T)/Cyp1 flies are indicated: #, $P \leq 0.05$. All data were analyzed by one-way ANOVA followed by Bonferonni's post hoc test.

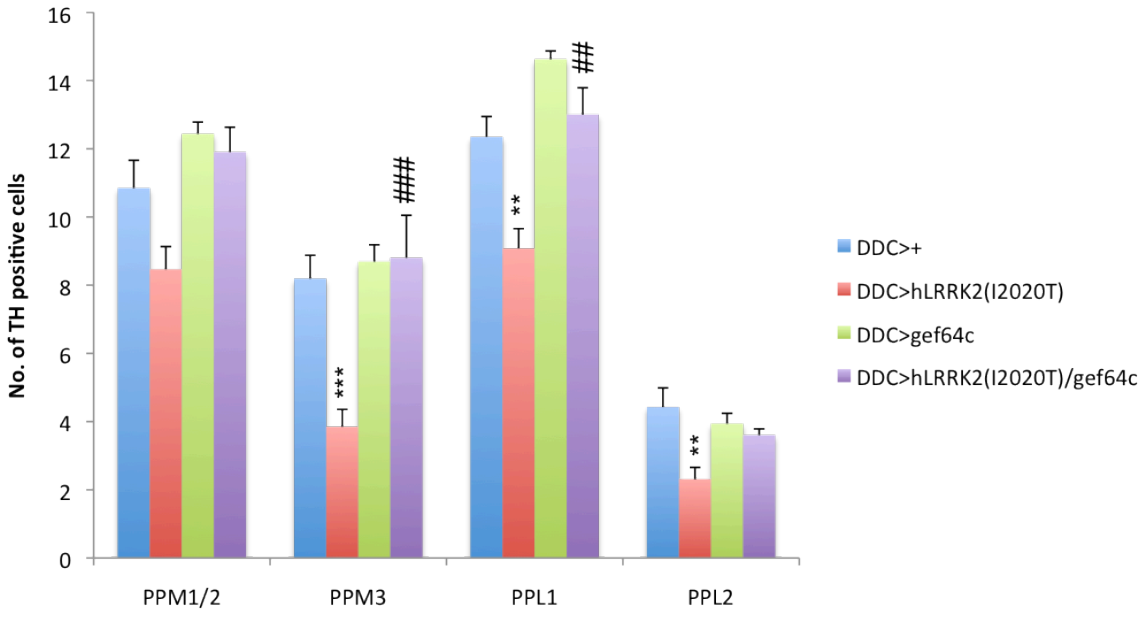

Figure 13 Rescue of DA cell loss mediated by gef64c. Quantification of TH-positive neurons per DA cluster in 10-day-old flies of the indicated genotypes. Rescue of DA loss in the PPM3 and PPL1 DA cluster of Ddc>hLRRK2(I2020T)/gef64c (n=5). The same Ddc>+ (n=13) and Ddc>hLRRK2(I2020T) (n=13) sample have been used among the several experiments. Statistically significant differences between the control Ddc>+ and Ddc>hLRRK2(I2020T) transgenic lines are indicated: *, $P \leq 0.05$, **if $P \leq 0.01$, *** if $P \leq 0.001$. Statistically significant differences between Ddc>hLRRK2(I2020T) and Ddc>hLRRK2(I2020T)/gef64c flies are indicated: #, $P \leq 0.05$, ##if $P \leq 0.01$, ### if $P \leq 0.001$. All data were analyzed by one-way ANOVA followed by Bonferonni's post hoc test.

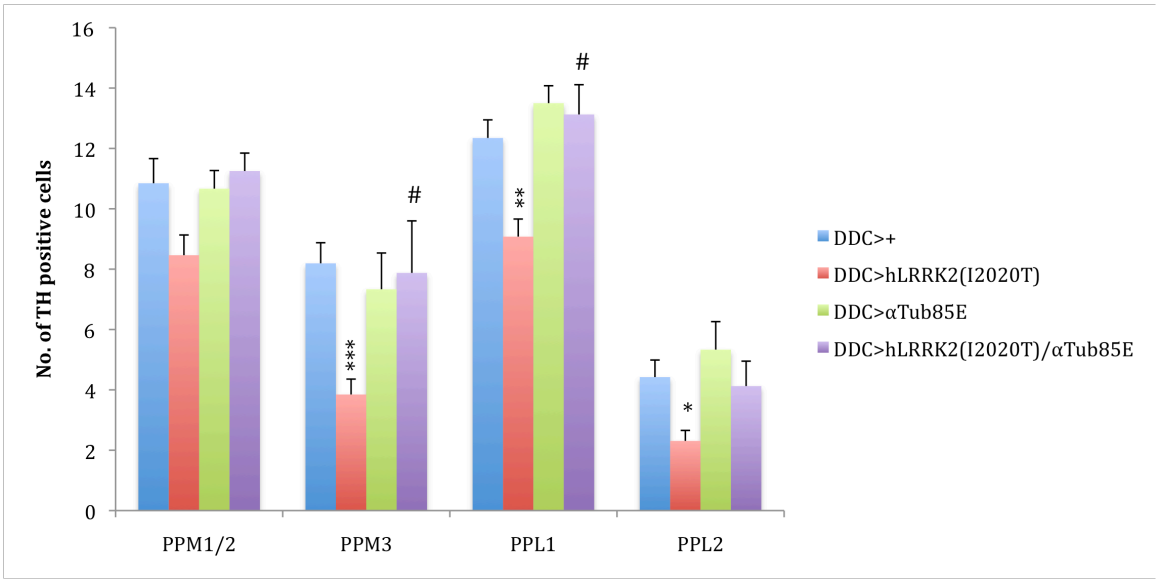

Figure 14 Rescue of DA cell loss mediated by αTub85E. Quantification of TH-positive neurons per DA cluster in 10-day-old flies of the indicated genotypes. Rescue of DA loss in the PPM3 and PPL1 DA cluster of Ddc>hLRRK2(I2020T)/αTub85E (n=4). The same Ddc>+ (n=13) and Ddc>hLRRK2(I2020T) (n=13) sample have been used among the several experiments. Statistically significant differences between the control Ddc>+ and Ddc>hLRRK2(I2020T) transgenic lines are indicated: *, $P \leq 0.05$, ** if $P \leq 0.01$, *** if $P \leq 0.001$. Statistically significant differences between Ddc>hLRRK2(I2020T) and Ddc>hLRRK2(I2020T)/αTub85Eflies are indicated: #, $P \leq 0.05$. All data were analyzed by one-way ANOVA followed by Bonferonni's post hoc test.

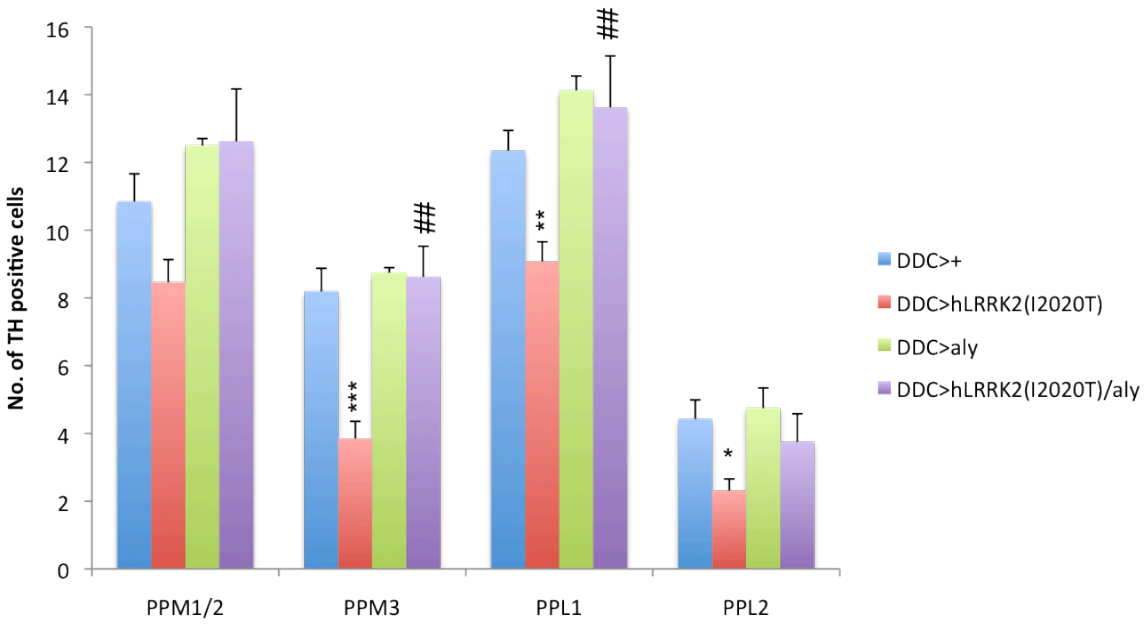

Figure 15 Rescue of DA cell loss mediated by aly. Quantification of TH-positive neurons per DA cluster in 10-day-old flies of the indicated genotypes. Rescue of DA loss in the PPM3 and PPL1 DA cluster of Ddc>hLRRK2(I2020T)/aly (n=4). The same Ddc>+ (n=13) and Ddc>hLRRK2(I2020T) (n=13) sample have been used among the several experiments. Statistically significant differences between the control Ddc>+ and Ddc>hLRRK2(I2020T) transgenic lines are indicated: *, $P \leq 0.05$, ** if $P \leq 0.01$, *** if $P \leq 0.001$. Statistically significant differences between Ddc>hLRRK2(I2020T) and Ddc>hLRRK2(I2020T)/alyflies are indicated: #, $P \leq 0.05$, ## if $P \leq 0.01$. All data were analyzed by one-way ANOVA followed by Bonferonni's post hoc test.

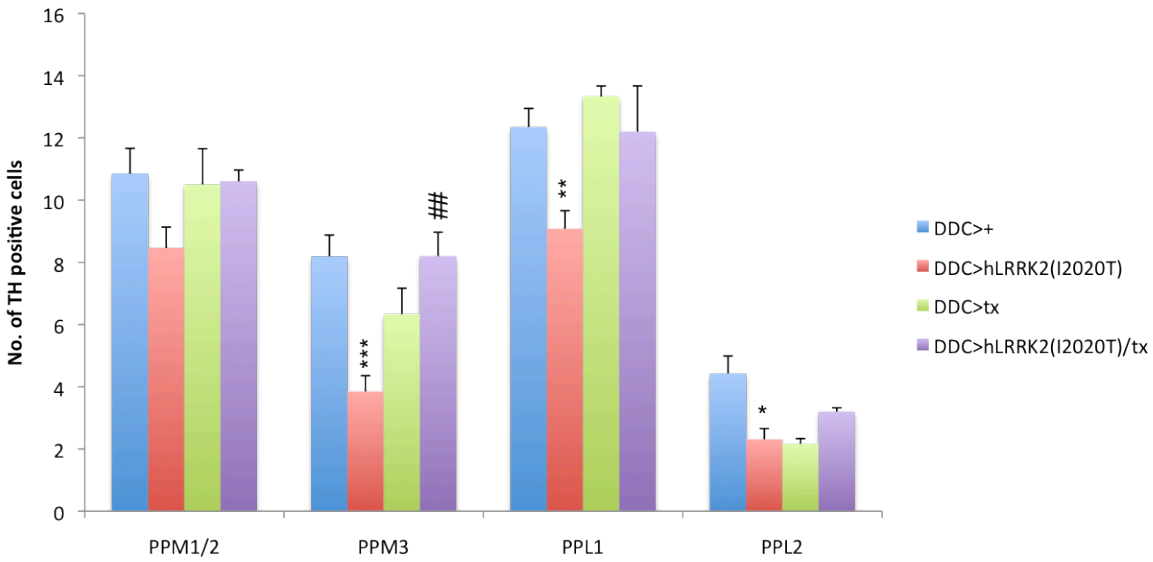

Figure 16 Rescue of DA cell loss mediated by tx. Quantification of TH-positive neurons per DA cluster in 10-day-old flies of the indicated genotypes. Rescue of DA loss in the PPM3 DA cluster of Ddc>hLRRK2(I2020T)/tx (n=5). The same Ddc>+ (n=13) and Ddc>hLRRK2(I2020T) (n=13) sample have been used among the several experiments. Statistically significant differences between the control Ddc>+ and Ddc>hLRRK2(I2020T) transgenic lines are indicated: *, $P \leq 0.05$, ** if $P \leq 0.01$, *** if $P \leq 0.001$. Statistically significant differences between Ddc>hLRRK2(I2020T) and Ddc>hLRRK2(I2020T)/txflies are indicated: #, $P \leq 0.05$, ## if $P \leq 0.01$. All data were analyzed by one-way ANOVA followed by Bonferonni's post hoc test.

