THE ANALYSIS OF PARKIN IN DROSOPHILA MELANOGASTER

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THE ANALYSIS OF *PARKIN* IN *DROSOPHILA MELANOGASTER*

A thesis presented in partial fulfillment of the requirements for the degree of
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Abstract

Parkinson's disease (PD) is a highly prevalent neurodegenerative disease characterized by loss of motor control and resting tremor. Mutations in a number of genes, including *α-synuclein* and *parkin*, have been associated with inherited forms of PD and many of these genes are linked to the ubiquitin/proteasome degradation system (UPS). Studies of the effects of mutations in these genes suggest that impairment of the UPS is central to PD. Expression of wild-type or PD-associated forms of human *α-synuclein* in *Drosophila melanogaster* neurons recapitulates some of the symptoms of PD such as the loss of motor control, development of neuronal inclusions, and degeneration of dopaminergic neurons. Parkin, an E3 ubiquitin protein ligase, may be involved in targeting α-synuclein for degradation. To analyse this interaction I generated transgenic flies expressing *parkin* under the control of the yeast enhancer upstream activating sequence (UAS). The *α-synuclein* and *parkin* transgenes were expressed in combination to examine their interaction *in vivo*. I showed that expresion of *parkin* prevents the toxic effects of both mutant and wild-type human α-synuclein. Although the yeast protein Gal4 is a key component of the UAS/Gal4 ectopic expression system in *Drosophila melanogaster*, I showed that this protein can be toxic. Transgenic flies that express high levels of *Gal4* in the developing eye show elevated apoptosis in the eye imaginal disc, which leads to a disorganised ommatidial array in the adult. Suppression of apoptosis by expression of the caspase inhibitor *p35* prevents this. High levels of *Gal4* expression in dopaminergic neurons produce larvae that have excessive apoptosis in the brain and reduced longevity in adult flies. I showed that *parkin* can suppress apoptosis and development defects in the eye. The ability of *parkin* to counter the toxicity of exogenous and endogenous proteins may provide great insight into our understanding of toxic protein-induced diseases.

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Dedication

This thesis is dedicated to my biggest supporter of my academic career, my Grandmother Margaret Laird or as I knew her Mardy, who sadly passed away in January 2002. I thank her for all her support throughout my budding academic career and I hope that she can see me now. I finally finished my Ph.D.

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List of Abbreviations

μL	microlitres
6-OHDA	6-hydroxydopamine
A30Pα-synuclein	alanine to proline amino acid exchange at position 30 of human α-synuclein
A53Tα-synuclein	alanine to threonine amino acid exchange at position 53 of human α-synuclein
ADPD	Autosomal Dominant Parkinson's disease
ANOVA	Analysis of Variance between groups
ap	apterous
ARJP	Autosomal Recessive Juvenile Parkinsonian
BDGP	Berkley Drosophila Genome Project
CHIP	carboxy terminus of the HSP/HSC70 interacting protein
CSPα	cystein string protein α
CyO	Curly of Oscar – a multiply inverted balancer chromosme II in Drosophila
dADP	Deoxyadenosine diphosphate
dATP	deoxyadenosine triphosphate
Ddc	DOPA decarboxylase
DIAP 2	Drosophila inhibitor of apoptosis 2
DIG	Digoxigenin
DNA	Deoxyribonucleic acid
E1	ubiquitin activating enzyme
E2	ubiquitin conjugating enzyme
E3	ubiquitin ligase enzyme
ER	endoplasmic reticulum
ey	eyeless
GFP	Green Fluorescent Protein
GMR	glass mediated receptor
Gprk2	G-protein coupled receptor kinase
H_2O_2	hydrogen peroxide
HSP	Heat Shock Protein
IBR	inbetween ring domain
InR	insulin receptor
K	lysine
LRRK2	gene name for dadarin
mL	millilitres
MPP^+	1-methly-4-phenyl-2,3-diydropyridinium
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
NAC	non-β amyloid component
NaOH	sodium hydroxide
NCBI	National Center for Biotechnolgy Information
Pael-R	Pael receptor
PBS	Phosphate-buffered saline
PBT	phosphate buffered saline with 0.1% Tween20
PCR	polymerase chain reaction

PD	Parkinson's disease
RB	ringbox
RING	Really interesting new gene
ROCO	Roc (Ras of complex proteins)/COR (c-terminal of Roc)
ROS	reactive oxygen species
rpr	reaper
selegiline	L-deprenyl
SEM	scanning electron micrograph
SNARE	soluble N-ethylmaleimide-sensitive factor attachment protein (SNAP) receptor
SNCA	Gene name for α-synuclein
TM3	third chromosome multiply inverted – a Drosophila balance chromosome
Tween-20	Polyoxyethylenesorbitan monolaurate
UAS	upstream activating sequence
UbcH7or 8	ubiquitin conjugating enzyme human 7 or 8
UBL	ubiquitin binding domain
Uch-L1	Ubiquitin carboxy-terminal hydrolase- L1
UPD	unique parkin domain
UPR	unfolded protein response
WTα-synuclein	wild-type human α-synuclein

Chapter 1: Models of Parkinson's disease

1.1 Neurodegenerative diseases and *Drosophila melanogaster*

As the mean age of the population increases, age-related diseases such as Parkinson's disease (PD), Alzheimer's disease and sporadic cancers are gaining in prominence. Most of these diseases have a genetic basis, or at the least a genetic bias, and to this end research has been undertaken to learn the genetic factors that contribute to age-related diseases. There is much to be gained by observation of the behaviour patterns of aging humans and by post-mortem analysis, however it is not practical nor ethical to conduct experiments with chemicals that have unknown toxicologies, to do invasive procedures, or to conduct selective breeding studies to look at genetic interactions. The development of disease models in an organism that is short-lived, and easy to manipulate, analyse, and sacrifice, is a reasonable approach to understanding age-related disease. Evidence supports that study of disease models is an extremely valuable approach in the investigation of many health problems. In order to understand the pathology of a disease and to test potential therapeutic compounds, a disease should be modeled in an organism that facilitates ready analysis. Understanding the mechanisms involved in disease pathology enables rational selection of possible therapeutic agents.

Models of several neurodegenerative diseases have been developed using a range of methods and organisms (Chan, 2004; Marsh and Thompson, 2004). As an alternative to humans, disease phenotypes, both behavioural and morphological, can be replicated in other organisms. In order to develop disease models, both behavioural and biochemical analysis must be feasible and reproducible, and the findings must be translatable into implications for the disease. Model organisms, such as *Drosophila melanogaster,* have up to 77% of genes

involved in human diseases (Fortini *et al.*, 2000; Reiter *et al.*, 2001) and the basic cellular mechanisms, in terms of structure and function, are often highly conserved. Over-expression of human disease genes in flies has been successful in modelling the pathology of amyloid β_{1-42} in Alzheimer's disease, α-synuclein in PD and expanded polyglutamine tract proteins similar to those found in Huntington's disease (Driscoll and Gerstbrein, 2003). The generation of transgenic flies is relatively simple, and cost effective, and both molecular and behavioural studies are feasible, which make them ideal model organisms to find or evaluate potential therapeutic strategies of many neurodegenerative diseases.

1.2 Introduction to Parkinson's disease

PD is a neurodegenerative disorder, first described nearly two centuries ago (Parkinson, 1817) that is characterized by muscle tremors in stationary limbs, bradykinesia and rigidity. As the disease develops, the balance and memory of the affected person becomes progressively impaired until a premature death (Spacey and Wood, 1999). Post-mortem analysis of affected patients reveals the selective loss of dopaminergic neurons and the presence of filamentous protein inclusions (Lewy bodies) within the cell bodies and neurites of the substantia nigra pars compacta region of the brain (Spacey and Wood, 1999). Notably, not all PD patients have Lewy bodies (Hayashi *et al.*, 2000; Takahashi *et al.*, 1994) and conversely, some non-PD-symptomatic patients were shown to have Lewy bodies (Forno and Langston, 1993; Gibb and Lees, 1988; Goldberg and Lansbury, 2000). In recent years, many forms of PD, particularly those with early onset, have been shown to have an inherited basis. Approximately 5-10% of all disease cases of PD have an inherited basis (Mizuno *et al.*, 2001).

Animal models, many generated by chemical means and, more recently, by genetic methods have been invaluable in the study of PD (Betarbet *et al.*, 2002). For example, mice or rats treated with methamphetamine show a reduction of dopamine production, while treatments with 6-hydroxydopamine (6-OHDA), 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), paraquat, rotenone or 3-nitrotyrosine, result in the loss of dopaminergic neurons. The loss of these neurons is generally through oxidative stress-induced apoptosis, a feature of PD. A genetic model through over-expression of human wild-type *α-synuclein* in mice results in the accumulation of α-synuclein-rich protein-inclusions, loss of dopaminergic neurons and impaired motor performance (Masliah *et al.*, 2000). A primate model of PD was recently developed via specific expression of human *α-synuclein* in the substantia nigra of adult marmosets (Kirik *et al.*, 2003). Directed expression of mutant *α-synuclein*, the form associated with familial PD (human α-synuclein A53T), in *Drosophila melanogaster* led to degeneration of dopaminergic neurons (Feany and Bender, 2000; Giasson *et al.*, 2002; Lee *et al.*, 2002b). *Drosophila parkin* null mutants have reduced life spans and locomotor defects along with increased sensitivity to oxidative stress and overall smaller cell size (Greene *et al.*, 2003; Pesah *et al.*, 2004). Recent reports have described *Drosophila DJ-1* mutants that have oxidative stress-induced locomotor dysfunction and disrupted oxidative stress responses along with increased sensitivity to environmental toxins (Menzies *et al.*, 2005; Meulener *et al.*, 2005; Park *et al.*, 2005; Yang *et al.*, 2005). While all of these models incorporate features of PD, none of them completely captures the all aspects of the disease; nevertheless much insight has been gained from each model.

1.3 Non-genetic Models of Parkinson's disease

Several animal models of PD were developed through the exposure to various chemical compounds in a variety of well-studied organisms. While many chemicals were explored, only three, MPTP, rotenone, and 6-OHDA, produced an approximate reproduction of disease symptoms when model organisms were exposed to these substances. Notably, the examination of each of these models has led to revision of the theories of potential causes of PD and evaluation of therapeutic agents.

An illicit drug preparation that left three human subjects with severe permanent PD like symptoms was found to accidentally contain MPTP (Langston *et al.*, 1999; Weingarten, 1988). MPTP crosses the blood-brain barrier and is metabolized to 1-methyl-4-phenyl-2,3-diydropyridinium (MPP^+) by monoamine oxidase-B in astrocytes. MPP+ is then selectively taken into dopaminergic neurons via dopamine transporters (Figure 1-1) (Javitch *et al.*, 1985). These patients were shown to be responsive to levodopa treatment, a typical treatment for PD, in the earliest stages of their illness. Rhesus monkeys treated with MPTP showed symptoms similar to those of human PD patients that were subsequently relieved by treatment with levodopa (Burns *et al.*, 1983). Pathologically, loss of nerve cells in the pars compacta of the substantia nigra and reduced dopamine content in the striatum of the brain were observed. Mice treated with MPTP showed an increase in reactive oxygen species, dopaminergic neuron loss and *NADPH oxidase* up-regulation, traits that are also associated with PD (Wu *et al.*, 2003). MPTP-treated mice show an increase in the pro-apoptotic protein, Bax, and mutant mice that lack *Bax* are resistant to MPTP induced PD (Vila *et al.*, 2001). The MPTP model has given credence to the idea that oxidative stress plays an important role in PD pathogenesis

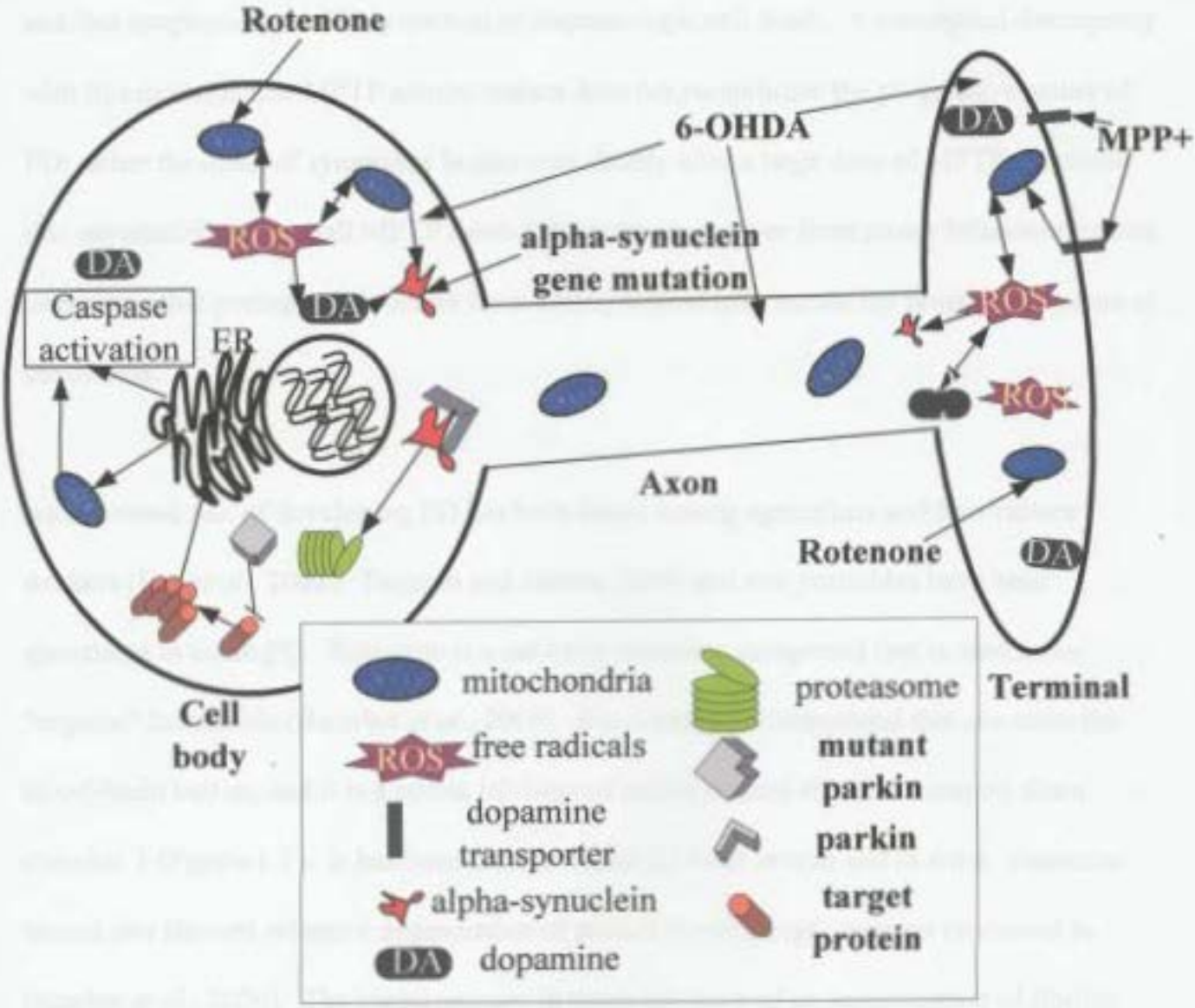

Figure 1-1: Schematic diagram of a dopaminergic neuron showing the cellular targets of toxic compounds in PD models.