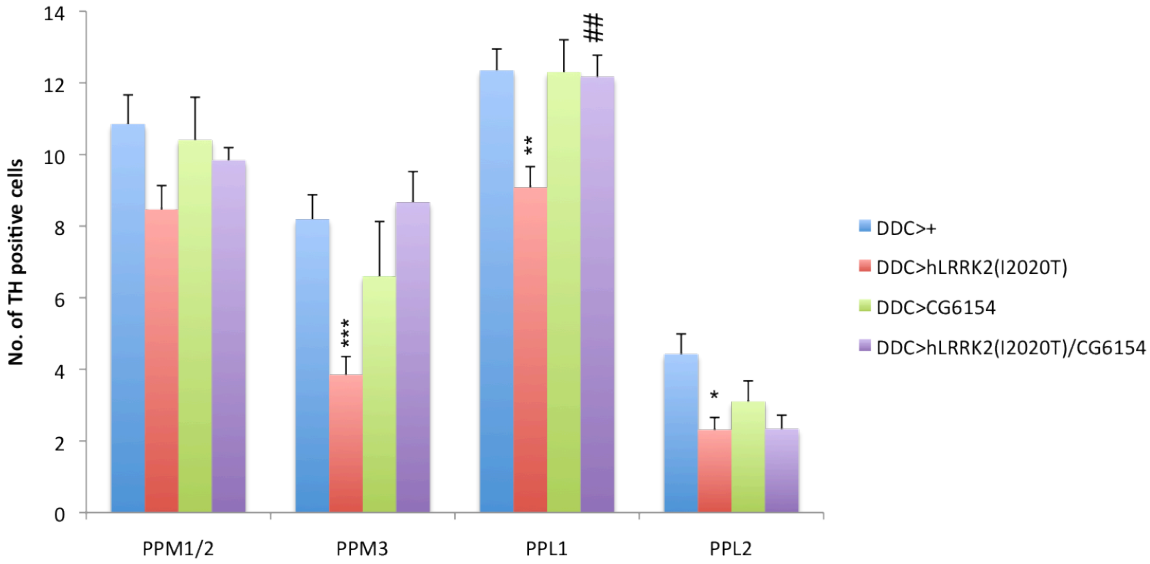

Figure 17 Rescue of DA cell loss mediated by CG6154. Quantification of TH-positive neurons per DA cluster in 10-day-old flies of the indicated genotypes. Rescue of DA loss in the PPL1 DA cluster of Ddc>hLRRK2(I2020T)/CG6154 (n=6). The same Ddc>+ (n=13) and Ddc>hLRRK2(I2020T) (n=13) sample have been used among the several experiments. Statistically significant differences between the control Ddc>+ and Ddc>hLRRK2(I2020T) transgenic lines are indicated: *, $P \leq 0.05$, ** if $P \leq 0.01$, *** if $P \leq 0.001$. Statistically significant differences between Ddc>hLRRK2(I2020T) and Ddc>hLRRK2(I2020T)/CG6154 flies are indicated: #, $P \leq 0.05$, ## if $P \leq 0.01$. All data were analyzed by one-way ANOVA followed by Bonferonni's post hoc test.

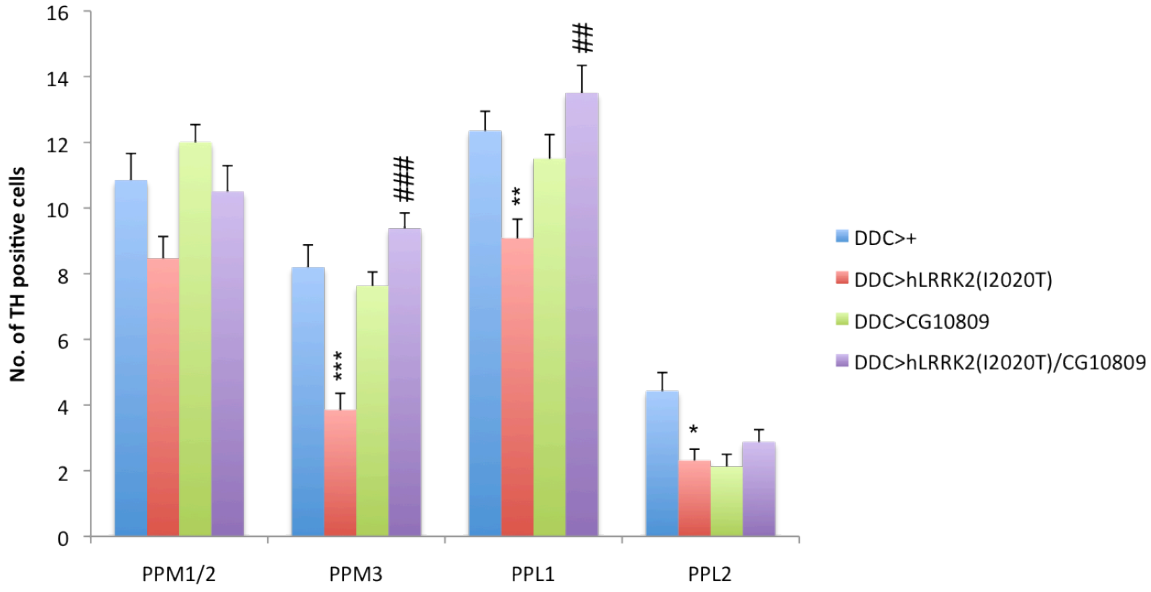

Figure 18 Rescue of DA cell loss mediated by CG10809. Quantification of TH-positive neurons per DA cluster in 10-day-old flies of the indicated genotypes. Rescue of DA loss in the PPM3 and PPL1 DA cluster of Ddc>hLRRK2(I2020T)/CG10809 (n=4). The same Ddc>+ (n=13) and Ddc>hLRRK2(I2020T) (n=13) sample have been used among the several experiments. Statistically significant differences between the control Ddc>+ and Ddc>hLRRK2(I2020T) transgenic lines are indicated: *, $P \leq 0.05$, **if $P \leq 0.01$, *** if $P \leq 0.001$. Statistically significant differences between Ddc>hLRRK2(I2020T) and Ddc>hLRRK2(I2020T)/CG10809 flies are indicated: #, $P \leq 0.05$, ##if $P \leq 0.01$, ### if $P \leq 0.001$. All data were analyzed by one-way ANOVA followed by Bonferonni's post hoc test.

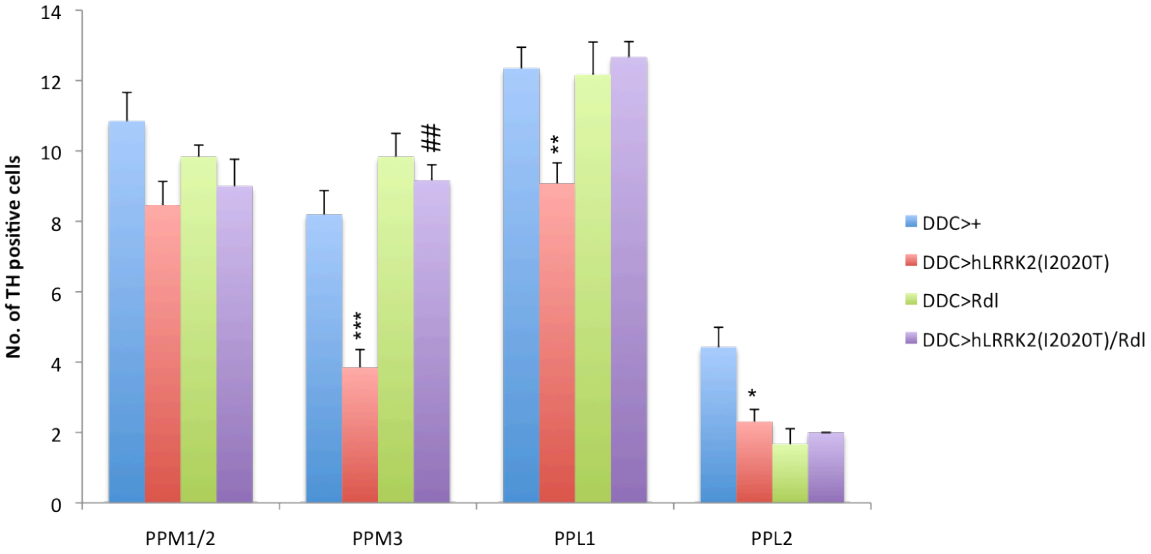

Figure 19 Rescue of DA cell loss mediated by Rdl. Quantification of TH-positive neurons per DA cluster in 10-day-old flies of the indicated genotypes. Rescue of DA loss in the PPM3 DA cluster of Ddc>hLRRK2(I2020T)/Rdl (n=3). The same Ddc>+ (n=13) and Ddc>hLRRK2(I2020T) (n=13) sample have been used among the several experiments. Statistically significant differences between the control Ddc>+ and Ddc>hLRRK2(I2020T) transgenic lines are indicated: *, $P \leq 0.05$, ** if $P \leq 0.01$, *** if $P \leq 0.001$. Statistically significant differences between Ddc>hLRRK2(I2020T) and Ddc>hLRRK2(I2020T)/Rdl flies are indicated: #, $P \leq 0.05$, ## if $P \leq 0.01$. All data were analyzed by one-way ANOVA followed by Bonferonni's post hoc test.

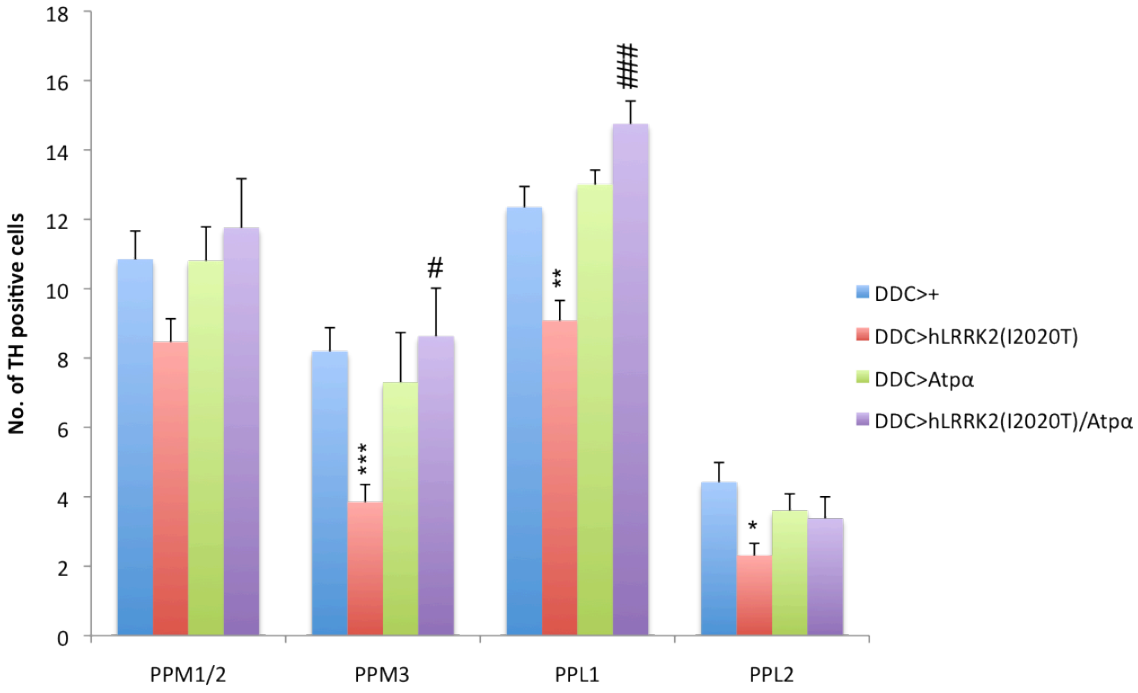

Figure 20 Rescue of DA cell loss mediated by Atpα. Quantification of TH-positive neurons per DA cluster in 10-day-old flies of the indicated genotypes. Rescue of DA loss in the PPM3 and PPL1 DA cluster of Ddc>hLRRK2(I2020T)/Atpα (n=4). The same Ddc>+ (n=13) and Ddc>hLRRK2(I2020T) (n=13) sample have been used among the several experiments. Statistically significant differences between the control Ddc>+ and Ddc>hLRRK2(I2020T) transgenic lines are indicated: *, $P \leq 0.05$, ** if $P \leq 0.01$, *** if $P \leq 0.001$. Statistically significant differences between Ddc>hLRRK2(I2020T) and Ddc>hLRRK2(I2020T)/Atpα flies are indicated: #, $P \leq 0.05$, ## if $P \leq 0.01$, ### if $P \leq 0.001$. All data were analyzed by one-way ANOVA followed by Bonferonni's post hoc test.

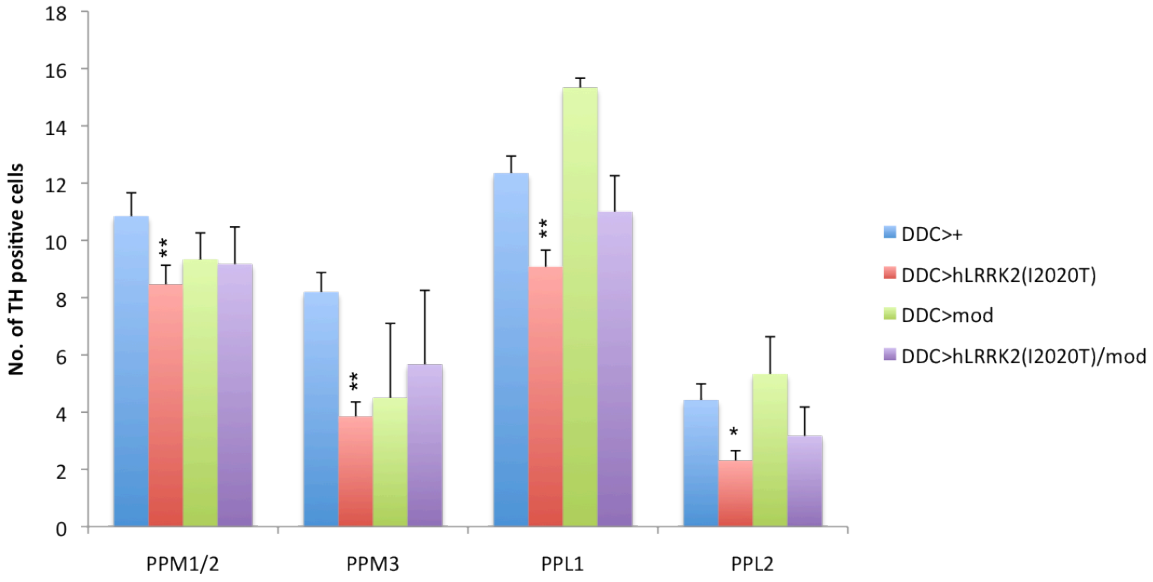

Figure 21 No significant modification of DA cell loss mediated by mod. Quantification of TH-positive neurons per DA cluster in 10-day-old flies of the indicated genotypes. The same Ddc>+ (n=13) and Ddc>hLRRK2(I2020T) (n=13) sample have been used among the several experiments. Statistically significant differences between the control Ddc>+ and Ddc>hLRRK2(I2020T) transgenic lines are indicated: *, $P \leq 0.05$, ** if $P \leq 0.01$. All data were analyzed by one-way ANOVA followed by Bonferonni's post hoc test.

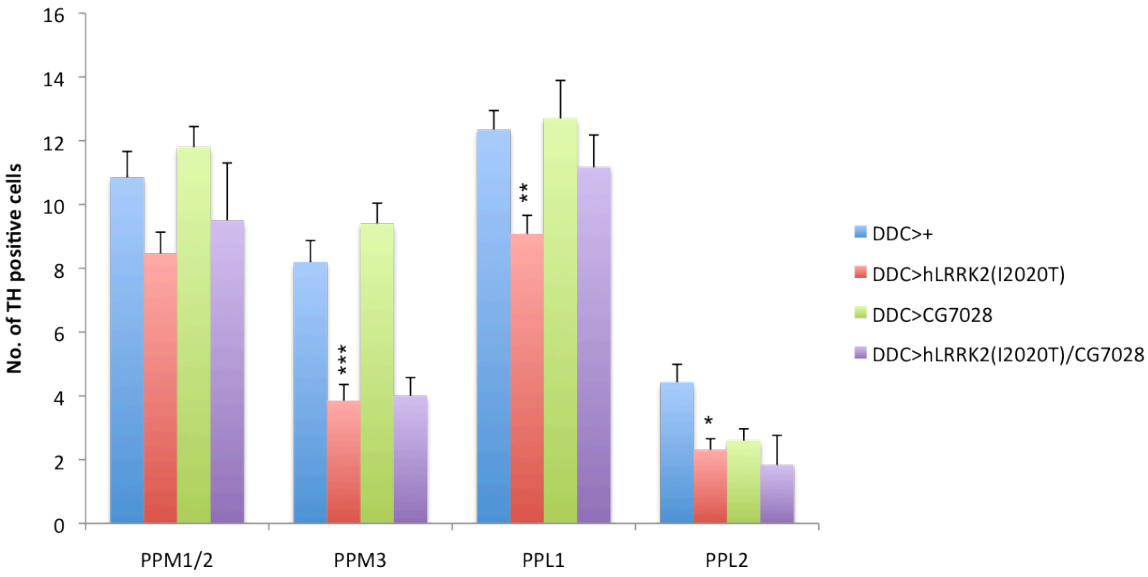

Figure 22 No significant modification of DA cell loss mediated by CG7028.

Quantification of TH-positive neurons per DA cluster in 10-day-old flies of the indicated genotypes. The same Ddc>+ and Ddc>hLRRK2(I2020T) sample have been used among the several experiments. Statistically significant differences between the control Ddc>+ and Ddc>hLRRK2(I2020T) transgenic lines are indicated: *, $P \leq 0.05$, ** if $P \leq 0.01$. All data were analyzed by one-way ANOVA followed by Bonferonni's post hoc test.

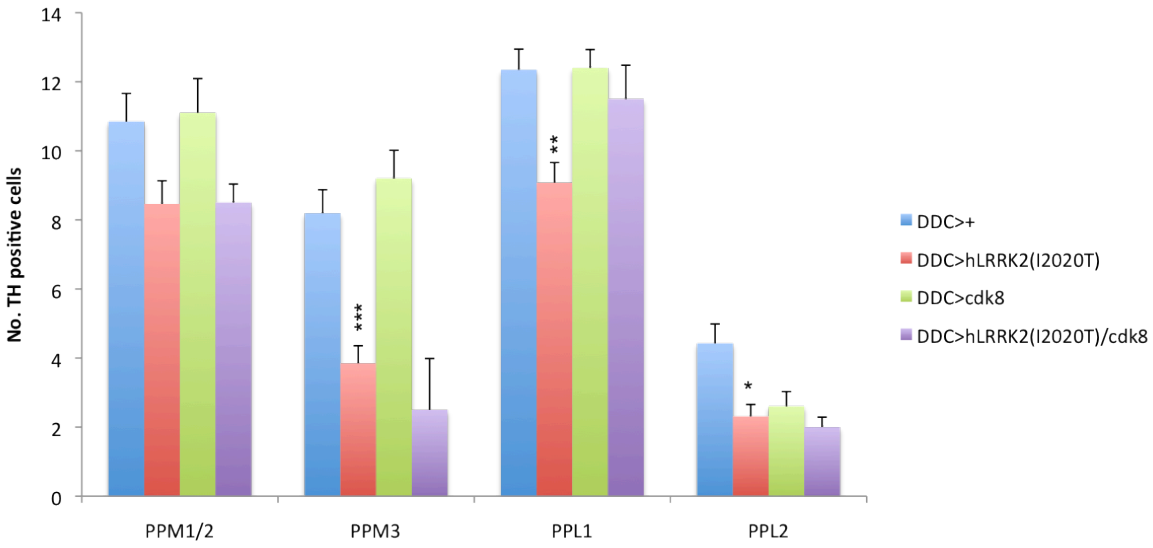

Figure 23 No significant modification of DA cell loss mediated by cdk8. Quantification of TH-positive neurons per DA cluster in 10-day-old flies of the indicated genotypes. The same Ddc>+ and Ddc>hLRRK2(I2020T) sample have been used among the several experiments. Statistically significant differences between the control Ddc>+ and Ddc>hLRRK2(I2020T) transgenic lines are indicated: *, $P \leq 0.05$, ** if $P \leq 0.01$. All data were analyzed by one-way ANOVA followed by Bonferonni's post hoc test.

Discussion

PD is a depletive disease affecting the health and the quality of life of patients. The discovery of several Parkinson's genes has been a major step in understanding the pathogenesis of the disease. Mutations in LRRK2 contribute to both familial and sporadic cases making it the most common PD linked gene. Several abnormalities have been reported in both *in vitro* and *in vivo* models of LRRK2. However, the function(s) of LRRK2 have yet to be elucidated.

This study offers an excellent approach to identify novel LRRK2 genetic interactors, which will be of great advantage to develop a better understanding of LRRK2 functioning in PD pathogenesis. *Drosophilamelanogaster* have long been used as a tool in studying neurodegenerative diseases *invivo*. Several LRRK2 *Drosophila* models that have been published have recapitulated PD pathology unlike many murine models (Liu *et. al.*, 2008, Venderova *et. al.*, 2009, Kanao *et. al.*, 2010, Ng *et. al.*, 2009; Yang *et. al.*, 2012).

In the Venderova *et. al.* (2009) study, the Park lab were able to establish a recognizable eye phenotype in transgenic flies that overexpressed hLRRK2. The compound eye of the fly is made of ommatidia that are composed of variety of cell types including photoreceptors. The roughness of the eye surface, loss of pigmentation and presence of black lesions all indicate degeneration of these cells, as has been reported in previous studies (Wang *et. al.*, 2006; Yarosh *et. al.*, 2008). It is important to keep in mind that these defects are observed at a higher temperature where the GAL4/UAS system is more active causing an increased expression of hLRRK2 as shown by western blotting in Venderova *et. al.* (2009). Liu *et. al.* (2008) have failed to detect these defects in their LRRK2 transgenic flies, perhaps due to insufficient levels of LRRK2 expression. Interestingly, Hindle *et. al.* (2013) also utilized the eyes of *Drosophila* as a model system to study LRRK2 function. In this instance,

they explored the effects of hLRRK2(G2019S) overexpressed in DA neurons innervating the eyes and reported decreased electroretinogram (ERG) recordings, anatomical degeneration accompanied with increased apoptosis and autophagy, and mitochondrial abnormalities.

Using the eye as a model system of neurodegeneration, the present study utilized the overt degeneration observed in the fly eye in a suppressor/enhancer genetic screen. The hLRRK2(I2020T) transgenic fly was initially used in this screen since it showed the most prominent DA loss and locomotor impairment when driven under the TH driver (Venderova *et. al.*, 2009), when compared to other LRRK2 expressors including hLRRK2(WT), hLRRK2(I1122V), and hLRRK2(Y1699C). Importantly, the hLRRK2(I2020T) transgenic flies show comparable levels of DA degeneration when compared to the hLRRK2(G2019S) transgenic flies generated by Liu *et. al.* (2008). While much attention has focused on the G2019S mutation due to its relatively high prevalence in LRRK2 PD families, the study of other pathogenic LRRK2 mutations offers critical insights into LRRK2 function.

In this study we were able to screen the full 3rd chromosome deficiency kit that covers up to 97% of the chromosome, while my colleague Paul Marcogliese screened the 2nd chromosome deficiency kit, which covers 94% of the chromosome. These deficiency kits are haplosufficient deletions of euchromatic genes in *Drosophila*. In total, both the 3rd and the 2nd chromosomes provide an adequate coverage of about 12X10^3 genes out of approximately 15X10^3 of the *Drosophila*euchromatic genes. Twenty-two strong and sixteen weak modifiers have been identified from the 3rd chromosome screen. Identifying weak interactors of hLRRK2(I2020T) is essential because these interactions could show stronger modification when tested with the other hLRRK2 transgenic flies carrying the different mutations.

The above interactors were further evaluated as potential modifiers of the LRRK2 phenotype utilizing both the GMR>hLRRK2 (WT) and the GMR>hLRRK2 transgenic flies carrying LRR susceptibility mutation (I1122V*)*. The interaction between the different hLRRK2 transgenic flies and the identified genes showed discrepancies similar to reports by Venderova *et. al.* (2009) when these transgenic flies where used in a biased genetic screen with the recessive PD genes. Out of the strong interactors, only three genes (*CG9614*, *CG32236*, and *tx*) showed consistent results among all three different transgenic flies. In addition one gene, *CG10809*, showed opposing effects in the GMR>hLRRK2 (I1122V*)* transgenic fly when compared to GMR>hLRRK2 (I2020T*)*. From the weak interacting genes only *sprouty* showed consistent results among the different transgenic flies. As expected, other interactors showed stronger modification when tested with GMR>hLRRK2(WT) and GMR>hLRRK2(I1122V) including *Heph,CG5484* and *CG6420.* Consistent interaction among the different transgenic flies adds strength to the interaction between the gene and LRRK2, however, the lack of consistency does not rule out the interaction because the interaction could be mediated through the presence of the I2020T mutation and its effects on LRRK2, which differs from the WT and I1122V mutation. In addition, the effect of the I1122V mutation on LRRK2 is not fully understood except that it does not cause any conformational changes to LRRK2 and it is most likely to effect the inter- and intramolecular interactions of LRRK2 (Vancraenenbroeck *et. al.*, 2012). Therefore, we could not draw a full explanation of the discrepancies noted in this study. However, examining the interaction with transgenic flies of the other pathogenic mutations (Y1699C and R1441G), which are currently being generated, would add more confidence to these putative interactions.