The cell represents a substantia nigral dopaminergic neuron with the cell body in the substantia and terminal in the striatum. 6-OHDA can be targeted to the striatum and its action is through oxidative stress mechanism. MPTP is taken up through the dopamine transporters and inhibits complex 1 of the electron transport chain. Rotenone inhibits complex 1 also. Mutations in *α-synuclein* can result in toxic oligomers of the protein. Mutations in *parkin* lead to endoplasmic reticulum stress induced apoptosis. Adapted from Bertarbet *et al.*, (2002).

and that apoptosis is the likely method of dopaminergic cell death. A conceptual discrepancy with this model is that MPTP administration does not recapitulate the progressive nature of PD; rather the onset of symptoms begins very shortly after a large dose of MPTP. Animals that are administered small MPTP doses long-term can recover from motor behaviour defects, suggesting that perhaps, a moderate toxic dosing regime may mimic the progressive nature of the disease.

An increased risk of developing PD has been found among agriculture and horticulture workers (Lee *et al.*, 2002a; Tuchsen and Jensen, 2000) and two pesticides have been speculated to cause PD. Rotenone is a naturally occurring compound that is used as an "organic" insecticide (Betarbet *et al.*, 2000). It is a lipophilic compound that can cross the blood-brain barrier, and it is a potent inhibitor of mitochondrial electron transport chain complex 1 (Figure 1-1). Ir has been used to model PD both *in vivo* and *in vitro*. Rotenone-treated rats showed selective degeneration of striatal dopaminergic neurons (reviewed in Betarbet *et al.*, 2000). The nigral neurons in these rats showed an accumulation of fibrillar cytoplasmic inclusions that contain both ubiquitin and α-synuclein. The resulting behavioural phenotypes included a flexed or stooped posture, rigidity, and spontaneously shaking paws similar to the resting tremor seen in PD. *In vitro* experiments have shown that rotenone can induce a conformational change in α-synuclein and increase the rate of fibril formation (Uversky *et al.*, 2001), a pathological feature of α-synuclein-induced PD. This particular model reproduces all the main features of PD, including the progressive and systematic degeneration of the nigrostriatal pathway. For non-inherited PD, this model may become a

very powerful model of PD and be an ideal testing ground for preliminary drug trials, as most of the pathogenic mechanisms of the disease are captured.

A specific neurotoxin, 6-OHDA, uses the same catecholamine transport system as dopamine, and affects catecholaminergic pathways through an oxidative stress mechanism to induce PD-like symptoms (Figure 1-1) (Ungerstedt, 1968). While 6-OHDA has been used to induce PD-like symptoms in a wide range of model organisms, from *Caenorhabditis elegans* to non-human primates, the majority of research has been conducted with rats. As 6-OHDA is unable to cross the blood-brain barrier, this chemical must be injected into the brain in order to target nigrostriatal dopaminergic neurons. The dopaminergic neurons start to degrade within 24 hours and dopamine is fully depleted from the striatum 2-3 days later (reviewed in Betarbet *et al.*, 2002). The extent of the lesion is dependent upon the amount of 6-OHDA injected and it is possible to have a slower degeneration of neurons by injecting into the striatum as opposed to the nigrostriatal tract (Przedborski *et al.*, 1995). This model has been used to test alternative non-drug therapies such as acupuncture, which was demonstrated to have protective effects against neuronal death (Park *et al.*, 2003). The interesting feature of this model is the routine experimental protocol of injecting 6-OHDA into one hemisphere and leaving the other hemisphere as an internal control. While this model has some limitations, it has been effectively used to test the behavioural effects of widely used pharmacological challenges such as amphetamines, apomorphine, selective receptor agonists, and levodopa, and the effects of treatments that can promote recovery like neuropeptides and neurotrophins (reviewed in Schwarting and Huston, 1996). The 6-OHDA model produces sudden death of neurons instead of the progressive degeneration of neurons seen in PD. In addition, neither Lewy bodies nor other cytoplasmic inclusions have been observed in this model. In other

words, although this model has some valid applications, the progressive nature of PD is not recapitulated.

While the MPTP, rotenone and 6-OHDA models are not the only models of PD they have been the most heavily studied and also appear to have the closest similarity to the pathology of non-inherited PD of the chemical-induced models. The progressive nature of the rotenone model of PD is of particular interest as this is a feature that was not captured in either the MPTP or 6-OHDA models. While the lack of Lewy bodies in the 6-OHDA model questions whether this is indeed a suitable model of PD, it has been successfully used to test important aspects of PD, such as the effects of selective receptor agonists, levodopa and neurotrophins (reviewed in Schwarting and Huston, 1996). These models all have limitations and should be used with great caution in the study of PD. Using the knowledge of the genetic basis of some PD to generate gentic models of the disease appears to be the next logical step in our understanding of PD.

1.4 The genetic basis of Parkinson's disease

Mutations in a number of genes including α-synuclein, parkin, ubiquitin carboxy-terminal hydrolase 1 (UCH-L1), PTEN induced kinase 1(PINK1), DJ-1 and leucine rich repeat kinase-2 (LRRK2) are known to be associated with PD and several more chromosomal loci have been implicated (Table 1-1) (reviewed in Cookson, 2005; Gasser, 2005; Healy et al., 2004; Morris, 2005). There are two underlying common features among some of these genes. Firstly, several of the genes listed encode proteins that are involved in the ubiquitin proteasome

Table 1-1: Genes associated with Parkinson's disease

Locus (inheritance)	Gene name	Map position	Gene Function	Phenotype
Park1/4[1] (dominant)	*SNCA*[2]	4q21	Synaptic? Lipid binding	PD/DLBD[3], onset - 30-60 yrs[4], rapid course
Park 2 (recessive)	*Parkin*	6q25	E3 ubiquitin ligase	Parkinsonism, onset - teens-40 yrs, slow course, no LB[3] except one case
Park 3 (dominant)	Unknown	2p13	___	PD, dementia, onset – 50-60 yrs, LB tangles and plaques
Park 5 (dominant)	*UCHL1*[5]	4p14	Ubiquitin hydrolase/ligase	Typical PD, onset ~50 yrs, pathology unknown
Park 6 (recessive)	*PINK1*[6]	1p35-37	Protein kinase Oxidative stress Regulates *parkin*	Parkinsonism, onset 30-50 yrs, pathology unknown
Park 7 (recessive)	*DJ-1*	1p38	Oxidative stress response	Parkinsonism, onset 20-40 yrs, slow course, pathology unknown
Park 8 (dominant)	*LRRK2*[7]	12cen	___	Parkinsonism, onset 40-60 yrs, variable tau and α-synuclein pathology
Park 10 (dominant?)	Unknown	1p32	___	Unknown pathology
Park 11 (dominant?)	Unknown	2p34	___	Unknown pathology

[1]Park 4 – is a *SNCA* triplication; [2] *SNCA* – gene name for α-synuclein; [3] DLBD – dementia with Lewy body disease, LB – Lewy bodies; [4]yrs- years old, [5]Ubiquitin C-terminal hydrolase L1; [6] *PTEN induced kinase* 1; [7] *LRRK2* – gene name for dadarin. Adapted from Cookson, M.R. (2005) and Gasser, T. (2005).

degradation system (UPS). The UPS is a mechanism that the cell uses to rid itself of damaged, misfolded or otherwise unwanted proteins by tagging and targeting these proteins to the proteasome, where they are degraded to amino acids (reviewed in Hershko and Ciechanover, 1998). Secondly, two of the genes have been implicated in oxidative stress mechanisms. Overall, both oxidative stress mechanisms and the UPS have been shown to play a role in the typical pathology of PD.

1.4.1 The structure and functions of α-synuclein

In a large Italian family, a single base pair change in *SNCA* was found to be associated with Autosomal Dominant PD (ADPD) (Polymeropoulos *et al.*, 1996; Polymeropoulos *et al.*, 1997). This original mutation results in an amino acid change from alanine to threonine at position 53 (A53T) in α-synuclein (Polymeropoulos *et al.*, 1997). Two further mutations, an alanine to proline exchange at amino acid 30 (A30P) (Kruger *et al.*, 1998) and a glutamic acid to lysine (E46K) (Zarranz *et al.*, 2004) have also been identified. Finally, triplication of *SNCA* was found to be associated with ADPD (Singleton *et al.*, 2003). These mutations are all relatively rare and do not seem to be involved in non-inherited cases of PD (Chan *et al.*, 1998). Nevertheless understanding how these mutations are associated with PD is necessary.

The α-synuclein protein is an abundant 140 amino acid cytosolic protein found mostly at the pre-synaptic terminal of neurons in either a vesicle bound or soluble form (Clayton and George, 1998; Jakes *et al.*, 1994). Mutant forms of the protein are generally found in the same locations but they have been also identified in the neuronal cell body and neurites throughout

the brain (Kahle *et al.*, 2000). Originally α-synuclein was identified as a non-β amyloid component of Alzheimer's disease amyloid plaques (Ueda *et al.*, 1993).

Immunocytochemistry has revealed α-synuclein to be a major component of Lewy bodies and indeed staining for α-synuclein is more extensive than for ubiquitin, which has previously been used as a marker for Lewy bodies (Spillantini *et al.*, 1997; Spillantini *et al.*, 1998). There are three basic structural domains in α-synuclein: an acidic carboxy-terminal domain, an amino-terminal domain organised around 7 copies of an 11 amino acid motif and a hydrophobic centre region, identified in amyloid plaques (Borden, 1998). The repeated motif in the amino-terminal domain, an amphipathic α-helical structure, and is also called class A2 lipid-binding domain. α-Synuclein has a mainly unfolded tertiary structure but it undergoes large conformational changes in the presence of phospholipids and binds to synaptic vesicles (Davidson *et al.*, 1998; Weinreb *et al.*, 1996). This abundant protein is likely to be directly involved in protein-lipid interactions and may be involved in reuptake of dopamine.

α-Synuclein acts to reduce the activity of tyrosine hydroxylase, the rate-limiting enzyme in dopamine biosynthesis (Perez *et al.*, 2002). This suggests that loss of α-synuclein, either through loss-of-function mutations or aggregation, would reduce the regulation of dopamine synthesis and the resulting increase of dopamine would increase deleterious reactive dopamine metabolite levels. A more recent study has shown the α-synuclein can ameliorate the neurodegeneration caused by cysteine-string-protein-α (CSP-α) deficiency in mice (Chandra *et al.*, 2005). The data suggests that α-synuclein acts downstream of CSPα in a cell-autonomous manner that also requires phospholipid-binding activity of α-synuclein (Chandra

et al., 2005). These recent studies indicate that α-synuclein has a role in the protection of nerve terminals from injury, possibly in combination with CSPα and SNARE proteins on the presynaptic membrane interface.

Phosphorylation of tyrosine residues in α-synuclein is thought to be important in regulating synaptic function and plasticity (Gurd, 1997; O'Dell *et al.*, 1991). α-Synuclein is phosphorylated on both serine and tyrosine residues (Ellis *et al.*, 2000; Nakamura *et al.*, 2001; Okochi *et al.*, 2000; Pronin *et al.*, 2000). The G protein-coupled receptor kinase 2 (Gprk2) phosphorylates Ser129 in α-synuclein *in vivo* and enhances α-synuclein toxicity (Chen and Feany, 2005). Other reports show that co-expression of *parkin* with *α-synuclein* and *synphilin-1* in cell culture formed inclusions that had ubiquitin tags, which were decreased in number with the expression of S129A *α-synuclein* (Smith *et al.*, 2005). These reports indicate that phosphorylation of α-synuclein at S129 may be important for the formation of inclusions in PD or that it alters targeting to the proteasome.

1.4.2 Protein toxicity and *α-synuclein*

Over-expression studies of wild type and mutant *α-synuclein* in human neuroblastoma (SH-SY5Y) cells and other cultured neurons show that these α-synuclein species can induce apoptosis (El-Agnaf *et al.*, 1998a; Saha *et al.*, 2000). Wild type α-synuclein forms an insoluble fibrillar aggregate with an antiparallel β-sheet structure *in vitro*, which is greatly enhanced by both the A30P and A53T amino acid substitutions (Conway *et al.*, 1998; El-Agnaf *et al.*, 1998b; Narhi *et al.*, 1999). The *in vitro* fibril formation of α-synuclein does not

follow a one-step transition rather there appears to be an increase in β-sheet conformation of the otherwise unfolded α-synuclein and oligomerization of the protein into protofibrils (Figure 1-2). Subsequently, the protofibrils may either take on a spherical protofibril/oligomer bodies (Conway *et al.*, 2000; Goldberg and Lansbury, 2000; Harper *et al.*, 1997a; Harper *et al.*, 1997b, 1999). Interestingly, solutions of mouse and human α-synuclein form fibrils at a slower rate in the presence of the human A30P and A53T forms of α-synuclein, which leads to an increase of non-fibrillar oligomers (Rochet *et al.*, 2000). The unanswered question remains, which, if any, forms of α-synuclein are toxic? It remains unknown whether it is simply the aggregation of the protein that leads to cell death or whether there are other factors involved.

Pathological examinations of human brains have found two observations that do not correlate with the theory that Lewy bodies are the species that cause PD. Firstly, the substantia nigra dopaminergic neurons that contained Lewy bodies appear 'healthier', using both morphological and biochemical analysis, compared to the surrounding neurons (Tompkins *et al.*, 1997; Tompkins and Hill, 1997). Indeed, quantitative analysis revealed aggresomes in 60% of non-apoptotic cells but only in 10% of apoptotic cells that express *α-synuclein*, and α-synuclein-induced apoptosis was not coupled with increased prevalence of aggresome-bearing cells (Tanaka *et al.*, 2004). Secondly, incidental Lewy bodies have been identified in the brains of aged individuals who had no symptoms of PD or any other neurodegenerative disease (Forno and Langston, 1993; Gibb and Lees, 1988; Goldberg and Lansbury, 2000). Experimental perturbations that reduce the number of nuclear inclusions (detected by light

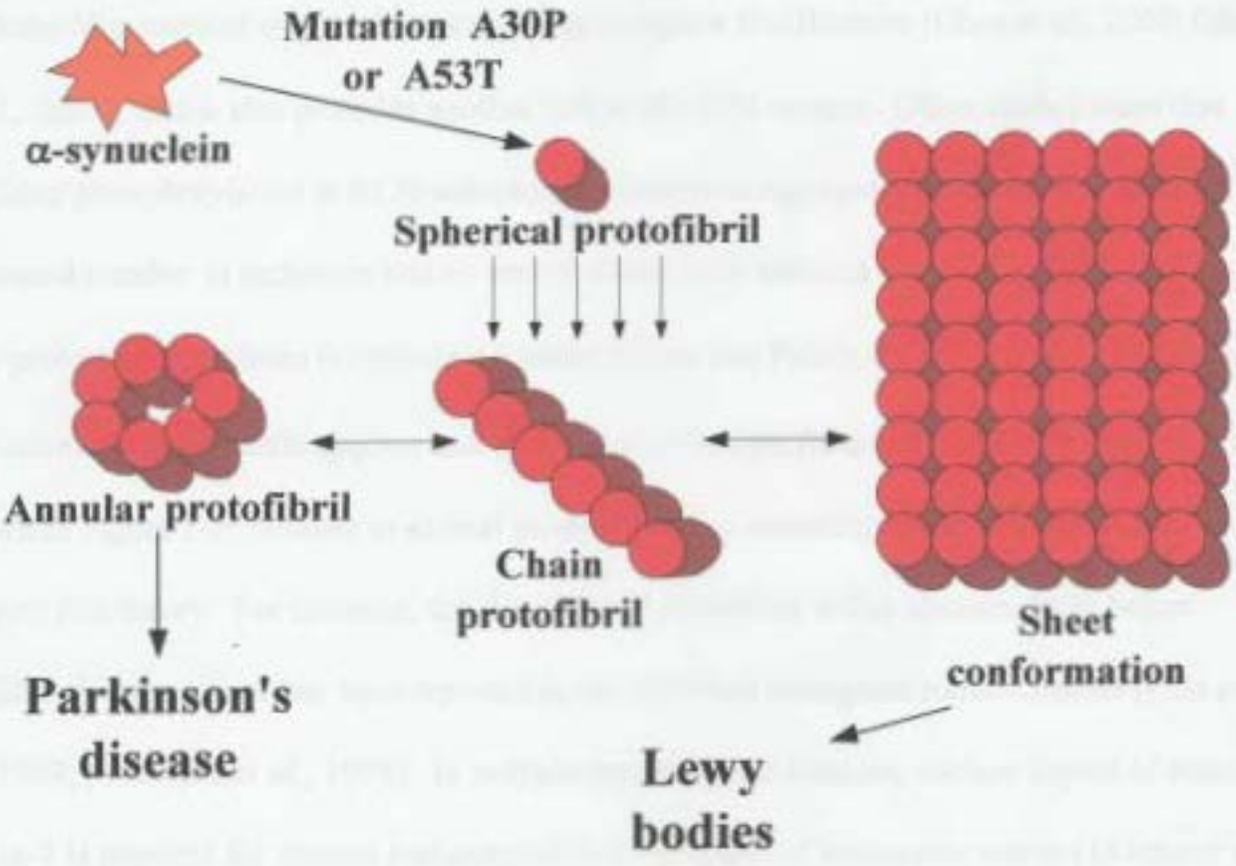

Figure 1-2: Diagrammatic representation of a possible explanation for the formation of Lewy bodies.
Unfolded α-synuclein could change conformation to β-sheet, which can form oligomers/protofibrils or form fibrils and continue on to form Lewy bodies. Adapted from Goldberg and Langbury (2000).

conformation, or full fibrillization takes place followed by further aggregation into Lewy microscopy) actually increase the severity of disease-related abnormalities (Goldberg and Lansbury, 2000). PA700, the regulatory complex of the 26S proteasome, is likely to interact with the oligomers of α-synuclein preventing complete fibrillisation (Ghee *et al.*, 2000; Ghee *et al.*, 2005), which also provides another link to the UPS system. Other studies show that blocking phosphorylation at S129 substantially increases aggregate formation and because increased number of inclusion bodies are correlated with reduced toxicity, inclusion bodies may protect neurons from α-synuclein toxicity (Chen and Feany, 2005). Perhaps the fibrillar inclusions sequester toxic species and/or divert α-synuclein from toxic assembly pathways as shown in Figure 1-2. Studies in animal models of other neurodegenerative diseases also support this theory. For instance, the detection of Alzheimer's-like abnormalities before fibrillar plaques appear has been reported in two different transgenic mouse models (Hsia *et al.*, 1999; Moechars *et al.*, 1999). In polyglutamine-repeat diseases, nuclear import of mutant ataxin-1 is required for disease pathogenesis but formation of inclusions was not (Klement *et al.*, 1998). In Huntington's disease, Huntingtin protein acts in the nucleus to induce apoptosis but this neuronal death does not correlate with the inclusion formation (Saudou *et al.*, 1998). It appears that soluble oligomers are the principal pathogenic species that drive neuronal dysfunction (reviewed in Walsh and Selkoe, 2004) and it is generally accepted that the oligomeric species of α-synuclein are indeed the toxic forms of the protein.

1.4.3 Parkin functions as an E3 ubiquitin ligase

A variety of point mutations and large deletions in the *parkin* gene have been associated with Autosomal Recessive Juvenile Parkinsonism (ARJP), the second inherited form of PD

identified (Kitada *et al.*, 1998). This form of the disease is specifically characterized by an early age of onset (~40 years old) and the relative absence of Lewy bodies (Hayashi *et al.*, 2000; Ishikawa and Tsuji, 1996; Mori *et al.*, 1998). Autopsy findings have rarely shown typical Lewy body pathology in PD patients that possess mutant forms of *parkin* (Farrer *et al.*, 2001). The parkin protein has been localized to the neurons in the substantia nigra but also to unaffected regions of the brain such as the cerebellum and glial cells (Gu *et al.*, 2000). This widespread distribution of the protein does not correlate with the specific loss of dopaminergic neurons in the substantia nigra when certain mutations occur in *parkin*. Lysates of specific brain segments showed *parkin* expression in the substantia nigra, putamen and frontal brain segments of control patients but a complete loss of *parkin* expression in the substantia nigra from ARJP patients. However, it should be noted that the expression of *parkin* in parts of the brain (putamen, frontal cortex) was significantly lower than in dopaminergic neurons and was not decreased in ARJP patients (Shimura *et al.*, 1999). On the sub-cellular level, dopaminergic neurons in the substantia nigra were immuno-reactive for parkin protein in the cytoplasm, granular structure (golgi) and the neuronal processes (Shimura *et al.*, 1999). Why do mutations in parkin lead to the selective loss of dopaminergic neurons and yet does not appear to affect other parts of the brain.

The *parkin* gene encodes a 465 amino acid protein (Kitada *et al.*, 1998) that functions as an ubiquitin ligase, which is an integral part of the protein degradation machinery (Shimura *et al.*, 2000). Ubiquitin ligases act as mediators between a specific target protein to be degraded and an ubiquitin-conjugating enzyme, which tags the target protein with ubiquitin in a process called ubiquitination (Figure 1-3). Ubiquitination is a post-translational modification that uses ATP and three enzymes, ubiquitin activating enzyme (E1), ubiquitin conjugating enzyme (E2)

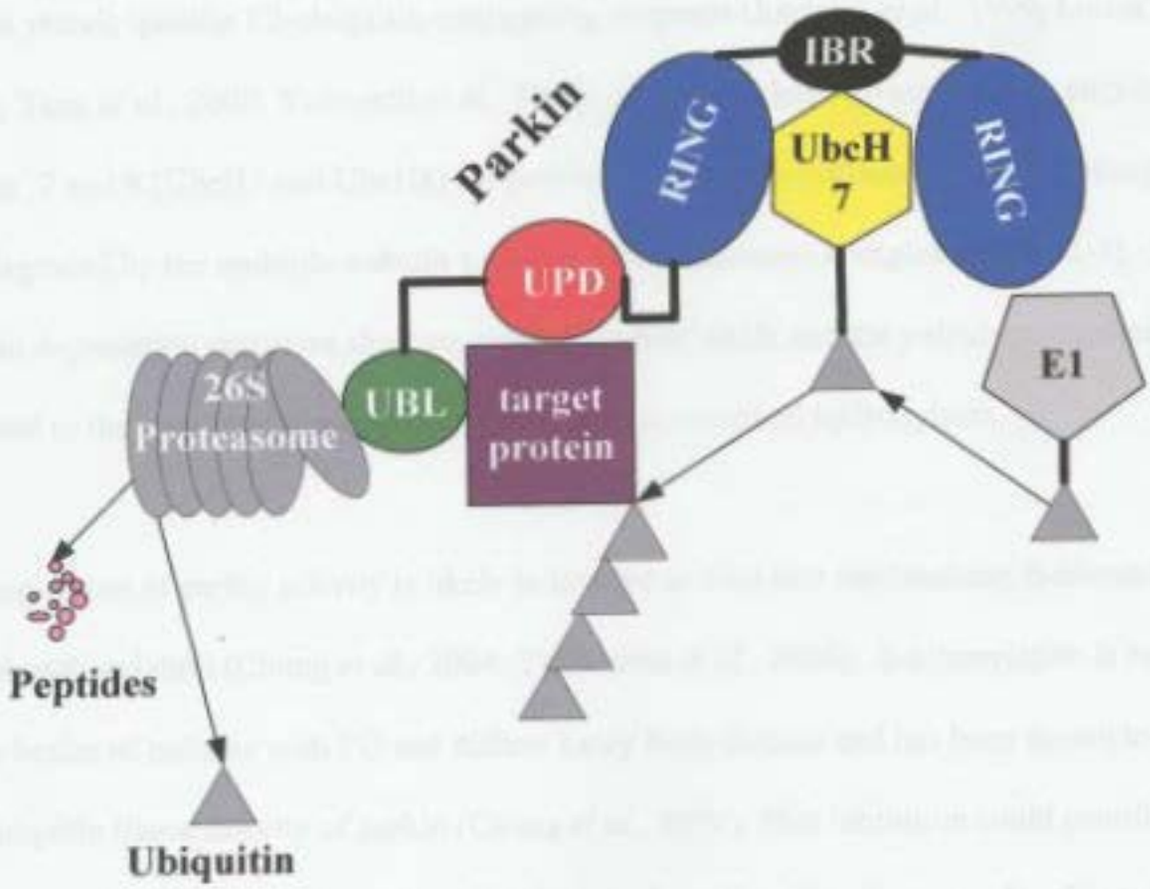

Figure 1-3: Schematic diagram of ubiquitin/proteasome pathway.
UPD-unique parkin domain; *E1*-Ub-activating enzyme; *E2*-Ub-conjugatin enzyme; *UBL*-Ub-like domain. Parkin is a RING-type E3 ubiquitin-protein ligase that binds to E2 ubiquitin-conjugating enzymes, through its RING-IBR-RING motif. The UBL/UPD region of parkin segment binds the target protein to be degraded. Adapted from Tanaka *et al.*, (2001) and Shimura *et al.*, (2000).

and ubiquitin ligase (E3) (Hershko and Ciechanover, 1998). Parkin contains a Really interesting new gene (RING) and inbetween RING (IBR) finger motif (RING-IBR-RING) (Imai *et al.*, 2000), which has been found in other E3 ligases (Lorick *et al.*, 1999). This motif acts to recruit specific E2-ubiquitin-conjugating enzymes (Joazeiro *et al.*, 1999; Lorick *et al.*, 1999; Yang *et al.*, 2000; Yokouchi *et al.*, 1999), including ubiquitin conjugating enzyme human 7 and 8 (UbcH7 and UbcH8) for parkin. The polyubiquitinated protein is recognized and degraded by the multiple-subunit-protease 26S-proteasome complex (Figure 1-3). This protein degradation produces short peptides and amino acids and the polyubiquitin chains are returned to the monomeric state by ubiquitin carboxy-terminal hydroxylases.