In order to examine the effects on the DA system of the fly, we also determined whether the interactors identified in the eye 12020T screen would also affect DA degeneration. To do this, we first generated Ddc>hLRRK2(I2020T) flies. Although TH>hLRRK2(I2020T) were available and used in the Venderova *et. al.* (2009) publication, they could not be used for the 3rd chromosome screen due to the position of the TH driver on the 3rd chromosome along with hLRRK2. Therefore, we instead made the decision to drive LRRK2 using the Ddc driver. We first confirmed that hLRRK2(I2020T) overexpression by the Ddc driver gave similar effects with that reported by the TH driver line. In 10-day-old flies, significant loss of TH staining was observed in DA clusters. In this case however, we observed degeneration in all DA clusters whereas Venderova *et. al.* (2009) only observed death in PPM1/2 and PPL1, and not PPL2 or PPM3. Our present reports are consistent to that of Liu *et. al.* (2008) also reported DA loss in all clusters when using the Ddc driver.

Next, we explored the interaction of the originally identified gene interactors with hLRRK2(I2020T) driven in the DA system. Out of 14 lines that have been analysed thus far, eleven genes showed modification of the TH loss phenotype in the DA system. A rescue was only detected in the PPM3 and/or PPL1 clusters. The function of these clusters is not fully understood, however it has been shown that a subtype of PPL1 neurons and PPM3 neurons project to the Central Complex, which is a region of the fly brain that controls locomotion (White *et. al.*, 2010; Mao and Davis, 2009). It will be interesting to further study the effects of loss and rescue of these clusters on the locomotor behaviour of the Ddc>hLRRK2(I2020T) and Ddc>hLRRK2(I2020T)/GD flies in future studies. Surprisingly, assessmnet of the interaction of the identified genes in the fly brain showed only rescue of the DA cell loss caused by hLRRK2(I2020T), which is opposite of the effect observed in the

eye screen in many of these interactors. The eye and the brain are two different systems and they might react differently to gene manipulations by compensatory processes, especially that LRRK2 is being expressed in larval stages when using the Ddc driver, while LRRK2 is being expressed only in the adult fly when using the GMR driver. Also, the different modifiers might have different functions in these two systems leading perhaps to different outcomes. In addition, these flies were aged for 10 days, since TH>hLRRK2(I2020T) caused similar degree of DA cell loss compared to older flies (Venderova *et. al.*, 2009). However, assessing the identified interactors in older flies (i.e. 50-day-old) could be a better assessment since PD is an aging disorder and these interactions need the stress caused by aging to have a more prominent effects on the DA system. This discussion will focus on five interacting genes that showed modification in both clusters indicating a stronger interaction.

One of the genes that showed DA modification was the *Atpα* line, an RNAi line that showed rescue of both the eye defects of the GMR>hLRRK2(I2020T) and GMR>hLRRK2(WT) and the DA cell loss observed in Ddc>hLRRK2(I2020T). *Atpα* protein is 99% homologous to human Na+/K+ ATPase. This protein is an ATP-dependent antiporter enzyme that exchanges Na+ for K+ to maintain the membrane potential of many cell types. Interestingly, loss of function mutations in this gene were reported to cause rapid-onset dystonia Parkinsonism, an autosomal dominant movement disorder characterized by sever dystonia and signs of Parkinsonism (de Carvalho Aguiar el. al., 2004). In addition, inhibition of the pump by ouabain has several effects on DA neurons. Blockage of the protein with low concentrations of ouabain was reported to increase survival of DA cells in a mesencephalic culture by increasing the excitability of these neurons (Salthun-Lassalle *et. al.*, 2004). Additionally, inhibition was reported to increase Na^+ levels in cells that lead to increasing

DA transmission through dopamine active transporter (DAT) (Milusheva *et. al.*, 1995; Fairbrother *et. al.*, 1990). Although many murine models of mutant LRRK2 have not reported DA cell loss, there is evidence of impaired neurotransmission in these animals (Tong *et. al.*, 2009; Li *et. al.*, 2010; Melrose *et. al.*, 2010). The above evidence makes this an interesting candidate for further study, particularly in the context of PD. It would be interesting to examine the effect of blocking Na+/K+ ATPase on the neurotransmission of brain slices obtained from the WT and mutant LRRK2 transgenic mice. Further, examining the effect on the survival of neurons obtained from these LRRK2 transgenic mice in cell culture.

An over-expression line of the *Drosophila* gene, *CG10809,* caused a rescue of the eye defects in GMR>hLRRK2(I2020T) and DA cell loss in Ddc>hLRRK2(I2020T) in both the PPM3 and PPL1 while it enhanced the eye defects in the GMR>hLRRK2(I1122V) fly. These two different mutations lie at different domains of the protein which might have different effects on LRRK2 function and its genetic interactors. The I2020T mutation is found on the kinase domain, while I1122V mutation lie on the protein-protein interaction domain, LRR. The effects of these mutations on the LRRK2's function is still unknown which limit or interpretation of the observed results. When BLASTed to its human homolog, *CG10809* was 53% similar toankyrin repeat domain-containing protein 54 (Ankrd54), also known as Lyn-interacting ankyrin repeat protein (Liar). This novel protein was first identified in a yeast two hybrid screen where it interacts with the tyrosine protein kinase Lyn mediating erythroid differentiation (Samuels *et. al.*, 2009). Liar functions as a nuclear/cytoplasmic shuttling protein for both Lyn and Bruton's tyrosine kinase (Btk), which are both involved in the regulation of B-lymphocyte activation (Samuels *et. al.*, 2009; Gustafsson *et. al.*, 2012). There

is strong evidence indicating LRRK2's involvement in the immune system in both a PD context and in inflammatory diseases such as Crohn'sdisease (Hakimi *et. al.*, 2011; Umeno *et. al.*, 2011). Furthermore, several studies reported the expression of LRRK2 in different immune cells including B cells (Hakimi *et. al.*, 2011, Thévenet *et. al.*, 2011). In order to study the interaction between LRRK2 and Liar, a better characterization of the latter gene is needed, as there are only two reports of Liar available in the literature thus far (Samuels *et. al.*, 2009; Gustafsson *et. al.*, 2012). Examining the expression patterns of Liar in the immune system of the WT and mutant LRRK2 transgenic mice and its response to an inflammatory agent could be an initial step to understand the nature of the interaction.

RNAi line of αTub85E showed enhancement of the eye phenotype and a rescue of DA cell loss. αTub85E is 100% homologous to α-tubulin-1C subunit. This interaction validates our screen since several studies have already reported an interaction between LRRK2 and β-tubulin and microtubules dynamics (Gandhi *et. al.*, 2008; Kawakami *et. al*., 2012; Caesar *et. al*., 2013). LRRK2 was reported to interact with and phosphorylate β-tubulin increasing its stability (Gillardon, 2009). Further, LRRK2 was found to modulate microtubule stability through tau phosphorylation (Kawakami *et. al*., 2012; Melrose *et. al.,* 2010; Lin *et. al.*, 2010). These evidences were suggested as the mechanism by which LRRK2 modulate the neurite morphology reported in LRRK2 mutants' over-expression in *in vitro* and *in vivo* models (Chan *et. al.*, 2011; Ramonet *et. al.*, 2011; Kawakami *et. al.*, 2012). Although the identified gene was α-tubulin, which forms dimers with β-tubulin and essential for microtubules formation, it is still an intersting gene to be pulled out from the screen as it validates the screen because LRRK2 is already shown to interact with its close partner β-tubulin affecting tubulins function. This interactor is a great example to also demonstrate the

limitation of this study as this screen only identifies genetic interactions and does not reveal the biochemical interactions.

In addition to the previous interactors, RNAi lines of both *aly* and *gef64c* caused an enhancement in the eye and a suppression in the DA system and rescued cell loss. These genes might play different roles in these different tissues explaining the different results we observed. Lin-9 showed 59% homology to *aly* and is one of the components of the DREAM complex involved in the regulation of cell cycle and early development through its interaction with RB proteins (Esterlechner *et. al.*,2013). Its function is not fully characterized and more studies are needed to understand its role in neurons. *Gef64c* when BLASTed to its human homolog showed a low homology of 9% to intersectin 1, specifically its SH3 domain. Intersectin protein family is scaffolding/adaptor proteins involved endocytosis and cytoskeleton rearrangement (Tsyba *et. al.*, 2011). This is an interesting interactor to further study since there is strong evidence of LRRK2 involvement in vesicular trafficking and cytoskeletal dynamics. A true homolog of intersectin 1 is known in *Drosophila* asdynamin associated protein 160 (Dap160). We could confirm this interaction by testing the true homolog in our LRRK2 fly model for modification. Another possible interpretation of this interaction could be that the GTPase domain of LRRK2 is interacting with *gef64c*, as it is a guanine nucleotide exchange factor, mediating the GTPase activation and subsequent signaling.

Although multiple genes have not shown modification in the DA system, these results are not conclusive, as some of these interactors need to be more carefully examined with larger sample sizes and older flies. Also, lack of modification in the invertebrate DA system may not preclude an interaction between LRRK2 and the interactors in the mammalian

system. This is particularly important since LRRK2 has been suggested to function in non-nervous system contexts including the immune system. In addition, several other *Drosophila* phenotypes have been reported upon overexpression of WT and/or mutantsof hLRRK2 in different systems of the fly, including mitochondrial defects in flight muscles, dendrite degeneration of DA neurons in larvae, and impaired endocytosis in larval neuromuscular junctions (Ng *et. al.*, 2012; Lin *et. al.*, 2010; Matta *et. al.*, 2012). Thus, depending on the pathway the interacting gene is involved in, modification of the other phenotypes reported in LRRK2 flies could be more appropriate to assess.

In conclusion, we have used a *Drosophila* model of LRRK2 exhibiting both pathological and behavioral PD features that are particularly lacking in mammalian models. With the use of easily identifiable eye defects we were able to screen the 3rd chromosome deficiency kit and identify several genetic interactors. These interactors may shed light on the pathways that are affected by LRRK2. A closer examination of these interactors is required depending on their function in either the fly model, cell culture or in the murine models especially that this screen only indicate genetic interaction which could be indirect interaction and just give clues of a cellular pathway upstream or down stream of LRRK2 and does not reveal any evidence of a physical biochemical interaction modulating LRRK2 functioning.

Material and Methods

Fly stocks

The flies were maintained on a standard cornmeal/agar medium at room temperature and crossed at 25°C or 29°C. GMR-Gal4/Cyo;hLRRK2(I2020T)/TM6B transgenic flies were generated in Dr. Park's lab (Vendorova *et. al.*, 2009). The 3rd chromosome deficiency (Def) kit was purchased from Bloomington Drosophila stock centre. RNAi, gene deletion, and overexpression lines were obtained from either Bloomington Drosophila stock center or Vienna Drosophila RNAi center.

Fly Genetics

GMR-Gal4/Cyo;UAS-hLRRK2/TM2 transgenic flies were generated by crossing GMR-Gal4/Bc;+/+ and +/+;UAS-hLRRK2;+/+ with Sp/Cyo;TM2/TM6B double balanced fly and the progeny of that cross GMR-Gal4/Sp;+/TM2 and Cyo/+;UAS-hLRRK2/TM6B are then crossed to get the final cross.

The suppressor/enhancer screen was performed by crossing GMR-Gal4/Cyo;UAS-hLRRK2(I2020T)/TM6B transgenic fly with the deficiency lines as the experimental cross to obtain GMR-Gal4/+;UAS-hLRRK2(I2020T)/Def. Three control crosses were made using GMR-Gal4/BC;+/+ and the deficiency lines, +/+; hLRRK2(I2020T)/hLRRK2(I2020T) transgenic flies, and W1118;+/+;+/+ wild type flies. The same crosses were performed with other hLRRK2 mutant transgenic flies.

Ddc/Cyo;UAS-hLRK2(I2020T)/TM2 flies were generated by crossing Ddc-Gal4/+;+/+ with double balancer fly SP/Cyo;TM2/TM6B to obtain Ddc-Gal4/Cyo;TM2/+ fly which was then crossed with Sp/Cyo;UAS-hLRRK2(I2020T)/TM6B to get the Ddc-Gal4/Cyo;UAS-hLRRK2(I2020T)/TM2 fly.

Scoring of eye phenotype

GMR-Gal4/+;UAS-hLRRK2(I2020T)/Def flies are anesthetized by placing them on a carbon dioxide pad and the eyes were visualized under a stereo microscope. The hLRRK2 transgenic flies show no phenotype at 25°C and this is scored as zero and only enhancements are expected in case of an interaction increasing from 1- survival. This modification is very clear and easily identified. Score 1 is for mild roughness of the eye surface, 2 addition of loss of pigmentation, 3 significant loss of pigmentation and presence of some black lesions, 4 prominent presence of black lesions, and 5 severely deformed eye. On the other hand, the transgenic flies show a baseline phenotype at 29°C characterized by rough eye, loss of pigmentation and presence of black lesions at level 3. An enhancement of the phenotype is scored from 3 – 5, while suppression is scored by 2 – 0 (Figure 5).

DA staining and quantification

RNAi, gene deletion, or over-expression lines of the different interacting gene candidates are crossed with Ddc/Cyo;hLRRK2/TM2 transgenic flies. Two crosses are made to obtain control flies, Ddc/+;Def/+ and Ddc/+;hLRRK2/Def. 10-day-old flies are raised at 25° and then dissected and fixed in 4% paraformaldehyde overnight. The brains are then washed in PBST and incubated overnight in blocking solution (5% normal goat serum (NGS)). Following that, the samples are incubated in primary antibody (1:300 rabbit anti-TH (Millipore) in 5% NGS) overnight and then washed in PBST. Later, the brains are incubated in secondary antibody (1:3000 donkey anti-rabbit conjugated Alexa 488 (Invitrogen) in 5% NGS) overnight. The brains are mounted onto slides following a PBST wash and an overnight incubation in Vectashield mounting medium (Vector Laboratory). The slides are

visualized using confocal microscopy (Zeiss LSM510) under the 20X objective and a Z-stack of 1-μm-thick-sections is carried out; the image is then visualized in 3D configuration and the DA clusters are quantified.

Statistical analysis

The data were analyzed as specified, expressed as means ± standard error of means.

References

- Alegre-Abarrategui J, Christian H, Lufino MM, Mutihac R, Venda LL, Ansorge O, Wade-Martins R. LRRK2 regulates autophagic activity and localizes to specific membrane microdomains in a novel human genomic reporter cellular model. Hum Mol Genet. 2009 Nov 1;18(21):4022-34.
- Barrett LE, Van Bockstaele EJ, Sul JY, Takano H, Haydon PG, Eberwine JH. Elk-1 associates with the mitochondrial permeability transition pore complex in neurons. Proc Natl Acad Sci U S A. 2006 Mar 28;103(13):5155-60.
- Bekris LM, Mata IF, Zabetian CP. The genetics of Parkinson disease. J Geriatr Psychiatry Neurol. 2010 Dec;23(4):228-42.
- Berardelli A, Rothwell JC, Thompson PD, et al. Pathophysiology of bradykinesia in Parkinson's disease. Brain 2001;124:2131–46.
- Bhidayasiri, Roongroj. "Parkinson's Disease: Hoehn and Yahr Scale." *Movement Disorders: A Video Atlas*. Ed. Daniel Tarsy. New York: Humana, 2012.
- Biskup S, Moore DJ, Celsi F, Higashi S, West AB, Andrabi SA, Kurkinen K, Yu SW, Savitt JM, Waldvogel HJ, Faull RL, Emson PC, Torp R, Ottersen OP, Dawson TM, Dawson VL. Localization of LRRK2 to membranous and vesicular structures in mammalian brain. Ann Neurol. 2006 Nov;60(5):557-69.
- Biskup S, West AB: Zeroing in on LRRK2-linked pathogenic mechanisms in Parkinson's disease. Biochim Biophys Acta 2009, 1792:625–633.
- Bonifati V, Rizzu P, van Baren MJ, et al. Mutations in the DJ-1 gene associated with autosomal recessive early-onset parkinsonism. Science 2003;299(5604):256–259
- Braak H, Del Tredici K, Rüb U, de Vos RA, Jansen Steur EN, Braak E. Staging of brain pathology related to sporadic Parkinson's disease. Neurobiol Aging. 2003 Mar-Apr;24(2):197-211.
- Brand, A., and Perrimon, N. (1993). Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. Development 118, 401–415.
- Bravo-San Pedro JM, Niso-Santano M, Gómez-Sánchez R, Pizarro-Estrella E, Aiastui-Pujana A, Gorostidi A, Climent V, López de Maturana R, Sanchez-Pernaute R, López de Munain A, Fuentes JM, González-Polo RA. The LRRK2 G2019S

mutant exacerbates basal autophagy through activation of the MEK/ERK pathway. Cell Mol Life Sci. 2013 Jan;70(1):121-36.

- Canadian Institute for Health Information, The Burden of Neurological Diseases, Disorders and Injuries in Canada (Ottawa: CIHI, 2007)
- Caesar M, Zach S, Carlson CB, Brockmann K, Gasser T, Gillardon F. Leucine-rich repeat kinase 2 functionally interacts with microtubules and kinase-dependently modulates cell migration. Neurobiol Dis. 2013 Jun;54:280-8.
- Chan D, Citro A, Cordy JM, Shen GC, Wolozin B. Rac1 protein rescues neurite retraction caused by G2019S leucine-rich repeat kinase 2 (LRRK2). J Biol Chem. 2011 May 6;286(18):16140-9.
- Chen CY, Weng YH, Chien KY, Lin KJ, Yeh TH, Cheng YP, Lu CS, Wang HL. (G2019S) LRRK2 activates MKK4-JNK pathway and causes degeneration of SN dopaminergic neurons in a transgenic mouse model of PD. Cell Death Differ. 2012 Oct;19(10):1623-33.
- Clark IE, Dodson MW, Jiang C, Cao JH, Huh JR, Seol JH, Yoo SJ, Hay BA, Guo M. Drosophila pink1 is required for mitochondrial function and interacts genetically with parkin. Nature. 2006 Jun 29;441(7097):1162-6.
- Clark, David L; Boutros, Nashaat N; Mendez, Mario F. (2010). The Brain and Behavior: An Introduction to Behavioral, New York : Cambridge University Press.
- Coelho M, Ferreira JJ. Late-stage Parkinson disease. Nat Rev Neurol. 2012 Aug;8(8):435-42. doi: 10.1038/nrneurol.2012.126. Epub 2012 Jul 10. Review. PubMed PMID: 22777251.
- Cooper O, Seo H, Andrabi S, Guardia-Laguarta C, Graziotto J, Sundberg M, McLean JR, Carrillo-Reid L, Xie Z, Osborn T, Hargus G, Deleidi M, Lawson T, Bogetofte H, Perez-Torres E, Clark L, Moskowitz C, Mazzulli J, Chen L, Volpicelli-Daley L, Romero N, Jiang H, Uitti RJ, Huang Z, Opala G, Scarffe LA,Dawson VL, Klein C, Feng J, Ross OA, Trojanowski JQ, Lee VM, Marder K, Surmeier DJ, Wszolek ZK, Przedborski S, Krainc D, Dawson TM, Isacson O. Pharmacological rescue of mitochondrial deficits in iPSC-derived neural cells from patients with familial Parkinson's disease. Sci Transl Med. 2012 Jul 4;4(141):141ra90.
- Davis, G.C., Williams, A.C., Markey, S.P., Ebert, M.H., Caine, E.D., Reichert, C.M.,

Kopin, I.J., 1979. Chronic Parkinsonism secondary to intravenous injection of meperidine analogues. Psychiatry Res. 1, 249–254.

- de Carvalho Aguiar P, Sweadner KJ, Penniston JT, Zaremba J, Liu L, Caton M, Linazasoro G, Borg M, Tijssen MA, Bressman SB, Dobyns WB, Brashear A, Ozelius LJ. Mutations in the Na+/K+ -ATPase alpha3 gene ATP1A3 are associated with rapid-onset dystonia parkinsonism. Neuron. 2004 Jul 22;43(2):169-75.
- De Lau LLL, Breteler MMB. Epidemiology of Parkinson's disease. Lancet Neurol 2006;5:525Y535.
- Deep Brain Stimulation for PD Study Group, 2001. Deep-brain stimulation of the subthalamic nucleus or the pars interna of the globus pallidus in Parkinson's disease. N. Engl. J. Med. 345, 956–963.
- Dodson MW, Zhang T, Jiang C, Chen S, Guo M. Roles of the Drosophila LRRK2 homolog in Rab7-dependent lysosomal positioning. Hum Mol Genet. 2012 Mar 15;21(6):1350-63.
- Elliott DA, Kim WS, Gorissen S, Halliday GM, Kwok JB. Leucine-rich repeat kinase 2 and alternative splicing in Parkinson's disease. Mov Disord. 2012 Jul;27(8):1004-11.
- Esterlechner J, Reichert N, Iltzsche F, Krause M, Finkernagel F, Gaubatz S. LIN9, a Subunit of the DREAM Complex, Regulates Mitotic Gene Expression and Proliferation of Embryonic Stem Cells. PLoS One. 2013 May 7;8(5):e62882.
- Fairbrother IS, Arbuthnott GW, Kelly JS, Butcher SP. In vivo mechanisms underlying dopamine release from rat nigrostriatal terminals: I. Studies using veratrine and ouabain. J Neurochem. 1990 Jun;54(6):1834-43.
- Farrer M, Kachergus J, Forno L, Lincoln S, Wang DS, Hulihan M, Maraganore D, Gwinn-Hardy K, Wszolek Z, Dickson D, Langston JW. Comparison of kindreds with parkinsonism and alpha-synuclein genomic multiplications. Ann Neurol. 2004 Feb;55(2):174-9.
- Ferrer I, Martinez A, Blanco R, Dalfó E, Carmona M. Neuropathology of sporadic Parkinson disease before the appearance of parkinsonism: preclinical Parkinson disease. J Neural Transm. 2011 May;118(5):821-39. doi: 10.1007/s00702-010-0482-8. Epub 2010 Sep 23. Review. PubMed PMID: 20862500.

- Funayama M, Hasegawa K, Kowa H, Saito M, Tsuji S, Obata F. A new locus for Parkinson's disease (PARK8) maps to chromosome 12p11.2 q13.1. Ann Neurol. 2002 Mar;51(3):296-301.
- Gandhi PN, Wang X, Zhu X, Chen SG, Wilson-Delfosse AL. The Roc domain of leucine-rich repeat kinase 2 is sufficient for interaction with microtubules. J Neurosci Res. 2008 Jun;86(8):1711-20.
- Gao HM, Hong JS. Gene-environment interactions: key to unraveling the mystery of Parkinson's disease. Prog Neurobiol. 2011 Jun;94(1):1-19.
- Garcia Ruiz PJ, Catalán MJ, Fernández Carril JM. Initial motor symptoms of Parkinson disease. Neurologist. 2011 Nov;17(6 Suppl 1):S18-20. doi:10.1097/NRL.0b013e31823966b4. Review. PubMed PMID: 22045320.
- Gardet A, Benita Y, Li C, Sands BE, Ballester I, Stevens C, Korzenik JR, Rioux JD, Daly MJ, Xavier RJ, Podolsky DK. LRRK2 is involved in the IFN-gamma response and host response to pathogens. J Immunol. 2010 Nov 1;185(9):5577-85.
- Gillardon F. Leucine-rich repeat kinase 2 phosphorylates brain tubulin-beta isoforms and modulates microtubule stability—a point of convergence in parkinsonian neurodegeneration? J Neurochem. 2009 Sep;110(5):1514-22.
- Gloeckner CJ, Kinkl N, Schumacher A et al.: The Parkinson disease causing LRRK2 mutation I2020T is associated with in- creased kinase activity. Hum Mol Genet 2006, 15:223–232.
- Goetz CG. Charcot on Parkinson's disease. Mov Dis. 1986; 1: 27-32.
- Goetz CG, LeWitt PA, Weidenman M. Standardized training tools for the UPDRS activities of daily living scale: newly available teaching program. Mov Disord. 2003 Dec;18(12):1455-8. PubMed PMID: 14673881.
- Gómez-Suaga P, Luzón-Toro B, Churamani D, Zhang L, Bloor-Young D, Patel S, Woodman PG, Churchill GC, Hilfiker S. Leucine-rich repeat kinase 2 regulates autophagy through a calcium-dependent pathway involving NAADP. Hum Mol Genet. 2012 Feb 1;21(3):511-25.
- Greggio E, Cookson MR. Leucine-rich repeat kinase 2 mutations and Parkinson's disease: three questions. ASN Neuro. 2009 Apr 14;1(1).
- Greggio E, Jain S, Kingsbury A, Bandopadhyay R, Lewis P, Kaganovich A, van der

Brug MP, Beilina A, Blackinton J, Thomas KJ, Ahmad R, Miller DW, Kesavapany S, Singleton A, Lees A, Harvey RJ, Harvey K, Cookson MR. Kinase activity is required for the toxic effects of mutant LRRK2/dardarin. Neurobiol Dis. 2006 Aug;23(2):329-41.