The regulation of parkin activity is likely to involve at least two mechanisms, S-nitrosylation and phosphorylation (Chung *et al.*, 2004; Yamamoto *et al.*, 2004). S-nitrosylation is evident in the brains of patients with PD and diffuse Lewy body disease and has been shown to inhibit the ubiquitin ligase activity of parkin (Chung *et al.*, 2004). This inhibition could contribute to the degenerative process in these disorders by impairing the ubiquitination of parkin substrates (Chung *et al.*, 2004). A recent study showed that induction of stress due to the presence of unfolded proteins in cells resulted in reduced parkin phosphorylation; unphosphorylated parkin has increased ubiquitin ligase activity compared with phosphorylated (Yamamoto *et al.*, 2004). Overall levels of phosphorylation and nitrosylation appear to contribute to the level of ubiquitin ligase activity that parkin has in the cell.

1.4.4 Parkin and protein detoxification

The accumulation of proteins due to the failure of ubiquitin-dependant processes of protein degradation has been proposed as a major factor in the destruction of neurons in sporadic and familial PD (Cookson, 2005; McNaught and Jenner, 2001; McNaught *et al.*, 2001). The absence of parkin protein in the substantia nigra of ARJP patients combined with the neuronal cell death in this area of the brain suggests a possible cell-protective role of parkin. As one line of defence there is an increase in parkin protein in response to endoplasmic reticulum (ER) stress (Imai *et al.*, 2000). ER stress, caused by the accumulation of misfolded proteins in the ER, can initiate cell death, however, parkin can prevent this via its E3 activity (Imai *et al.*, 2000; Mori, 2000). Specifically, a substrate of parkin, the Pael receptor (Pael-R) [a G-protein coupled receptor], when over-expressed in cells becomes unfolded, insoluble, and leads to ER stress-induced cell death (reviewed in Takahashi and Imai, 2003 and Takahashi *et al.*, 2003). Parkin ubiquitinates a number of substrates, including, most notably, itself (Imai *et al.*, 2000; Zhang *et al.*, 2000b), a glycosylated form of α-synuclein (Shimura *et al.*, 2001), the Pael-R (Imai *et al.*, 2001), CDCrel-1 (Zhang *et al.*, 2000b) and the α-synuclein-binding protein, synphilin-1 (Chung *et al.*, 2001). Moreover, CHIP (carboxyl terminus of the HSP/HSC70 interacting protein), which acts as an E4 multiubiquitin assembly protein, forms a complex with parkin, heat shock protein 70 (HSP70) and Pael-R, and positively regulates the ubiquitin ligase activity of parkin (Imai *et al.*, 2002). CHIP and HSP70 suppress cell death due to the presence of unfolded Pael-R through coordination of ubiquitination and the molecular chaperone system. Over-expression of *parkin* in the presence of proteasome inhibitors leads to the accumulation of large protein inclusions that stain for α-synuclein, synphilin-1, parkin,

molecular chaperones and proteasome subunits (Junn *et al.*, 2002). The role of parkin in assigning proteins for breakdown seems to be at the heart of ARJP cellular defects.

1.4.5 Mutations in *PTEN induced kinase 1* are associated with Parkinson's disease

Mutations in *PINK1* lead to early-onset Parkinson's disease (Hatano *et al.*, 2004; Rohe *et al.*, 2004; Valente *et al.*, 2004a; Valente *et al.*, 2004b). Initially two mutations, in the sequence that codes for the kinase domain, a nonsense mutation, and missence mutation were identified. A number of novel mutations have since been discovered in unrelated families (Hatano *et al.*, 2004; Rohe *et al.*, 2004). PINK1 protein is localized to the mitochondria and may phosphorylate mitochondrial proteins in response to cellular stress, protecting against apoptotic mitochondrial dysfunction. However the altered forms of PINK1 do not have this protective effect (Valente *et al.*, 2004a). Consistent with their role in PD for example, a significantly higher number of sporadic early-onset PD patients were identified as carriers of single heterozygous mutations in *PINK1* (Valente *et al.*, 2004b). Mutations in *PINK1* are relatively common and in a variety of ethnic populations and appear to have an important role in inherited PD.

1.4.6 Mutations in *DJ-1* are associated with Parkinson's disease

Mutations in the highly conserved gene, *DJ-1*, are associated with autosomal recessive early onset Parkinson's disease (Bonifati *et al.*, 2003). *DJ-1* is ubiquitously expressed throughout the mouse central nervous system (Shang *et al.*, 2004) and functions as a homodimer; one of the mutations found in the gene prevents dimerization of the protein (Gorner *et al.*, 2004;

Moore *et al.*, 2003; Olzmann *et al.*, 2004). *DJ-1* appears to be involved in anti-oxidative stress as mutations in this gene produce proteins that show reduced or no ability to prevent hydrogen peroxide (H_2O_2)-induced cell death (Taira *et al.*, 2004; Takahashi-Niki *et al.*, 2004). There are conflicted views as to whether DJ-1 is degraded via the proteasomal pathway (Gorner *et al.*, 2004; Miller *et al.*, 2003). Nevertheless understanding the function and breakdown of the DJ-1 protein is paramount in determining the prevention of PD associated with *DJ-1* mutations.

1.4.7 Mutations in *Ubiquitin C-terminal hydrolase-L1* are associated with Parkinson's disease

Uch-L1 is a thiol protease that is involved in the cleavage of ubiquitin from ubiquitin-tagged proteins and facilitates the recycling of ubiquitin tags (Chung and Baek, 1999). This enzyme shows dimerization-dependent, ubiquitin ligase activity (Liu *et al.*, 2002). *Uch-L1* is expressed in nerve cells and testes/ovaries in mice where it co-localizes with monoubiquitin and can elongate the half-life of ubiquitin (Osaka *et al.*, 2003). Mutations in *Uch-L1* are associated with ADPD (Leroy *et al.*, 1998). Aggresomes of Uch-L1 and other proteins are formed in response to proteasome impairment (Ardley *et al.*, 2004). Interestingly, a polymorphism in *Uch-L1* appears to confer protection against sporadic PD in Caucasian and Asian populations (Lincoln *et al.*, 1999; Satoh and Kuroda, 2001; Zhang *et al.*, 2000a). Once again mutations in a gene that is associated with the proteasome degradation pathway are also associated with PD.

1.4.8 Mutations in *leucine rich repeat kinase-2* are associated with Parkinsonism

A genome-wide linkage analysis of a Japanese family with ADPD pinpointed a locus mapped to 12p11.2-q13.1 and called *Park 8* (Funayama *et al.*, 2002). Two groups subsequently determined that the gene, which causes this form of PD, that encodes a large, multifunctional protein called leucine-rich repeat kinase 2 (LRRK2) also known as dadarin (Paisan-Ruiz *et al.*, 2004; Zimprich *et al.*, 2004). In patients with ADPD, mutations in *LRRK2* are the most frequently found mutation (reviewed in Gasser, 2005). Clinically, patients with these genetic alterations have symptoms that look like typical PD, however the pathology of this form of PD is variable. Along with the normal pathological changes seen in Lewy body PD, diffuse Lewy body disease, nigral degeneration without distinctive histopathology and progressive supranuclear palsy-like tau were also observed and it is thought that mutations in *LRRK2* may be involved in other neurodegenerative disorders such as Alzheimer's (Zimprich *et al.*, 2004). The protein product of *LRRK2* has been assigned to a group of recently identified ROCO [Roc (Ras of complex proteins)/COR (c-terminal of Roc)] family of proteins (Bosgraaf and Van Haastert, 2003) and it contains a protein kinase domain.

1.5 Genetic models of Parkinson's disease

With the discovery that some forms of PD have a genetic basis, a plethora of genetic models of the disease have been developed. To date these models have been based largely upon mutation of the *α-synuclein*, *parkin* or *DJ-1* genes. The generation of animals that have a non-functional gene or "knock outs" has resulted in further understanding of PD progression and provides excellent models in which potential therapeutic strategies can be tested. These

models have been developed in a variety of organisms ranging from *Drosophila melanogaster* to non-human primates. Of particular note is a series of models in *Drosophila* developed by ectopic expression of normal or mutant forms of *α-synuclein.*

1.5.1 Mutant or altered *DJ-1* models of PD

Most studies on the effects of *DJ-1* mutations suggest that it functions in oxidative stress responses. The recent development of mice with *DJ-1* null mutations corroborates this theory. In one study *DJ-1* null mice show increased sensitivity to oxidative insults, such as H_2O_2, which is abrogated by the over-expression of *DJ-1* (Kim *et al.*, 2005). In another study *DJ-1* null mice did not show significant loss of dopaminergic neurons but did show a change in the function of these neurons (Chen *et al.*, 2005; Goldberg *et al.*, 2005). These mice also showed a loss of motor skill in an age-dependent manner (Chen *et al.*, 2005; Goldberg *et al.*, 2005). In *C. elegans*, when *DJ-1* is "knocked down", the nematodes are more sensitive to mitochondrial complex I inhibitors, such as rotenone, although no increase in sensitivity was noted with paraquat (a nonselective herbicide), or etoposide, a topoisomerase inhibitor (Ved *et al.*, 2005). A series of groups have examined the effect of mutations in *DJ-1* in *Drosophila*. All of these groups showed that homozygous mutant flies had increased sensitivity to oxidative stress (Menzies *et al.*, 2005; Meulener *et al.*, 2005; Park *et al.*, 2005; Yang *et al.*, 2005). Furthermore, these flies had locomotor deficiencies and many of the other hallmarks of mutant *DJ-1*-induced PD (Menzies *et al.*, 2005; Meulener *et al.*, 2005; Park *et al.*, 2005; Yang *et al.*, 2005). With these relatively new PD models, many insights into the biology of *DJ-1*-induced PD, such as the role of oxidative stress, are coming to light.

1.5.2 Mutant or altered *α-synuclein* models of PD

Mice with loss-of-function mutations in *α-synuclein* appear to develop normally. These mice do not show any dopaminergic neuron or synaptic loss, nor are any Lewy bodies observed. However, these mice do have slightly altered stimulus dependent dopamine release (Abeliovich *et al.*, 2000). The data suggests that α-synuclein is a negative regulator of dopamine release, in agreement with the observations that α-synuclein is likely to regulate dopamine biosynthesis (Perez *et al.*, 2002).

In another study of mice, neuronally expressed human *α-synuclein* formed both cytoplasmic inclusions and intranuclear deposits in the neocortex, substantia nigra and hippocampus, although human PD patients have been noted to only have cytoplasmic inclusions (Masliah *et al.*, 2000). A loss of dopaminergic terminals and motor abnormalities were also observed in the human α-synuclein mice, indicating a possible causal effect of the accumulation of α-synuclein inclusions. In particular, this difference has highlighted that lines that express lower levels of human *α-synuclein* do not show the same dopaminergic neuronal and behavioural deficits (Masliah *et al.*, 2000). Interestingly, expression of either wild type or mutant forms of human *α-synuclein* in mice resulted in no neuronal inclusions although altered dopaminergic neuron terminals were observed (Richfield *et al.*, 2002). In addition, mice that express mutant *α-synuclein* showed a reduced locomotor response with exposure to repeated doses of amphetamines, a response that may be due to a reduction of dopamine transporters and a resulting progressive motor impairment (Richfield *et al.*, 2002). These resistant animals were characterized by increased levels of HSP70, a chaperone protein that has been shown to counteract paraquat toxicity in other PD experimental models and could

therefore contribute to neuroprotection in *α-synuclein* transgenic mice. These results indicate a differentiation between toxic-induced α-synuclein aggregation and neurodegeneration. These experiments support a role for α-synuclein in toxic insults and suggest that its involvement in human neurodegenerative processes may arise not only from a gain of toxic function, as previously proposed, but also from a loss of defensive properties (Manning-Bog *et al.*, 2003). An α-synuclein rat model of PD (Lo Bianco *et al.*, 2002) was used to analyse the ability of parkin to suppress symptoms of the disease (Lo Bianco *et al.*, 2004). In these experiments, lentiviral vector delivered parkin prevented dopaminergic degeneration.

A recently developed primate model of PD forced the expression of human *α-synuclein* in the substantia nigra of adult marmosets (Kirik *et al.*, 2003). In this model, a severe neuronal pathology was observed, that included α-synuclein-positive cytoplasmic inclusions and granular deposits as well as swollen, dystrophic, and fragmented neurites. At a time 16 weeks post-transduction of the human gene, 30-60% of dopaminergic neurons were lost and severe motor impairments had developed, which indicates the progressive nature of this model, a feature missing from most other PD models (Kirik *et al.*, 2003). As non-human primates are so similar to humans, this model offers exciting new opportunities for the exploration of new therapeutic targets.

Models of PD have been generated in *Drosophila melanogaster* by generating transgenic flies that conditionally express wild type, A53T or A30P forms of human *α-synuclein* (Feany and Bender, 2000). In these models, the expression of the *α-synuclein* transgenes is dependent on transcriptional activation by the yeast protein, Gal4 (Figure 1-4) (Brand and Perrimon, 1993).