- Gru¨newald A, Voges L, Rakovic A, et al. Mutant parkin impairs mitochondrial function and morphology in human fibroblasts. PLoS ONE 2010;5(9):e12962
- Guo L, Gandhi PN, Wang W, Petersen RB, Wilson-Delfosse AL, Chen SG. The Parkinson's disease-associated protein, leucine-rich repeat kinase 2 (LRRK2), is an authentic GTPase that stimulates kinase activity. Exp Cell Res. 2007 Oct 1;313(16):3658-70.
- Gustafsson MO, Hussain A, Mohammad DK, Mohamed AJ, Nguyen V, Metalnikov P, Colwill K, Pawson T, Smith CI, Nore BF. Regulation of nucleocytoplasmic shuttling of Bruton's tyrosine kinase (Btk) through a novel SH3 dependent interaction with ankyrin repeat domain 54 (ANKRD54). Mol Cell Biol. 2012 Jul;32(13):2440-53.
- Ha AD, Jankovic J. Pain in Parkinson's disease. Mov Disord. 2012 Apr;27(4):485-91.
- Hakimi M, Selvanantham T, Swinton E, Padmore RF, Tong Y, Kabbach G, Venderova K, Girardin SE, Bulman DE, Scherzer CR, LaVoie MJ, Gris D, Park DS, Angel JB, Shen J, Philpott DJ, Schlossmacher MG. Parkinson's disease-linked LRRK2 is expressed in circulating and tissue immune cells and upregulated following recognition of microbial structures. J Neural Transm. 2011 May;118(5):795-808.
- Hatano T, Kubo S, Imai S, Maeda M, Ishikawa K, Mizuno Y, Hattori N. Leucine-rich repeat kinase 2 associates with lipid rafts. Hum Mol Genet. 2007 Mar 15;16(6):678-90.
- Hayashi S, Wakabayashi K, Ishikawa A, Nagai H, Saito M, Maruyama M, Takahashi T, Ozawa T, Tsuji S, Takahashi H. An autopsy case of autosomal recessive juvenile parkinsonism with a homozygous exon 4 deletion in the parkin gene. Mov Disord. 2000 Sep;15(5):884-8.
- Healy DG, Falchi M, O'Sullivan SS, Bonifati V, Durr A, Bressman S, Brice A, Aasly

J, Zabetian CP, Goldwurm S, Ferreira JJ, Tolosa E, Kay DM, Klein C, Williams DR, Marras C, Lang AE, Wszolek ZK, Berciano J, Schapira AH, Lynch T, Bhatia KP, Gasser T, Lees AJ, Wood NW; International LRRK2 Consortium. Phenotype, genotype, and worldwide genetic penetrance of LRRK2 associated Parkinson's disease: a case-control study. Lancet Neurol. 2008 Jul;7(7):583-90.

- Heikkinen H, Nutt JG, LeWitt PA, et al. The effects of different repeated doses of entacapone on the pharmacokinetics of L- dopa and on the clinical response to L dopa in Parkinson's disease. Clin Neuropharmacol 2001; 24 (3): 150-7
- Higashi S, Biskup S, West AB, Trinkaus D, Dawson VL, Faull RL, Waldvogel HJ, Arai H, Dawson TM, Moore DJ, Emson PC. Localization of Parkinson's disease-associated LRRK2 in normal and pathological human brain. Brain Res. 2007 Jun 25;1155:208-19.
- Higashi S, Moore DJ, Colebrooke RE, Biskup S, Dawson VL, Arai H, Dawson TM, Emson PC. Expression and localization of Parkinson's disease-associated leucine-rich repeat kinase 2 in the mouse brain. J Neurochem. 2007 Jan;100(2):368-81.
- Hindle S, Afsari F, Stark M, Middleton CA, Evans GJ, Sweeney ST, Elliott CJ. Dopaminergic expression of the Parkinsonian gene LRRK2-G2019S leads to non-autonomous visual neurodegeneration, accelerated by increased neural demands for energy. Hum Mol Genet. 2013 Feb 20.
- Hirsch, M.A., Toole, T., Maitland, C.G., Rider, R.A., 2003. The effects of balance training and high-intensity resistance training on persons with idiopathic Parkinson's disease. Arch. Phys. Med. Rehabil. 84, 1109–1117.
- Ho CC, Rideout HJ, Ribe E, Troy CM, Dauer WT. The Parkinson disease protein leucine-rich repeat kinase 2 transduces death signals via Fas-associated protein with death domain and caspase-8 in a cellular model of neurodegeneration. J Neurosci. 2009 Jan 28;29(4):1011-6.
- Huang Y, Deng T, Winston BW. Characterization of hPRP4 kinase activation: potential role in signaling. Biochem Biophys Res Commun. 2000 May 10;271(2):456-63.
- Hummel T, Klämbt C. P-element mutagenesis. Methods Mol Biol. 2008;420:97-117.
- Iaccarino C, Crosio C, Vitale C, Sanna G, Carrì MT, Barone P. Apoptotic

mechanisms in mutant LRRK2-mediated cell death. Hum Mol Genet. 2007 Jun 1;16(11):1319-26.

- Ibanez P, Bonnet AM, Debarges B, Lohmann E, Tison F, Pollak P, et al. Causal relation between alpha-synuclein gene duplication and familial Parkinson's disease. Lancet 2004;364:1169e71.
- Ibáñez P, Lesage S, Lohmann E, Thobois S, De Michele G, Borg M, Agid Y, Dürr A, Brice A; French Parkinson's Disease Genetics Study Group. Mutational analysis of the PINK1 gene in early-onset parkinsonism in Europe and North Africa. Brain. 2006 Mar;129(Pt 3):686-94
- Imai Y, Gehrke S, Wang HQ, Takahashi R, Hasegawa K, Oota E, Lu B. Phosphorylation of 4E-BP by LRRK2 affects the maintenance of dopaminergic neurons in Drosophila. EMBO J. 2008 Sep 17;27(18):2432-43.
- Imai, Y., Soda, M., Takahashi, R., 2000. Parkin suppresses unfolded protein stress-induced cell death through its E3 ubiquitin-protein ligase activity. J. Biol. Chem. 275, 35661–35664.
- Irrcher I, Aleyasin H, Seifert EL, Hewitt SJ, Chhabra S, Phillips M, Lutz AK, Rousseaux MW, Bevilacqua L, Jahani-Asl A, Callaghan S, MacLaurin JG, Winklhofer KF, Rizzu P, Rippstein P, Kim RH, Chen CX, Fon EA, Slack RS, Harper ME, McBride HM, Mak TW, Park DS. Loss of the Parkinson's disease linked gene DJ-1 perturbs mitochondrial dynamics. Hum Mol Genet. 2010 Oct 1;19(19):3734-46.
- Ito G, Okai T, Fujino G et al.: GTP binding is essential to the protein kinase activity of LRRK2, a causative gene product for familial Parkinson's disease†. Biochemistry 2007, 46:1380–1388.
- Jaleel M, Nichols RJ, Deak M, Campbell DG, Gillardon F, Knebel A, Alessi DR. LRRK2 phosphorylates moesin at threonine-558: characterization of how Parkinson's disease mutants affect kinase activity. Biochem J. 2007 Jul 15;405(2):307-17.
- Jankovic J. Parkinson's disease: clinical features and diagnosis. J Neurol Neurosurg Psychiatry. 2008 Apr;79(4):368-76. doi: 10.1136/jnnp.2007.131045.
- Jankovic J, Poewe W. Therapies in Parkinson's disease. Curr Opin Neurol. 2012 Aug;25(4):433-47.
- Jankovic J, Stacy M. Medical management of levodopa associated motor

complications in patients with Parkinson's disease. CNS Drugs. 2007;21(8):677-92.

- Jellinger KA. Formation and development of Lewy pathology: a critical update. J Neurol. 2009 Aug;256 Suppl 3:270-9. doi: 10.1007/s00415-009-5243-y. Review. PubMed PMID: 19711116.
- Jorgensen ND, Peng Y, Ho CC, Rideout HJ, Petrey D, Liu P, Dauer WT. The WD40 domain is required for LRRK2 neurotoxicity. PLoS One. 2009 Dec 24;4(12):e8463.
- Kahle PJ, Waak J, Gasser T. DJ-1 and prevention of oxidative stress in Parkinson's disease and other age-related disorders. Free Radic Biol Med. 2009 Nov 15;47(10):1354-61.
- Kanao T, Venderova K, Park DS, Unterman T, Lu B, Imai Y. Activation of FoxO by LRRK2 induces expression of proapoptotic proteins and alters survival of postmitotic dopaminergic neuron in Drosophila. Hum Mol Genet. 2010 Oct 1;19(19):3747-58.
- Kawakami F, Yabata T, Ohta E, Maekawa T, Shimada N, Suzuki M, Maruyama H, Ichikawa T, Obata F. LRRK2 phosphorylates tubulin associated tau but not the free molecule: LRRK2-mediated regulation of the tau-tubulin association and neurite outgrowth. PLoS One. 2012;7(1):e30834.
- Kim B, Yang MS, Choi D, Kim JH, Kim HS, Seol W, Choi S, Jou I, Kim EY, Joe EH. Impaired inflammatory responses in murine Lrrk2 knockdown brain microglia. PLoS One. 2012;7(4):e34693.
- Kitada T, Asakawa S, Hattori N, Matsumine H, Yamamura Y, Minoshima S, et al. Mutations in the parkin gene cause autosomal recessive juvenile par- kinsonism. Nature 1998;392:605e8.
- Klein C, Pramstaller PP, Kis B, Page CC, Kann M, Leung J, Woodward H, Castellan CC, Scherer M, Vieregge P, Breakefield XO, Kramer PL, Ozelius LJ. Parkin deletions in a family with adult-onset, tremor-dominant parkinsonism: expanding the phenotype. Ann Neurol. 2000 Jul;48(1):65-71.
- Kruger, R., Kuhn, W., Muller, T., Woitalla, D., Graeber, M., Kosel, S., Przuntek, H., Epplen, J.T., Schols, L., Riess, O., 1998. Ala30Pro mutation in the gene encoding alpha-synuclein in Parkinson's disease. Nat. Genet. 18, 106–108.
- Kumar KR, Djarmati-Westenberger A, Grünewald A. Genetics of Parkinson's disease. Semin Neurol. 2011 Nov;31(5):433-40.

- Lai BC, Marion SA, Teschke K, Tsui JK. Occupational and environmental risk factors for Parkinson's disease. Parkinsonism Relat Disord. 2002 Jun;8(5):297-309.
- Langston JW, Ballard PA Jr. Parkinson's disease in a chemist working with 1-methyl-4-phenyl-1,2,5,6-tetrahydropyridine. N Engl J Med. 1983 Aug 4;309(5):310.
- Lee SB, Kim W, Lee S, Chung J. Loss of LRRK2/PARK8 induces degeneration of dopaminergic neurons in Drosophila. Biochem Biophys Res Commun. 2007 Jun 29;358(2):534-9.
- Lesage S, Durr A, Tazir M, Lohmann E, Leutenegger AL, Janin S, et al. LRRK2 G2019S as a cause of Parkinson's disease in North African Arabs. N Engl J Med. 2006;354:422–3. 211.
- Lewis PA, Greggio E, Beilina A et al.: The R1441C mutation of LRRK2 disrupts GTP hydrolysis. Biochem Biophys Res Commun 2007, 357:668–671.
- Li X, Patel JC, Wang J, Avshalumov MV, Nicholson C, Buxbaum JD, Elder GA, Rice ME, Yue Z. Enhanced striatal dopamine transmission and motor performance with LRRK2 over-expression in mice is eliminated by familial Parkinson's disease mutation G2019S. J Neurosci. 2010 Feb 3;30(5):1788-97.
- Li X, Patel JC, Wang J, Avshalumov MV, Nicholson C, Buxbaum JD, Elder GA, Rice ME, Yue Z. Enhanced striatal dopamine transmission and motor performance with LRRK2 over-expression in mice is eliminated by familial Parkinson's disease mutation G2019S. J Neurosci. 2010 Feb 3;30(5):1788-97.
- Li X, Tan YC, Poulose S et al.: Leucine-rich repeat kinase 2 (LRRK2)/PARK8 possesses GTPase activity that is altered in familial Parkinson's disease R1441C/G mutants. J Neurochem 2007, 103:238–247.
- Li Y, Liu W, Oo TF, Wang L, Tang Y, Jackson-Lewis V, Zhou C, Geghman K, Bogdanov M, Przedborski S, Beal MF, Burke RE, Li C. Mutant LRRK2(R1441G) BAC transgenic mice recapitulate cardinal features of Parkinson's disease. Nat Neurosci. 2009 Jul;12(7):826-8.
- Lin CH, Tsai PI, Wu RM, Chien CT. LRRK2 G2019S mutation induces dendrite degeneration through mislocalization and phosphorylation of tau by recruiting autoactivated GSK3ß. J Neurosci. 2010 Sep 29;30(39):13138-49.
- Liu Z, Wang X, Yu Y, Li X, Wang T, Jiang H, Ren Q, Jiao Y, Sawa A, Moran T,

Ross CA, Montell C, Smith WW. A Drosophila model for LRRK2-linked parkinsonism. Proc Natl Acad Sci U S A. 2008 Feb 19;105(7):2693 8.

- Lu¨cking CB, Du¨rr A, Bonifati V, et al; French Parkinson's Disease Genetics Study Group; European Consortium on Genetic Susceptibility in Parkinson's Disease. Association between early-onset Parkinson's disease and mutations in the parkin gene. N Engl J Med 2000;342(21):1560–1567
- Lu YW, Tan EK. Molecular biology changes associated with LRRK2 mutations in Parkinson's disease. J Neurosci Res. 2008 Jul;86(9):1895-901.
- MacLeod D, Dowman J, Hammond R, Leete T, Inoue K, Abeliovich A. The familial Parkinsonism gene LRRK2 regulates neurite process morphology. Neuron. 2006 Nov 22;52(4):587-93.
- Mao Z, Davis RL. Eight different types of dopaminergic neurons innervate the Drosophila mushroom body neuropil: anatomical and physiological heterogeneity. Front Neural Circuits. 2009;3:5.
- Marchese, R., Diverio, M., Zucchi, F., Lentino, C., Abbruzzese, G., 2000. The role of sensory cues in the rehabilitation of parkinsonian patients: a comparison of two physical therapy protocols. Mov. Disord. 15, 879–883.
- Marttila RJ, Kaprio J, Koskenvuo M, Rinne UK. Parkinson'sdisease in a nationwide twin cohort. Neurology. 1988;38:1217–9.
- Mata IF, Wedemeyer WJ, Farrer MJ, Taylor JP, Gallo KA. LRRK2 in Parkinson's disease: protein domains and functional insights. Trends Neurosci. 2006 May;29(5):286-93.
- Matta S, Van Kolen K, da Cunha R, van den Bogaart G, Mandemakers W, Miskiewicz K, De Bock PJ, Morais VA, Vilain S, Haddad D, Delbroek L, Swerts J, Chávez-Gutiérrez L, Esposito G, Daneels G, Karran E, Holt M, Gevaert K, Moechars DW, De Strooper B, Verstreken P. LRRK2 controls an EndoA phosphorylation cycle in synaptic endocytosis. Neuron. 2012 Sep 20;75(6):1008-21.
- Melrose HL, Dächsel JC, Behrouz B, Lincoln SJ, Yue M, Hinkle KM, Kent CB, Korvatska E, Taylor JP, Witten L, Liang YQ, Beevers JE, Boules M, Dugger BN, Serna VA, Gaukhman A, Yu X, Castanedes-Casey M, Braithwaite AT, Ogholikhan S, Yu N, Bass D, Tyndall G, Schellenberg GD, Dickson DW, Janus C, Farrer MJ. Impaired dopaminergic neurotransmission and microtubule associated protein tau

alterations in human LRRK2 transgenic mice. Neurobiol Dis. 2010 Dec;40(3):503-17.

- Miklossy J, Arai T, Guo JP, Klegeris A, Yu S, McGeer EG, McGeer PL. LRRK2 expression in normal and pathologic human brain and in human cell lines. J Neuropathol Exp Neurol. 2006 Oct;65(10):953-63.
- Milusheva EA, Dóda M, Baranyi M, Vizi ES. Effect of hypoxia and glucose deprivation on ATP level, adenylate energy charge and [Ca2+]o-dependent and independent release of [3H]dopamine in rat striatal slices. Neurochem Int. 1996 May-Jun;28(5-6):501-7.
- Moehle MS, Webber PJ, Tse T, Sukar N, Standaert DG, DeSilva TM, Cowell RM, West AB. LRRK2 inhibition attenuates microglial inflammatory responses. J Neurosci. 2012 Feb 1;32(5):1602-11.
- Monastirioti M. Biogenic amine systems in the fruit fly Drosophila melanogaster. Microsc Res Tech. 1999 Apr 15;45(2):106-21.
- Mori H, Kondo T, Yokochi M, et al. Pathologic and biochemical studies of juvenile parkinsonism linked to chromosome 6q. Neurology 1998;51(3):890–892
- Mortiboys H, Johansen KK, Aasly JO, Bandmann O. Mitochondrial impairment in patients with Parkinson disease with the G2019S mutation in LRRK2. Neurology. 2010 Nov 30;75(22):2017-20.
- Myhre R, Steinkjer S, Stormyr A, Nilsen GL, Abu Zayyad H, Horany K, Nusier MK, Klungland H. Significance of the parkin and PINK1 gene in Jordanian families with incidences of young-onset and juvenile parkinsonism. BMC Neurol. 2008 Dec 16;8:47.
- Narhi L, Wood SJ, Steavenson S, Jiang Y, Wu GM, Anafi D, Kaufman SA, Martin F, Sitney K, Denis P, Louis JC, Wypych J, Biere AL, Citron M. Both familial Parkinson's disease mutations accelerate alpha-synuclein aggregation. J Biol Chem. 1999 Apr 2;274(14):9843-6. Erratum in: J Biol Chem 1999 May 7;274(19):13728.
- Ng CH, Guan MS, Koh C, Ouyang X, Yu F, Tan EK, O'Neill SP, Zhang X, Chung J, Lim KL. AMP kinase activation mitigates dopaminergic dysfunction and mitochondrial abnormalities in Drosophila models of Parkinson's disease. J Neurosci. 2012 Oct 10;32(41):14311-7.

- Niu J, Yu M, Wang C, Xu Z. Leucine-rich repeat kinase 2 disturbs mitochondrial dynamics via Dynamin-like protein. J Neurochem. 2012 Aug;122(3):650-8.
- Obeso JA, Rodriguez-Oroz MC, Goetz CG, Marin C, Kordower JH, Rodriguez M, Hirsch EC, Farrer M, Schapira AH, Halliday G. Missing pieces in the Parkinson's disease puzzle. Nat Med. 2010 Jun;16(6):653-61.
- Orenstein SJ, Kuo SH, Tasset I, Arias E, Koga H, Fernandez-Carasa I, Cortes E, Honig LS, Dauer W, Consiglio A, Raya A, Sulzer D, Cuervo AM. Interplay of LRRK2 with chaperone-mediated autophagy. Nat Neurosci. 2013 Apr;16(4):394-406.
- Paisán-Ruiz C. LRRK2 gene variation and its contribution to Parkinson disease. Hum Mutat. 2009 Aug;30(8):1153-60.
- Paisan-Ruiz C, Jain S, Evans EW, Gilks WP, Simon J, van der Brug M, et al. Cloning of the gene containing mutations that cause PARK8-linked Parkin- son's disease. Neuron 2004;44:595e600.
- Paisan-Ruiz C, Nath P, Washecka N, Gibbs JR, Singleton AB. Comprehensive analysis of LRRK2 in publicly available Par- kinson's disease cases and neurologically normal controls. HumMutat. 2008;29:485–90.
- Palacino JJ, Sagi D, Goldberg MS, et al. Mitochondrial dysfunction and oxidative damage in parkin-deficient mice. J Biol Chem 2004;279(18):18614–18622
- Papkovskaia TD, Chau KY, Inesta-Vaquera F, Papkovsky DB, Healy DG, Nishio K, Staddon J, Duchen MR, Hardy J, Schapira AH, Cooper JM. G2019S leucine-rich repeat kinase 2 causes uncoupling protein-mediated mitochondrial depolarization. Hum Mol Genet. 2012 Oct 1;21(19):4201-13.
- Parent M, Parent A. Substantia nigra and Parkinson's disease: a brief history of their long and intimate relationship. Can J Neurol Sci. 2010 May;37(3):313-9.
- Parisiadou L, Xie C, Cho HJ, Lin X, Gu XL, Long CX, Lobbestael E, Baekelandt V, Taymans JM, Sun L, Cai H. Phosphorylation of ezrin/radixin/moesin proteins by LRRK2 promotes the rearrangement of actin cytoskeleton in neuronal morphogenesis. J Neurosci. 2009 Nov 4;29(44):13971-80.
- Parkinson J. An essay on the shaking palsy. London: Sherwood, Neely and Jones; 1817.
- Piccini P, Burn DJ, Ceravolo R, Maraganore D, Brooks DJ. The role of inheritance in

sporadic Parkinson's disease: evidence from a longitudinal study of dopaminergic function in twins. Ann Neurol. 1999 May;45(5):577-82.

- Piccoli G, Condliffe SB, Bauer M, Giesert F, Boldt K, De Astis S, Meixner A, Sarioglu H, Vogt-Weisenhorn DM, Wurst W, Gloeckner CJ, Matteoli M, Sala C, Ueffing M. LRRK2 controls synaptic vesicle storage and mobilization within the recycling pool. J Neurosci. 2011 Feb 9;31(6):2225 37.
- Polymeropoulos MH, Lavedan C, Leroy E, Ide SE, Dehejia A, Dutra A, Pike B, Root H, Rubenstein J, Boyer R, Stenroos ES, Chandrasekharappa S, Athanassiadou A, Papapetropoulos T, Johnson WG, Lazzarini AM, Duvoisin RC, Di Iorio G, Golbe LI, Nussbaum RL. Mutation in the alpha-synuclein gene identified in families with Parkinson's disease. Science. 1997 Jun 27;276(5321):2045-7.
- Poulopoulos M, Cortes E, Vonsattel JP, Fahn S, Waters C, Cote LJ, Moskowitz C, Honig LS, Clark LN, Marder KS, Alcalay RN. Clinical and pathological characteristics of LRRK2 G2019S patients with PD. J Mol Neurosci. 2012 May;47(1):139-43.
- Puschmann A. Monogenic Parkinson's disease and parkinsonism: Clinical phenotypes and frequencies of known mutations. Parkinsonism Relat Disord. 2013 Apr;19(4):407-15.
- Ramonet D, Daher JP, Lin BM, Stafa K, Kim J, Banerjee R, Westerlund M, Pletnikova O, Glauser L, Yang L, Liu Y, Swing DA, Beal MF, Troncoso JC, McCaffery JM, Jenkins NA, Copeland NG, Galter D, Thomas B, Lee MK, Dawson TM, Dawson VL, Moore DJ. Dopaminergic neuronal loss, reduced neurite complexity and autophagic abnormalities in transgenic mice expressing G2019S mutant LRRK2. PLoS One. 2011 Apr 6;6(4):e18568.
- Reiter, L.T., Potocki, L., Chien, S., Gribskov, M., and Bier, E. A systemat- ic analysis of human disease-associated gene sequences in Drosophila melanogaster. *Genome Res*. **11**, 1114–1125 (2001).
- Saito Y, Ruberu NN, Sawabe M, Arai T, Kazama H, Hosoi T, Yamanouchi H, Murayama S (2004) Lewy body-related a-syn- ucleinopathy in aging. J Neuropathol Exp Neurol 63:742–749
- Salthun-Lassalle B, Hirsch EC, Wolfart J, Ruberg M, Michel PP. Rescue of

mesencephalic dopaminergic neurons in culture by low level stimulation of voltage-gated sodium channels. J Neurosci. 2004 Jun 30;24(26):5922-30.

- Samaranch L, Lorenzo-Betancor O, Arbelo JM, Ferrer I, Lorenzo E, Irigoyen J, et al. PINK1-linked parkinsonism is associated with Lewy body pathology. Brain 2010;133:1128e42.
- Samuels AL, Klinken SP, Ingley E. Liar, a novel Lyn binding nuclear/cytoplasmic shuttling protein that influences erythropoietin-induced differentiation. Blood. 2009 Apr 16;113(16):3845 56.
- Sánchez-Danés A, Richaud-Patin Y, Carballo-Carbajal I, Jiménez-Delgado S, Caig C, Mora S, Di Guglielmo C, Ezquerra M, Patel B, Giralt A, Canals JM, Memo M, Alberch J, López-Barneo J, Vila M, Cuervo AM, Tolosa E, Consiglio A, Raya A. Disease-specific phenotypes in dopamine neurons from human iPS-based models of genetic and sporadic Parkinson's disease. EMBO Mol Med. 2012 May;4(5):380-95.
- Shadrina MI, Slominsky PA, Limborska SA. Molecular mechanisms of pathogenesis of Parkinson's disease. Int Rev Cell Mol Biol. 2010;281:229-66.
- Sharma A, Callahan LM, Sul JY, Kim TK, Barrett L, Kim M, Powers JM, Federoff H, Eberwine J. A neurotoxic phosphoform of Elk-1 associates with inclusions from multiple neurodegenerative diseases. PLoS One. 2010 Feb 2;5(2):e9002.
- Shin N, Jeong H, Kwon J, Heo HY, Kwon JJ, Yun HJ, Kim CH, Han BS, Tong Y, Shen J, Hatano T, Hattori N, Kim KS, Chang S, Seol W. LRRK2 regulates synaptic vesicle endocytosis. Exp Cell Res. 2008 Jun 10;314(10):2055-65.
- Singleton AB, Farrer M, Johnson J, Singleton A, Hague S, Kachergus J, et al. alpha-Synuclein locus triplication causes Parkinson's disease. Science 2003; 302:841.
- Smith WW, Pei Z, Jiang H, Dawson VL, Dawson TM, Ross CA. Kinase activity of mutant LRRK2 mediates neuronal toxicity. Nat Neurosci. 2006 Oct;9(10):1231-3.
- Smith WW, Pei Z, Jiang H, Moore DJ, Liang Y, West AB, Dawson VL, Dawson TM, Ross CA. Leucine-rich repeat kinase 2 (LRRK2) interacts with parkin, and mutant LRRK2 induces neuronal degeneration. Proc Natl Acad Sci USA. 2005 Dec 20;102(51):18676-81.
- Solla P, Cannas A, Floris GL, Orofino G, Costantino E, Boi A, Serra C, Marrosu MG, Marrosu F. Behavioral, neuropsychiatric and cognitive disorders in Parkinson's

disease patients with and without motor complications. Prog Neuropsychopharmacol Biol Psychiatry. 2011 Jun 1;35(4):1009-13.