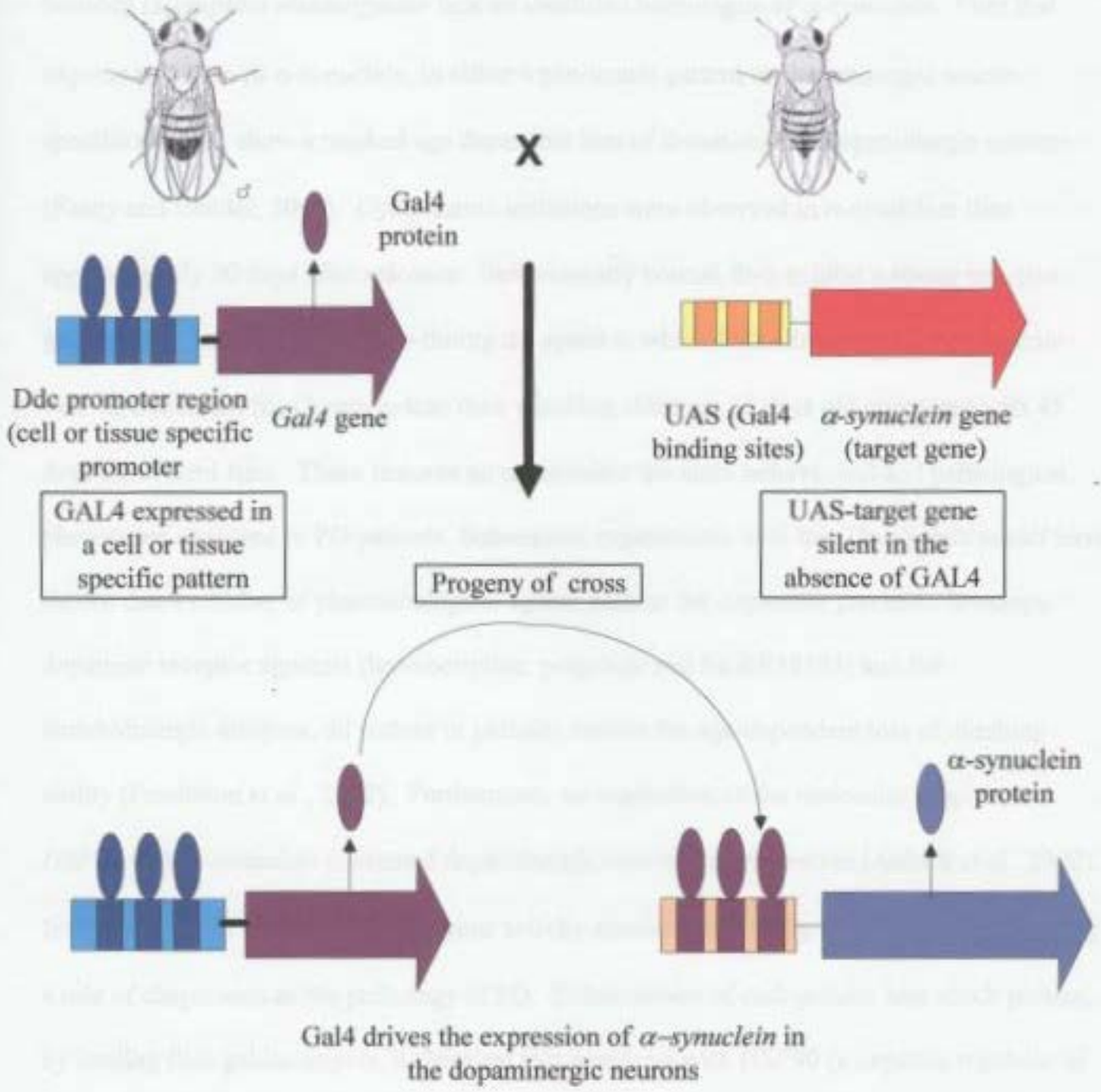

Figure 1-4: Schematic diagram of the UAS/Gal4 expression system in *Drosophila*.
This bipartite system uses two independent transgenic fly lines; one that contains the gene of interest cloned downstream from the UAS promoter which is silent in the absence of Gal4, the other that has a tissue specific promoter region fused to the *Gal4* gene such that Gal4 protein is produced in a tissue specific manner, in this case dopaminergic neruons. The resulting offspring from a cross of these two flies produce Gal4 protein in specific tissues; Gal4 in turn binds to the UAS sequence and activates transcription of the downstream gene. Adapted from Phelps and Brand (1998).

Notably *Drosophila melanogaster* lack an identified homologue of *α-synuclein.* Flies that express any form of *α-synuclein*, in either a pan-neural pattern or dopaminergic neuron specific manner, show a marked age dependent loss of dorsal-medial dopaminergic neurons (Feany and Bender, 2000). Cytoplasmic inclusions were observed in α-synuclein flies approximately 20 days after eclosure. Behaviourally normal flies exhibit a strong negative geotaxis that can be measured by timing the speed at which flies climb the sides of a sterile vial. α-Synuclein flies begin to lose their climbing ability at 25 days old compared with 45 days for control flies. These features all recapitulate the main behavioural and pathological phenotypes observed in PD patients. Subsequent experiments with this *Drosophila* model have shown that a number of pharmacological agents such as the dopamine precursor levodopa, dopamine receptor agonists (bromocriptine, pergolide and SK&F38393) and the anticholinergic atropine, all restore or partially restore the age-dependent loss of climbing ability (Pendleton *et al.*, 2002). Furthermore, co-expression of the molecular chaperone *HSP70* with *α-synuclein* prevented dopaminergic neuronal degeneration (Auluck *et al.*, 2002). Interference with endogenous chaperone activity accelerated toxicity of α-synuclein indicating a role of chaperones in the pathology of PD. Enhancement of endogenous heat shock protein, by feeding flies geldanamycin, a chemical that interferes with HSP90 (a negative regulator of some heat shock factors), prevented dopaminergic cell death but not the appearance of Lewy bodies (Auluck and Bonini, 2002). These results indicate that the *Drosophila* model is particularly good at capturing both biochemical and behavioural features of PD and so could be used as a first point of reference when testing out potential new drug therapies.

1.5.3 Mutant or altered *parkin* models of PD

Mice with loss-of-function *parkin* mutations have nigrostriatal deficits but there is no apparent degeneration in the substantia nigra, a result that is inconsistent with observations in human patients (Goldberg *et al.*, 2003). An increase in extracellular dopamine and no change in the levels of parkin substrates such as CDCrel-1, synphilin-1, and α-synuclein were also observed in the *parkin* deficient mice (Goldberg *et al.*, 2003), as was a decrease in the abundance and consequently activity of proteins involved in mitochondrial function or oxidative stress (Palacino *et al.*, 2004). These mice also show a delayed weight gain. Taken together, these findings show that parkin is intimately involved in dopamine regulation and mitochondrial function (Goldberg *et al.*, 2003; Palacino *et al.*, 2004). The mouse model of parkin-induced PD has many but not all of the features of the disease found in humans but not all. Nevertheless it is likely to be useful in understanding this form of PD.

The *Drosophila* homologue of *parkin* has been identified and the putative protein product is 59% similar to the human protein (Greene *et al.*, 2003). It carries both the target protein-binding domain and the ubiquitin-conjugating enzyme-binding domain and also probably functions as an ubiquitin ligase. Transcripts of *parkin* were found at all developmental stages of the fly life cycle. A recently developed fly PD model has a mutant allele of the *Drosophila* homologue of *parkin*, which was generated using P-element mutagenesis (Greene *et al.*, 2003). This model recapitulates some of the normal PD symptoms such as reduced lifespan and locomotor defects, however it has other defects such as male sterility, not normally seen in humans (Greene *et al.*, 2003). Interestingly, the locomotor defects resulted from severe disruption of muscle integrity and were not limited to the flight

muscles but affected climbing as well. Upon closer examination of the cellular structure, swollen mitochondria manifesting severe disruption and disintegration of the cristae were observed. Furthermore, the cells were undergoing apoptosis, a typical response of cells when mitochondrial dysfunction and release of cytochrome c is observed (Martin, 2001; Tatton and Olanow, 1999). As the sterility aspect of this model is not a feature of PD that is seen in humans and it is possible that *parkin* is involved in other cellular functions in *Drosophila* that are performed by a different ubiquitin ligase in humans. Examination of the *parkin* mutants reveals that they have a reduced cell size that results in a lower total mass. Furthermore they show a greater sensitivity to oxidative stress (Pesah *et al.*, 2004). More recent evidence suggests that the oxidative stress response is induced in *parkin* mutants rather than the ER stress pathway (Greene *et al.*, 2005). Mitochondrial dysfunction was a feature observed in some of the chemically induced models of PD and is one of the key features in the *Drosophila* model of ARJP. This feature of the disease has now been genetically induced and finding factors that initiate this dysfunction will be important.

A common pathway in inherited forms of PD has been revealed with the discovery that the α-synuclein protein physically interacts with parkin (Petrucelli *et al.*, 2002; Shimura *et al.*, 2001). Indeed *parkin* over-expression protects cells from toxicity associated with expression of mutant *α-synuclein* (Petrucelli *et al.*, 2002). The mutations in *α-synuclein* may be factors that prevent the protein from being targeted for degradation. Mutant parkin protein is unable to bind, and hence, mediate ubiquitination of αSp22 (a form of α-synuclein), which leads to accumulation of α-synuclein protein and, possibly, to the cells demise (Shimura *et al.*, 2001).

1.6 Thesis Objectives

Analysis of *Drosophila* models of PD may yet provide the most insight into the commonalities of inherited forms of PD. This organism is simple to genetically manipulate and yet complex enough to provide biochemical through to behavioural data. Importantly, the majority of the genes associated with PD have homologues in *Drosophila*. The overall goal in this thesis was to analyse *parkin* in *Drosophila melanogaster* and in particular in *Drosophila* models of PD. My interest in *parkin* came about, as, at the inception of this project, mutations in this gene appeared to be quite predominant in inherited forms of PD. When this project was initiated a genetic model of PD in *Drosophila* through ectopic expression of *α-synuclein* (Feany and Bender, 2000) had just been developed. I wanted to use this already established model of α-synuclein associated PD, to increase our understanding of the nature of the disease, and the mutations that are associated with the disease and to identify ways to prevent the progression of the disease. This particular model proved to be an invaluable tool in which to invoke the ectopic expression of *parkin* to study aspects of PD including prevention or suppression of the PD symptoms found in these flies.

The initial goals of the project were to clone and characterize the *Drosophila parkin* homologue and to generate transgenic *Drosophila* with *parkin* under the control of the UAS enhancer element. I analysed the effects of *parkin* expression in a variety of *Drosophila* tissues in terms of behavioural and physical phenotypes and life span. I hypothesized that the PD-like phenotypes found in the α-synuclein-induced *Drosophila* PD model could be suppressed through expression of *parkin*. Analysis of the effects of the *in vivo* interaction

between *parkin* and either wild type or PD associated mutant *α-synuclein* in terms of behaviour, life span and morphological changes was therefore undertaken.

Previous work has shown that excessive *Gal4* expression can cause developmental defects in the Drosophila eye (Kramer and Staveley, 2003). If increased expression of *Gal4* alone could cause development defects then perhaps excessive *Gal4* expression in the dopaminergic neurons could act as a toxic protein and be used as a model of toxic protein induced-PD. Further characterization of the *Ddc-Gal4* transgenic line that expresses *Gal4* in a dopaminergic neuronal pattern was initiated to analyse the effects of varying numbers of Gal4 transgenes. This analysis studied the behavioural effect of various levels of *Gal4* expression throughout the life span of the flies and also the expression patterns of the transgene.

If parkin can suppress the effects of *α-synuclein* expression then parkin may be able to suppress the phenotypes caused by excessive Gal4. Analysis of the effects of *parkin* expression in the developing eye with multiple copies of both transgenes was undertaken to see if parkin could suppress phenotypes caused by excessive expression of *Gal4*. If true this would suggest that parkin may prevent other defects caused by toxic proteins

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Chapter 2: Parkin counteracts symptoms in a *Drosophila* model of Parkinson's disease

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2.1 Introduction

Parkinson's disease (PD) is a neurodegenerative disorder that is characterized by muscle tremors in stationary limbs, bradykinesia (slowed movement) and difficulty initiating and sustaining movements, and affects 1–2% of the population older than sixty years of age (Dawson, 2000; Giasson and Lee, 2001; Lansbury and Brice, 2002; Parkinson, 1817; Spacey and Wood, 1999). As the disease progresses, both the sense of balance and the memory of the affected individual deteriorate. Post-mortem analysis reveals the selective loss of dopaminergic neurons from the substantia nigra region of the brain. Filamentous protein inclusions, known as Lewy bodies, are found within the neuronal cell bodies of the affected area in most but not all PD patients (Giasson and Lee, 2001). Although the majority of PD cases appear to be sporadic, about 5–15% have been determined to have an inherited basis (de Silva *et al.*, 2000; Mizuno *et al.*, 2001). Recently, mutations in a number of genes have been identified as causes of PD, and many of these genes are associated with the ubiquitin/proteasome protein degradation pathway.

Mutations in the gene encoding the α-synuclein protein lead to the development of Autosomal Dominant PD (ADPD) (Polymeropoulos *et al.*, 1996; Polymeropoulos *et al.*, 1997). The α-synuclein protein is an abundant 140 amino acid, cytosolic protein found at the pre-synaptic region of neurons (Clayton and George, 1998; Jakes *et al.*, 1994). α-synuclein appears to be involved in the biosynthesis of dopamine (Baptista *et al.*, 2003; Perez *et al.*, 2002). Mutations in the *α-synuclein* gene (Polymeropoulos *et al.*, 1996; Polymeropoulos *et al.*, 1997) may lead to enhanced oligomerization and fibril formation of the α-synuclein protein (Conway *et al.*, 1998; Conway *et al.*, 2000a; Conway *et al.*, 2000b).

Autosomal Recessive Juvenile Parkinson's disease (ARJP), another inherited form of PD, has been attributed to a number of point mutations and deletions of the *parkin* gene (Kitada *et al.*, 1998; Munoz *et al.*, 2000; Nisipeanu *et al.*, 1999). ARJP is specifically characterized by a very early age of onset, mostly before forty years of age, and the absence of Lewy bodies (Hayashi *et al.*, 2000; Ishikawa and Tsuji, 1996; Mori *et al.*, 1998). In humans, the *parkin* gene encodes a 465 amino acid protein (Kitada *et al.*, 1998) that functions as one of a number of E3 ubiquitin protein ligases, components of the ubiquitin/proteasome degradation pathway (Shimura *et al.*, 2000). Ubiquitin protein ligases act to identify damaged, misfolded, and short-lived proteins to mediate the ubiquitination (the sequential attachment of a number of ubiquitin monomers) of these proteins, which are targeted to the proteasome (Hershko and Ciechanover, 1998; Pickart, 2001). Experiments in tissue culture have demonstrated that parkin can ubiquitinate a number of substrates including a glycosylated form of α-synuclein (Shimura *et al.*, 2001), the Pael receptor (Imai *et al.*, 2001), CDCrel-1 (Zhang *et al.*, 2000), the α-synuclein-binding protein synphilin-1 (Chung *et al.*, 2001), and parkin itself (Imai *et al.*, 2000; Zhang *et al.*, 2000). The loss of *parkin* may lead to an accumulation of one or a number of proteins in sufficient quantities to cause neuronal cell death.

The interaction of parkin with α-synuclein suggests a common mechanism underlying inherited forms of PD. Indeed, elevated expression of *parkin* protects neuronal explants from the toxicity associated with expression of *α-synuclein* (Petrucelli *et al.*, 2002; Shimura *et al.*, 2001). The disease inducing-forms of α-synuclein may prevent its own degradation and result in toxic accumulation. In ARJP, functional parkin protein is lost along with the ability to mediate the ubiquitination of glycosylated α-synuclein and may lead to the accumulation of

this protein (Shimura *et al.*, 2001). Our working hypothesis is that aspects of parkin-mediated protein degradation are compromised in PD.

The first *Drosophila melanogaster* model of PD was generated by the conditional expression of human α-*synuclein* in transgenic *Drosophila* (Feany and Bender, 2000). Flies that express *α-synuclein*, in either a pan-neural or dopaminergic neuron specific manner, show a marked age-dependent loss of dorsal-medial dopaminergic neurons. Cytoplasmic inclusions were observed in *α-synuclein*-expressing flies approximately 20 days after eclosion. While control flies exhibit a strong negative geotaxis, these transgenic flies prematurely lose their climbing ability. In addition, expression of *α-synuclein* in the developing eye results in precocious degeneration of the retina. In this model expression of a number of genes are dysregulated prior to the onset of neurodegeneration (Scherzer *et al.*, 2003). These features recapitulate the main behavioural and pathological phenotypes of PD and provide an excellent model system to study the biological basis of the disease.

The *Drosophila α-synuclein* based model has been used to investigate a number of aspects of PD. Pharmacological agents, such as the dopamine precursor levodopa, dopamine receptor agonists (bromocriptine, pergolide and SK&F38393), and the anticholinergic atropine, were demonstrated to modify the age-dependent loss of climbing ability (Pendleton *et al.*, 2002). Co-expression of the molecular chaperone *HSP70* gene with *α-synuclein* prevented dopaminergic neuronal degeneration (Auluck *et al.*, 2002). Interference with endogenous chaperone activity accelerated the toxicity of α-synuclein demonstrating a role for chaperones in the pathology of the disease. Suppression of HSP90, a negative regulator of heat shock

factor 1, by feeding flies geldanamycin prevents dopaminergic neuronal cell death (Auluck and Bonini, 2002). Recently, the expression of human *parkin* has been shown to suppress the loss of dopaminergic neurons induced by *α-synuclein* in *Drosophila* (Yang *et al.*, 2003). This model has proven to be an effective tool in the investigation of the biological basis of PD.

To investigate the role of *parkin* in the *α-synuclein*-based model of Parkinson's disease in *Drosophila*, we have characterized and expressed the *Drosophila* homologue of *parkin* in this model. Our results demonstrate that *parkin* can counteract the effects of *α-synuclein* on climbing activity and retinal degeneration.

2.2 Materials and Methods

2.2.1 Bioinformatic and sequence analysis

The *Drosophila melanogaster* homologue of *parkin* was identified through a search of the Berkeley *Drosophila* Genome Project queried with the human parkin amino acid sequence, AB009973.1. A clone of the *Drosophila parkin* cDNA (SD01679) was obtained from Research Genetics (Stapleton *et al.*, 2002), sub-cloned and sequenced (Cortec DNA Service Laboratories Inc., Kingston, ON, Canada). The intron/exon map was constructed by comparison of the cDNA to the corresponding genomic region. Other homologues of *parkin* were identified with the tblastn algorithm (Altschul *et al.*, 1997) of the National Center for Biotechnology Information (NCBI) using the theoretical translation of SD01679 cDNA. The blast2 sequence comparison program (NCBI) was used to compare the *Rattus norvegicus* (NM_020093.1), *Mus musculus* (AB019558.1) and *Anopheles gambiae* (XM316606.1) parkin

protein sequences individually with the *D. melanogaster* parkin protein sequence (Altschul *et al.*, 1997). The multi-alignment of the five parkin homologues from *D. melanogaster*, *A. gambiae*, *R. norvegicus*, *M. musculus* and *Homo sapiens* was constructed by editing the results from the multialign ClustalW program from the Pôle Bio-Informatique Lyonnaise (Thompson *et al.*, 1994).

2.2.2 Fly stocks and culture

Dr. M. Feany (Harvard Medical School) and Dr. J. Hirsh (University of Virginia) generously provided *UAS-α-synuclein* (Feany and Bender, 2000) and *Ddc-Gal4*$^{4.36}$ flies (Li *et al.*, 2000) respectively. The *glass mediated receptor (GMR)-Gal4* flies (Freeman, 1996) were obtained from the Bloomington *Drosophila* Stock Center at University of Indiana, Bloomington. A *Bgl*II/*Xho*I fragment containing the *parkin* cDNA (SD01679) was subcloned into the *pUAST* vector to generate the *UAS-parkin* transgene. Two independent transgenic lines were generated using heat shock π as a source of transposase and standard injection techniques into w^{1118} embryos. Double transgenic lines with *UAS-α-synuclein;UAS-parkin*$^{1.1}$ and *UAS-α-synuclein;UAS-parkin*$^{2.1}$ were generated using standard techniques. To drive expression of the transgenes, *Ddc-Gal4* (for expression in the dopaminergic neurons) or *GMR-Gal4* (for expression in the eye) homozygous females were crossed to w^{1118} males (control) or *UAS-α-synuclein* males with or without *UAS-parkin*$^{1.1}$ or *UAS-parkin*$^{2.1}$. All flies were cultured on standard cornmeal/yeast/molasses/agar media at 25°C.

2.2.3 *In situ* hybridization analysis

Third instar larvae were dissected in phosphate buffered saline (PBS), fixed in 4% formaldehyde and dehydrated in methanol and ethanol. The carcases were probed with a DIG labelled anti-sense *parkin* RNA probe generated from a linear cut plasmid containing the entire *parkin* cDNA using the Roche Applied Science DIG Northern starter kit and reduced in size with carbonate buffer treatment. To visualize *parkin* RNA alkaline phosphatase labelled anti-DIG anti-bodies were incubated with the carcases and subjected to alkaline phosphate treatment as per the Roche Applied Science DIG application manual. The eye discs were dissected out completely and examined under light microscopy. The genotypes of the larvae examined were 1) w^{1118}; 2) w^{1118};*GMR-Gal4/+; UAS-parkin*$^{1.1}$/+; and 3) w^{1118};*GMR-Gal4/+;UAS-parkin*$^{2.1}$/+ and at least ten of each genotype were examined.

2.2.4 Aging analysis

Adult males were collected under gaseous carbon dioxide anaesthetic and aged in small groups (~10 or less per vial) upon standard cornmeal/yeast/molasses/agar media at 25°C in upright standard plastic shell vials. The flies were scored for viability every two to three days and transferred to fresh media without anaesthesia (Staveley *et al.*, 1990). The numbers of individuals aged are as follows: *UAS-α-synuclein/+; Ddc-Gal4/+* = 191; *UAS-α-synuclein/+; UAS-parkin*$^{2.1}$*/Ddc-Gal4* = 292; *UAS-α-synuclein/+; UAS-parkin*$^{1.1}$*/Ddc-Gal4* = 204; w^{1118}; *Ddc-Gal4/+* = 173; *UAS-parkin*$^{1.1}$*/Ddc-Gal4* = 262; and *UAS-parkin*$^{2.1}$*/Ddc-Gal4* = 227.

2.2.5 Locomotion assay

Flies were assayed for their ability to climb as described by Feany and Bender (Feany and Bender, 2000). Every five days, forty male flies from a cohort of flies were assayed for their ability to climb six centimetres in eighteen seconds in a sterile plastic vial. Twenty trials were carried out for each time point. Data shown represent the results from flies tested over ninety days.

2.2.6 Scanning electron microscopy of the *Drosophila* eye

Flies were of each genotype 1) w^{1118}; *UAS-α-synuclein/GMR-Gal4*; 2) w^{1118}; *UAS-α-synuclein/GMR-Gal4*; *UAS-parkin*$^{1.1}$/+; and 3) w^{1118}; *UAS-α-synuclein/GMR-Gal4*; *UAS-parkin*$^{2.1}$/+ aged and frozen in a -70°C ethanol bath. Whole flies were mounted, desiccated overnight and coated in gold before photography at 150 times magnification with a Hitachi S-570 SEM as per standard methods. For each condition at least six flies were analysed.

2.2.7 Histological examination of *Drosophila* adult retinas

Adult flies 1) w^{1118};*UAS-α-synuclein/GMR-Gal4*; 2) w^{1118};*UAS-α-synuclein/GMR-Gal4*;*UAS-parkin*$^{1.1}$/+; and 3) w^{1118};*UAS-α-synuclein/GMR-Gal4*;*UAS-parkin*$^{2.1}$/+ were aged (one or thirty days after eclosion), fixed in Karnovsky's fixative and embedded in epon. Tangential retinal sections were prepared at a thickness of 0.5 μm and stained with toluidine blue, examined by light microscopy and photographed at magnification of 800 times.

2.3 Results

2.3.1 Characterisation of *Drosophila melanogaster parkin*

The *Drosophila melanogaster parkin* homologue was identified through a search of the Berkeley Drosophila Genome Project (BDGP) utilizing the tBlastn search algorithm. The *parkin* gene is located on the left arm of the third chromosome, in the polytene chromosome section 78C within the genomic scaffolding region AE003593 (BDGP), and consists of 6 exons over 2.2 kb (Figure 2-1A). A search of the genome for additional *parkin* homologues revealed none. Our analysis confirmed the sequence of *parkin* to be identical to that reported by the BDGP (AY058754.1). Two potential initiation codons for the parkin protein are separated by 42 base pairs at the 5' region of the transcript. As the second potential start codon is preceded by CAAA, a match to the *Drosophila* Kozak consensus sequence (C/A)AA(A/C)ATG) of translation initiation (Cavener, 1987), we have assigned this as the most likely start codon. Furthermore, of the preceding fourteen potential codons only two use preferred codons (Moriyama and Powell, 1997) (data not shown). The *Drosophila parkin* gene was reported by Greene and colleagues (Greene *et al.*, 2003), while the current experiments were being conducted.

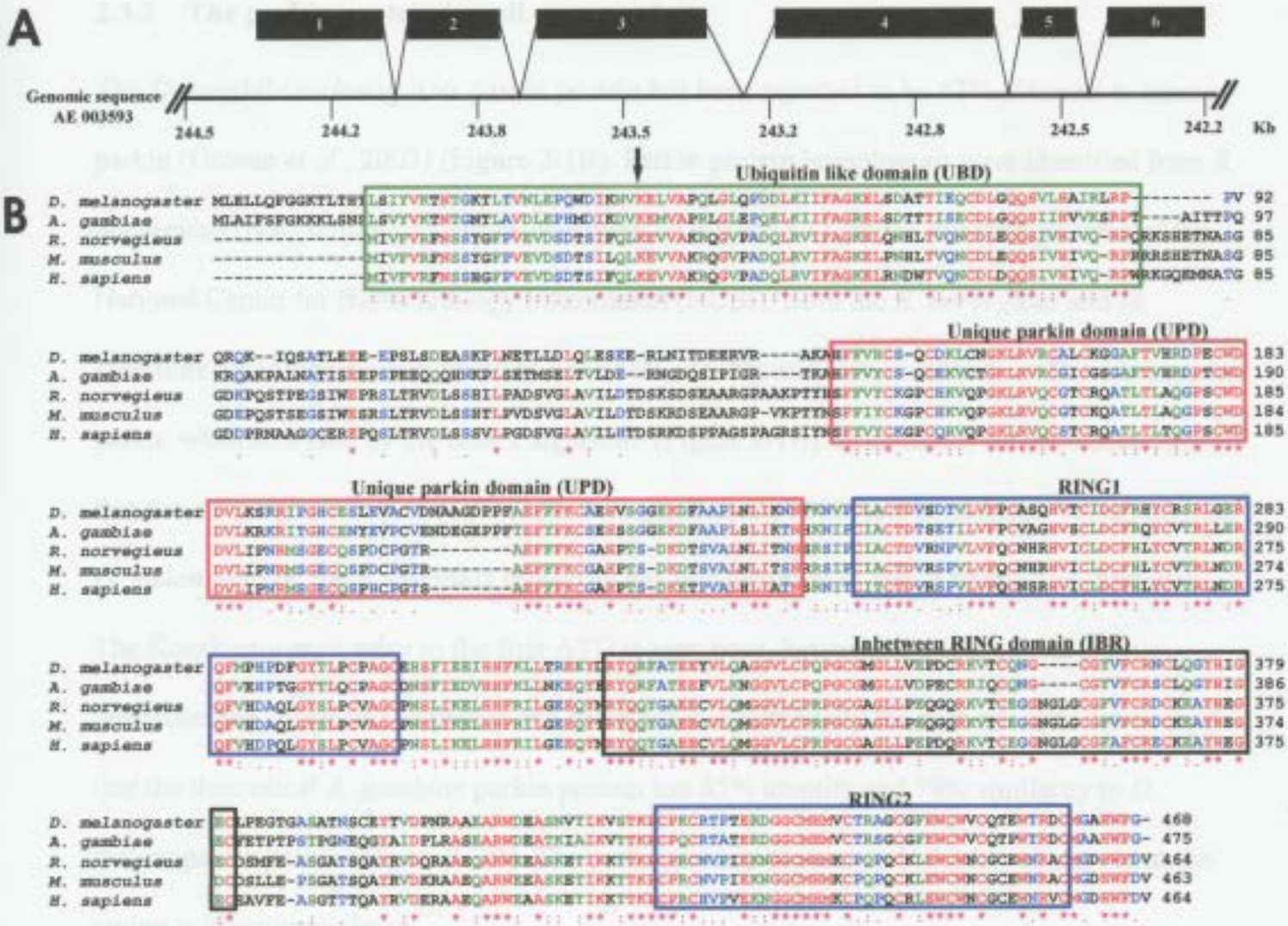

Figure 2-1: Dipteran and mammalian parkin proteins are well conserved.
A - Schematic representation of the *Drosophila melanogaster* parkin transcription unit and its location in the genomic scaffolding region AE003593. B - ClustalW alignment of the *Drosophila melanogaster* parkin with homologues from *Anopheles gambiae*, *Rattus norvegicus*, *Mus musculus* and *Homo sapiens*. Highlighted are the Ubiquitin-like Domain (UBL) (green box); the Unique Parkin Domain (UPD) (red box); RING1 and RING2 (blue boxes); In-Between Ring Domain (IRB) (black box). "*" and red lettering indicates amino acids that are identical in all sequences in the alignment. ":" and green lettering indicates conserved substitutions. "." and blue lettering indicates semi-conserved substitutions.