- Spillantini MG, Crowther RA, Jakes R, Cairns NJ, Lantos PL, Goedert M. Filamentous alpha-synuclein inclusions link multiple system atrophy with Parkinson's disease and dementia with Lewy bodies. Neurosci Lett. 1998 Jul 31;251(3):205-8.
- Stafa K, Trancikova A, Webber PJ, Glauser L, West AB, Moore DJ. GTPase activity and neuronal toxicity of Parkinson's disease associated LRRK2 is regulated by ArfGAP1. PLoS Genet. 2012;8(2):e1002526.
- Statistics Canada. *Table 105-1300 - Neurological conditions, by age group and sex, household population aged 0 and over, 2010/2011, occasional (number unless otherwise noted),* CANSIM (database). Accessed: 2013-04-20.
- Statistics Canada. *Table 102-0561 - Leading causes of death, total population, by age group and sex, Canada, annual,* CANSIM (database). Accessed: 2013-04-20.
- Steinlechner S, Stahlberg J, Vo¨lkel B, et al. Co-occurrence of affective and schizophrenia spectrum disorders with PINK1 mutations. J Neurol Neurosurg Psychiatry 2007;78(5): 532–535
- Tanner CM, Ottman R, Goldman SM, Ellenberg J, Chan P, Mayeux R, Langston JW. Parkinson disease in twins: an etiologic study. JAMA. 1999 Jan 27;281(4):341-6.
- Taymans JM, Vancraenenbroeck R, Ollikainen P et al.: LRRK2 kinase activity is dependent on LRRK2 GTP binding capacity but independent of LRRK2 GTP binding. PLoS One 2011, 6:e23207.
- Thévenet J, Pescini Gobert R, Hooft van Huijsduijnen R, Wiessner C, Sagot YJ. Regulation of LRRK2 expression points to a functional role in human monocyte maturation. PLoS One. 2011;6(6):e21519.
- Tong Y, Giaime E, Yamaguchi H, Ichimura T, Liu Y, Si H, Cai H, Bonventre JV, Shen J. Loss of leucine-rich repeat kinase 2 causes age-dependent bi-phasic alterations of the autophagy pathway. Mol Neurodegener. 2012 Jan 9;7:2.
- Tong Y, Pisani A, Martella G, Karouani M, Yamaguchi H, Pothos EN, Shen J. R1441C mutation in LRRK2 impairs dopaminergic neurotransmission in mice. Proc Natl Acad Sci U S A. 2009 Aug 25;106(34):14622-7.

- Tong Y, Yamaguchi H, Giaime E, Boyle S, Kopan R, Kelleher RJ 3rd, Shen J. Loss of leucine-rich repeat kinase 2 causes impairment of protein degradation pathways, accumulation of alpha-synuclein, and apoptotic cell death in aged mice. Proc Natl Acad Sci USA. 2010 May 25;107(21):9879-84.
- Toulouse A, Sullivan AM. Progress in Parkinson's disease-where do we stand? Prog Neurobiol. 2008 Aug;85(4):376-92.
- Trancikova A, Tsika E, Moore DJ. Mitochondrial dysfunction in genetic animal models of Parkinson's disease. Antioxid Redox Signal. 2012 May 1;16(9):896-919.
- Tsika E, Moore DJ. Mechanisms of LRRK2-mediated neurodegeneration. Curr Neurol Neurosci Rep. 2012 Jun;12(3):251-60.
- Tsyba L, Nikolaienko O, Dergai O, Dergai M, Novokhatska O, Skrypkina I, Rynditch A. Intersectin multidomain adaptor proteins: regulation of functional diversity. Gene. 2011 Mar 1;473(2):67-75.
- Umeno J, Asano K, Matsushita T, Matsumoto T, Kiyohara Y, Iida M, Nakamura Y, Kamatani N, Kubo M. Meta-analysis of published studies identified eight additional common susceptibility loci for Crohn's disease and ulcerative colitis. Inflamm Bowel Dis. 2011 Dec;17(12):2407-15.
- Valente EM, Abou-Sleiman PM, Caputo V, Muqit MM, Harvey K, Gispert S, et al. Hereditary early-onset Parkinson's disease caused by mutations in PINK1. Science 2004;304:1158e60.
- Valente EM, Salvi S, Ialongo T, et al. PINK1 mutations are associated with sporadic early-onset parkinsonism. Ann Neurol 2004;56(3):336–341.
- Vancraenenbroeck R, Lobbestael E, Weeks SD, Strelkov SV, Baekelandt V, et al. Expression, purification and preliminary biochemical and structural characterization of the leucine rich repeat namesake domain of leucine rich repeat kinase 2. Biochim Biophys Acta. 2012;1824:450–460.
- Venderova K, Kabbach G, Abdel-Messih E, Zhang Y, Parks RJ, Imai Y, Gehrke S, Ngsee J, Lavoie MJ, Slack RS, Rao Y, Zhang Z, Lu B, Haque ME, Park DS. Leucine-Rich Repeat Kinase 2 interacts with Parkin, DJ 1 and PINK-1 in a Drosophila melanogaster model of Parkinson's disease. Hum Mol Genet. 2009 Nov 15;18(22):4390-404.

- Wang D, Qian L, Xiong H, Liu J, Neckameyer WS, Oldham S, Xia K, Wang J, Bodmer R, Zhang Z. Antioxidants protect PINK1-dependent dopaminergic neurons in Drosophila. Proc Natl Acad Sci U S A. 2006 Sep 5;103(36):13520-5.
- Wang D, Tang B, Zhao G, Pan Q, Xia K, Bodmer R, Zhang Z. Dispensable role of Drosophila ortholog of LRRK2 kinase activity in survival of dopaminergic neurons. Mol Neurodegener. 2008 Feb 8;3:3.
- Wang X, Petrie TG, Liu Y, Liu J, Fujioka H, Zhu X. Parkinson's disease-associated DJ-1 mutations impair mitochondrial dynamics and cause mitochondrial dysfunction. J Neurochem. 2012 Jun;121(5):830-9.
- Wang X, Yan MH, Fujioka H, Liu J, Wilson-Delfosse A, Chen SG, Perry G, Casadesus G, Zhu X. LRRK2 regulates mitochondrial dynamics and function through direct interaction with DLP1. Hum Mol Genet. 2012 May 1;21(9):1931-44.
- Ward CD, Duvoisin RC, Ince SE, Nutt JD, Eldridge R, Calne DB. Parkinson's disease in 65 pairs of twins and in a set ofquadruplets. Neurology. 1983;33:815 24.
- Waters CH, Sethi KD, Hauser RA, et al. Zydis selegiline reduces off time in Parkinson's disease patients with motor fluctuations: a 3-month, randomized, placebo-controlled study. Mov Disord 2004; 19 (4): 426-32
- West AB, Moore DJ, Biskup S, Bugayenko A, Smith WW, Ross CA, Dawson VL, Dawson TM. Parkinson's disease-associated mutations in leucine-rich repeat kinase 2 augment kinase activity. Proc Natl Acad Sci U S A. 2005 Nov 15;102(46):16842-7.
- West AB, Moore DJ, Choi C, Andrabi SA, Li X, Dikeman D, Biskup S, Zhang Z, Lim KL, Dawson VL, Dawson TM. Parkinson's disease- associated mutations in LRRK2 link enhanced GTP-binding and kinase activities to neuronal toxicity. Hum Mol Genet. 2007 Jan 15;16(2):223-32.
- Westerlund M, Belin AC, Anvret A, Bickford P, Olson L, Galter D. Developmental regulation of leucine-rich repeat kinase 1 and 2 expression in the brain and other rodent and human organs: Implications for Parkinson's disease. Neuroscience. 2008 Mar 18;152(2):429-36.
- White KE, Humphrey DM, Hirth F. The dopaminergic system in the aging brain of Drosophila. Front Neurosci. 2010 Dec 8;4:205.
- Williams DR, Watt HC, Lees AJ. Predictors of falls and fractures in bradykinetic

rigid syndromes: a retrospective study. J Neurol Neurosurg Psychiatry 2006;77:468–73.

- Wirdefeldt K, Adami HO, Cole P, Trichopoulos D, Mandel J. Epidemiology and etiology of Parkinson's disease: a review of the evidence. Eur J Epidemiol. 2011 Jun;26
- Wood LD. Clinical review and treatment of select adverse effects of dopamine receptor agonists in Parkinson's disease. Drugs Aging. 2010 Apr 1;27(4):295-310.
- Xiong Y, Yuan C, Chen R, Dawson TM, Dawson VL. ArfGAP1 is a GTPase activating protein for LRRK2: reciprocal regulation of ArfGAP1 by LRRK2. J Neurosci. 2012 Mar 14;32(11):3877-86.
- Yahr MD, Duvoisin RC, Schear MJ, Barrett RE, Hoehn MM. Treatment of parkinsonism with levodopa. Arch Neurol. 1969 Oct;21(4):343-54.
- Yang D, Li T, Liu Z, Arbez N, Yan J, Moran TH, Ross CA, Smith WW. LRRK2 kinase activity mediates toxic interactions between genetic mutation and oxidative stress in a Drosophila model: suppression by curcumin. Neurobiol Dis. 2012 Sep;47(3):385-92.
- Yarosh W, Monserrate J, Tong JJ, Tse S, Le PK, Nguyen K, Brachmann CB, Wallace DC, Huang T. The molecular mechanisms of OPA1-mediated optic atrophy in Drosophila model and prospects for antioxidant treatment. PLoS Genet. 2008 Jan;4(1):e6.
- Yu HS, Kim SH, Park HG, Kim YS, Ahn YM. Intracerebroventricular administration of ouabain, a Na/K-ATPase inhibitor, activates tyrosine hydroxylase through extracellular signal-regulated kinase in rat striatum. Neurochem Int. 2011 Nov;59(6):779-86.
- Zarow, C., Lyness, S.A., Mortimer, J.A. & Chui, H.C. Neuronal loss is greater in the locus coeruleus than nucleus basalis and substantia nigra in Alzheimer and Parkinson diseases. Arch. Neurol. 60, 337–341 (2003).
- Zarranz, J.J., Alegre, J., Gomez-Esteban, J.C., Lezcano, E., Ros, R., Ampuero, I., Vidal, L., Hoenicka, J., Rodriguez, O., Atares, B., Llorens, V., Gomez Tortosa, E., del Ser, T., Munoz, D.G., de Yebenes, J.G., 2004. The new mutation, E46 K, of alpha- synuclein causes Parkinson and Lewy body dementia. Ann. Neurol. 55, 164–

173.

- Zhang FR, Huang W, Chen SM, Sun LD, Liu H, Li Y, Cui Y, Yan XX, Yang HT, Yang RD, Chu TS, Zhang C, Zhang L, Han JW, Yu GQ, Quan C, Yu YX, Zhang Z, Shi BQ, Zhang LH, Cheng H, Wang CY, Lin Y, Zheng HF, Fu XA, Zuo XB, Wang Q, Long H, Sun YP, Cheng YL, Tian HQ, Zhou FS, Liu HX, Lu WS, He SM, Du WL, Shen M, Jin QY, Wang Y, Low HQ, Erwin T, Yang NH, Li JY, Zhao X, Jiao YL, Mao LG, Yin G, Jiang ZX, Wang XD, Yu JP, Hu ZH, Gong CH, Liu YQ, Liu RY, Wang DM, Wei D, Liu JX, Cao WK, Cao HZ, Li YP, Yan WG, Wei SY, Wang KJ, Hibberd ML, Yang S, Zhang XJ, Liu JJ. Genomewide association study of leprosy. N Engl J Med. 2009 Dec 31;361(27):2609-18.
- Zimprich A, Biskup S, Leitner P, Lichtner P, Farrer M, Lincoln S, Kachergus J, Hulihan M, Uitti RJ, Calne DB, Stoessl AJ, Pfeiffer RF, Patenge N, Carbajal IC, Vieregge P, Asmus F, Müller-Myhsok B, Dickson DW, Meitinger T, Strom TM, Wszolek ZK, Gasser T. Mutations in LRRK2 cause autosomal dominant parkinsonism with pleomorphic pathology. Neuron. 2004 Nov 18;44(4):601-7.
- Zimprich A, Biskup S, Leitner P, Lichtner P, Farrer M, Lincoln S, et al. Muta- tions in LRRK2 cause autosomal-dominant parkinsonism with pleomorphic pathology. Neuron 2004;44:601e7.