2.3.2 The parkin protein is well conserved

The *Drosophila melanogaster* parkin protein has been reported to be 42% identical to human parkin (Greene *et al.*, 2003) (Figure 2-1B). Parkin protein homologues were identified from *R. norvegicus* (NM_020093.1) and *M. musculus* (AB019558.1) via the tblastn algorithm of the National Center for Biotechnology Information (NCBI). Both the *R. norvegicus* and *M. musculus* homologues were found to have 44% identity and 60% similarity to *D. melanogaster* parkin when analysed by the blast2 algorithm (Figure 2-1B). In addition, we have determined that the *A.s gambiae* sequence XM316606.1 (Holt *et al.*, 2002) is a homologue of *parkin*. Like *D. melanogaster*, the *A. gambiae* transcript has two potential in-frame translation start sites. The Kozak sequence prior to the first ATG is very poor, however the second site closely resembles the consensus sequence and therefore it is very likely the start site. We determined that the theoretical *A. gambiae* parkin protein has 65% identity and 79% similarity to *D. melanogaster* parkin (Figure 2-1B). The parkin protein appears to be highly conserved at the amino acid sequence level.

Alignment of *D. melanogaster* parkin protein sequences with the *A. gambiae*, *R. norvegicus*, *M. musculus* and *H. sapiens* homologues reveals conservation of the protein throughout a number of characteristic domains, including the Ubiquitin- like Domain (UBL), the Unique Parkin Domain (UPD), the Really Interesting New Gene finger 1 (RING1) domain, the In-Between Ring (IBR) domain, and the RING2 domain (Figure 2-1B). In the amino-terminal region of the proteins, the first 15 amino acids are well conserved between *A. gambiae* and *D. melanogaster*, but absent in the mammalian proteins. The human UBL shows very high similarity (62%) to human ubiquitin (Kitada *et al.*, 1998). Correspondingly the *Drosophila*

UBL (Figure 2-1B, green box) was found to have 43% identity and 67% similarity to *Drosophila* ubiquitin (AAA29007; data not shown). The second highly conserved region is unique to parkin and has been termed the unique parkin domain (UPD) (Kahle *et al.*, 2000) (Figure 2-1, red box). *D. melanogaster* and *A. gambiae* share a similar eight amino acid insertion in the UPD (Figure 2-1B). There are two RING-finger domains that are defined by the consensus sequence C-X_2-C-$X_{9...39}$-C-$X_{1...3}$-H-$X_{2...3}$-C/H-X_2-C-$X_{4...48}$-C-X_2-C where X can be any amino acid (Figure 2-1B, blue boxes) (Freemont, 2000). These RING-finger domains flank a cysteine-rich domain designated the In-Between Ring (IBR) domain (Figure 2-1B, black box) (Morett and Bork, 1999). These three domains are responsible for binding to specific E2 ubiquitin conjugating enzymes (Imai *et al.*, 2000; Shimura *et al.*, 2000; Zhang *et al.*, 2000). Between the RING1 and the IBR domains there is an eighteen amino acid stretch of high conservation with the sequence (N/H)S(L/F)I(K/E)(E/D)(I/L)HHF(K/R)(L/I)LX(R/E)E(E/Q)Y. A 41 amino acid segment separates the IBR and RING2 domains, and while the first half of this segment is not well conserved the second half is highly conserved with the sequence AX(E/Q)ARW(D/E)XA(S/T)(N/K)X(T/A)IKX(S/T)TKP. The carboxy-terminus is well conserved and has the following sequence M(G/A)XHWF(G/D)(-/V), suggesting a possible conserved function for the tail of the protein. As parkin undergoes self-ubiquitination (Zhang *et al.*, 2000), conserved potential ubiquitination sites (lysine residues) were identified. There is a lysine residue that is completely conserved at K-42 of the dipterans and this corresponding residue is K-27 in mammals (Figure 2-1B, black arrow). The mouse and rat *parkin* homologues have been recently compared to *Drosophila parkin* (Bae *et al.*, 2003), however a number of the above features were not discussed. Overall parkin appears to be highly

conserved between mammalian and dipteran species suggesting conservation of function among these species.

2.3.3 Parkin suppresses degeneration of the ommatidial array in flies that express α-*synuclein* in the eye

We generated stable transgenic flies that can conditionally express *parkin* when the UAS/Gal4 expression system is utilized (Brand and Perrimon, 1993). *In situ* hybridization was used to confirm *parkin* expression in transgenic *Drosophila* (data not shown). Expression of *parkin* was directed to the developing eye using the *GMR-Gal4* transgene resulting in no obvious alteration of the eye (data not shown). *In vitro* and cell culture research suggests that parkin can prevent α-*synuclein*-induced toxicity (Oluwatosin-Chigbu *et al.*, 2003; Petrucelli *et al.*, 2002). Expression of human α-*synuclein* in the *Drosophila* eye causes premature deterioration of the retina (Feany and Bender, 2000). To examine if parkin could prevent *α-synuclein*-induced degeneration, we co-expressed *parkin* with human *α-synuclein* in the developing eye. Cross-sections of the retinas of one-day-old flies that express *α-synuclein* appear to be intact, as previously described (Figure 2-2A) (Feany and Bender, 2000). The retinas of one-day-old flies that express both *α-synuclein* and *parkin* also appear normal (Figure 2-2B and C). As previously described, the retinas of thirty-day-old flies that express α-*synuclein* show signs of premature degeneration (Feany and Bender, 2000). Degeneration of the normal architecture of the eye is apparent (Figure 2-2D, black arrows) and reflects the disruption of the normal placement and alignment of the photoreceptors and supporting cells. In contrast, thirty-day-old flies that express α-*synuclein* and *parkin* maintain their ommatidial arrays and morphology

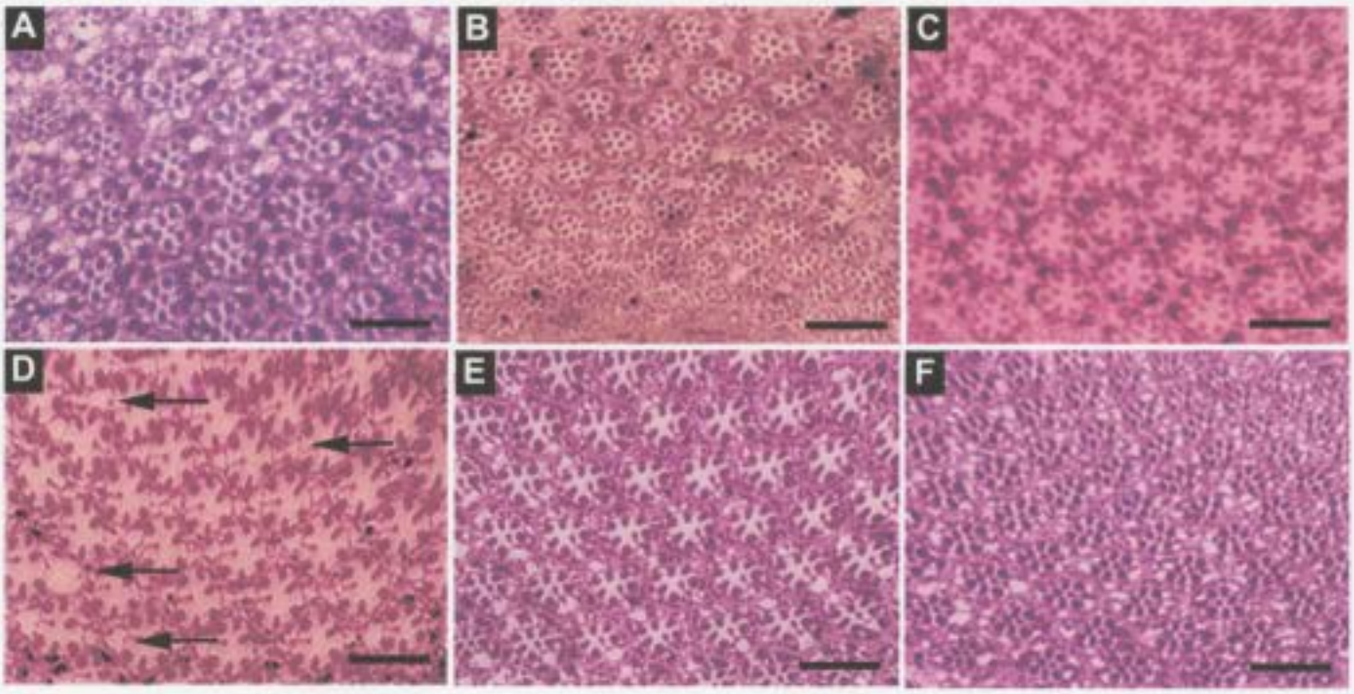

Figure 2-2: Expression of *parkin* suppresses *α-synuclein*-induced retinal degeneration. Flies that express *α-synuclein* with and without *parkin* were aged to 1 or 30 days old. They were fixed and embedded in epon. Tangential sections (0.5 μm thick) of the retina were cut, stained with toludine blue and examined by light microscopy. Panels A-C represent one-day-old flies and panels D-F represent thirty-day-old flies. Black arrows indicate degeneration of the ommatidial architecture. The genotypes are (A,D) w^{1118}; *UAS-α-synuclein/GMR-Gal4*, (B,E) w^{1118}; *UAS-α-synuclein/GMR-Gal4*; *UAS-parkin*$^{1.1}$/+, and (C,F) w^{1118}; *UAS-α-synuclein/GMR-Gal4*; *UAS-parkin*$^{2.1}$/+. Scale bar is 15 μm.

(Figure 2-2E and F). This observation demonstrates that directed expression of *parkin* suppresses *α-synuclein*-dependent degeneration of the ommatidial array.

Retinal damage can be observed by examining an optical effect termed the pseudopupil, which is lost in aged flies that express *α-synuclein* (Feany and Bender, 2000). We examined flies that co-express *α-synuclein* and *parkin*, and there appeared to be some retention of this optical effect compared with flies that express *α-synuclein* alone (data not shown). Scanning electron microscopy of eyes revealed no obvious deterioration of the surface in flies that express *α-synuclein* (Figure 2-3A and D) or flies that co-express *α-synuclein* and *parkin* (Figure 2-3B, C, E and F). Although *α-synuclein* causes degeneration of the ommatidial array, the external structure of the eye is unaffected.

2.3.4 Directed-expression of *parkin* to dopaminergic neurons does not affect climbing ability

Young wild-type adult *Drosophila* exhibit a strong negative geotaxis, which is increased by mechanical stimulation (Le Bourg and Lints, 1992; Miquel *et al.*, 1976). In order to measure climbing ability, flies are placed in a vial, gently tapped to the bottom and allowed to climb up the sides (Feany and Bender, 2000). When *parkin* is expressed in the dopaminergic neurons, these flies do not show any change in their climbing ability over their life span when compared with controls (Figure 2-4A). In addition, expression of *parkin* in dopaminergic neurons does not alter life span (Figure 2-4B). These results demonstrate that *parkin* expression in the dopaminergic neurons has little effect upon climbing ability or life span.

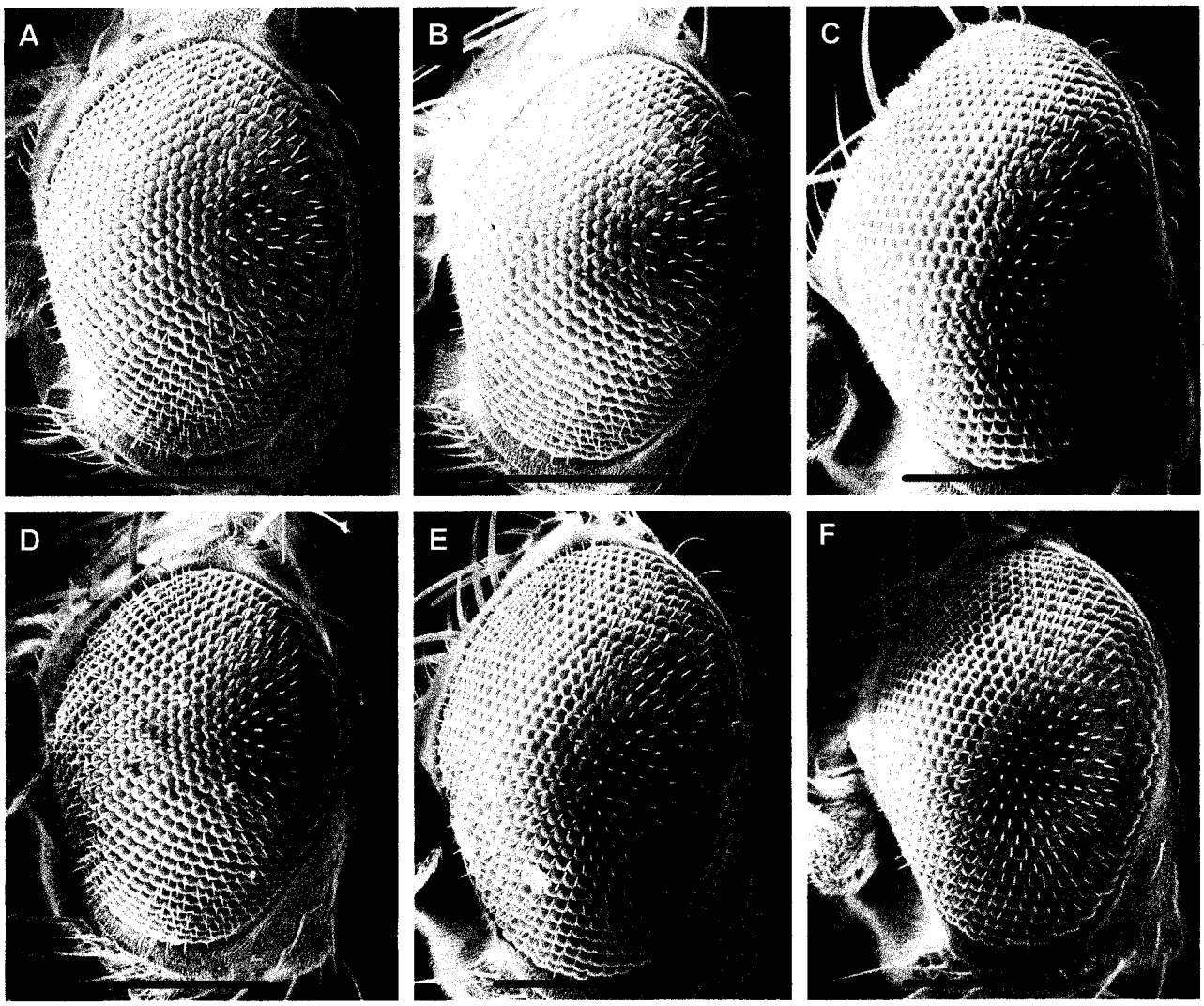

Figure 2-3: Expression of *α-synuclein* with and without *parkin* does not affect the external morphology of the eye.
Scanning electron microscopy of flies that express *α-synuclein* with and without *parkin* shows no change in their external morphology over thirty days. Panels A-C represent one-day-old flies and panels D-F represent thirty-day-old flies. The genotypes are (A, D) w^{1118}; *UAS-α-synuclein/GMR-Gal4*, (B, E) w^{1118}; *UAS-α-synuclein/GMR-Gal4*; *UAS-parkin*$^{1.1}$/+, and (C, F) w^{1118}; *UAS-α-synuclein/GMR-Gal4*; *UAS-parkin*$^{2.1}$/+. Scale bar indicates 200 μm.

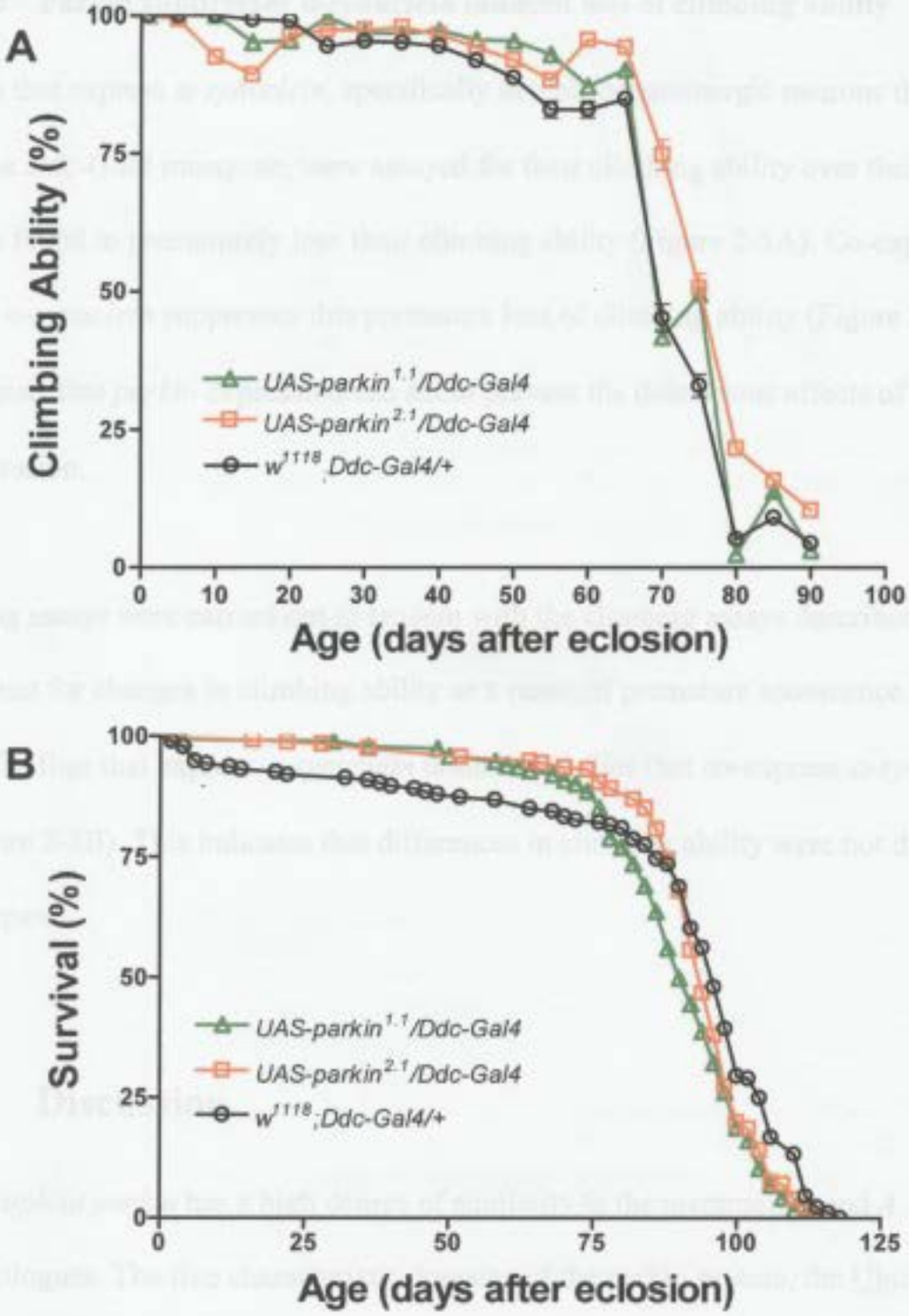

Figure 2-4: Expression of *parkin* does not affect climbing ability or life span.
A – Climbing ability of flies that express *parkin* does not differ from control flies. Genotypes are w^{1118}; *UAS-parkin*$^{1.1}$*/Ddc-Gal4* (green open triangle), w^{1118}; *UAS-parkin*$^{2.1}$*/Ddc-Gal4* (orange open square) and w^{1118}; *Ddc-Gal4/+* (black open circles). The error bars show the standard error of the mean of twenty trials at each point. Please note that the error bars are mostly within the symbols. B – The life span of flies that express *parkin* does not differ from the control. The genotypes are marked the same as in panel A.

2.3.5 Parkin suppresses *α-synuclein* induced loss of climbing ability

Flies that express *α-synuclein*, specifically in their dopaminergic neurons through the activity of the *Ddc-Gal4* transgene, were assayed for their climbing ability over their life span, and were found to prematurely lose their climbing ability (Figure 2-5A). Co-expression of *parkin* with α-*synuclein* suppresses this premature loss of climbing ability (Figure 2-5A). This suggests that *parkin* expression can act to prevent the deleterious effects of *α-synuclein* expression.

Aging assays were carried out in tandem with the climbing assays described above in order to account for changes in climbing ability as a result of premature senescence. Median survival age for flies that express *α-synuclein* is similar to flies that co-express *α-synuclein* with *parkin* (Figure 2-5B). This indicates that differences in climbing ability were not due to differences in life span.

2.4 Discussion

Drosophila parkin has a high degree of similarity to the mammalian and *A. gambiae* homologues. The five characteristic domains of the parkin protein, the Ubiquitin- like Domain (UBL), Unique Parkin Domain (UPD), Really Interesting New Gene finger 1 (RING1) domain, In- Between Ring (IBR) domain and RING2 all show a high degree of similarity. In addition, the two dipterans, *D. melanogaster* and *A. gambiae*, have a highly conserved extra segment of 15 amino acids at the amino-terminal of the protein. The regions between the three carboxy-terminus domains are also highly conserved, which may indicate conservation of

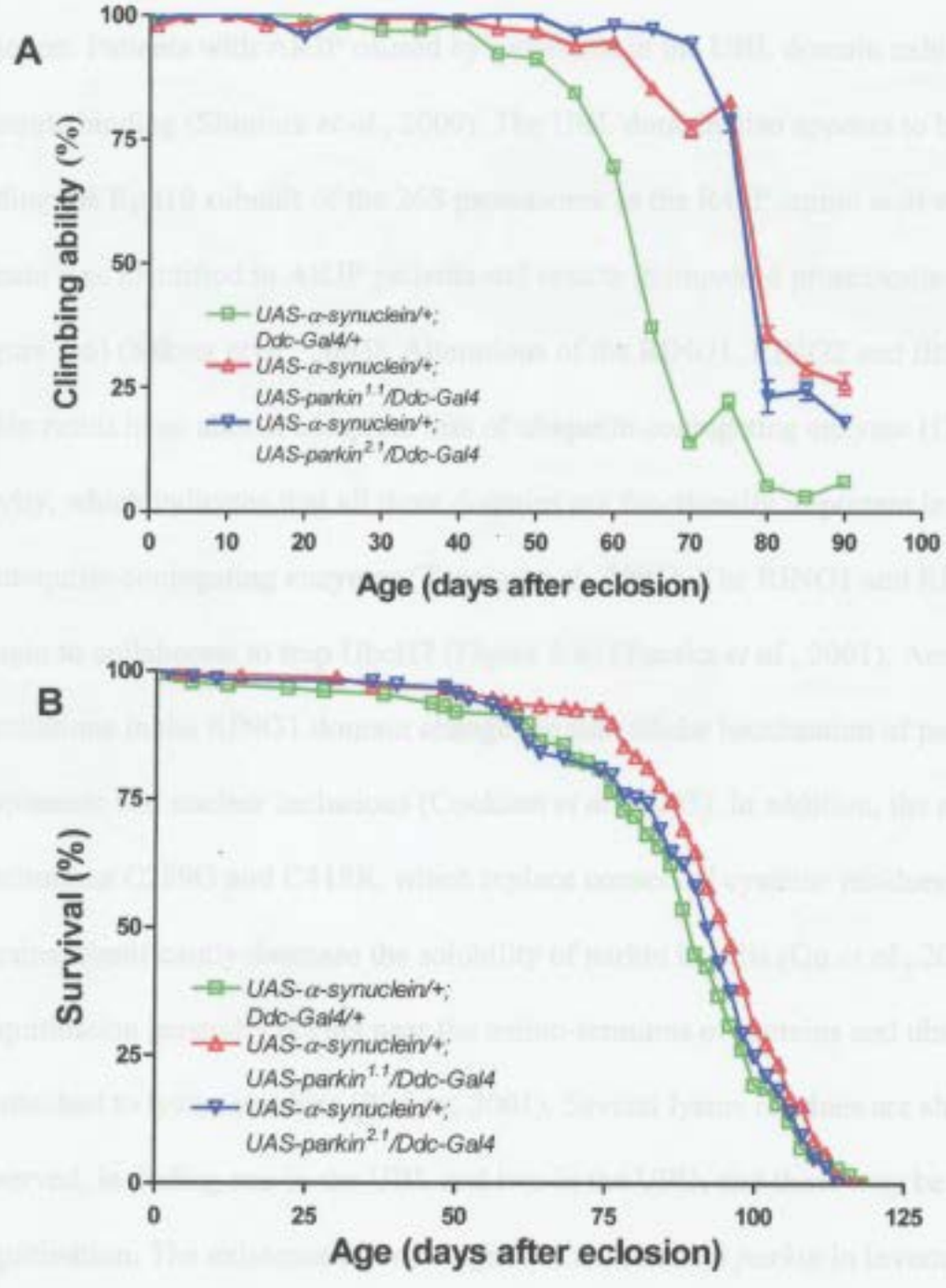

Figure 2-5: Expression of *parkin* suppresses α–synuclein-induced loss of climbing ability
Panel A - Aged flies that express *parkin* and α–*synuclein* climb significantly better than flies that express α–*synuclein* ($P<0.001$, one-way analysis of variance with supplementary Newman-Keuls test). Genotypes are w^{1118};*UAS-α–synuclein/+;Ddc-Gal4/+* (green open square), w^{1118};*UAS-α–synuclein/+;UAS-parkin*$^{1.1}$*/Ddc-Gal4* (red open triangle), w^{1118};*UAS-α–synuclein/+;UAS-parkin*$^{2.1}$*/Ddc-Gal4* (blue upside down open triangle). The error bars show the standard error of the mean of twenty trials at each point. Please note that the error bars are mostly within the symbols. B - The life span of flies that express α–*synuclein* with and without *parkin* does not differ. The genotypes are marked the same as in panel A.

function. Patients with ARJP caused by mutations in the UBL domain exhibit signs of lost substrate binding (Shimura *et al.*, 2000). The UBL domain also appears to be involved in binding the Rpn10 subunit of the 26S proteasome as the R42P amino acid substitution in this domain was identified in ARJP patients and results in impaired proteasome binding of parkin (Figure 2-6) (Sakata *et al.*, 2003). Alterations of the RING1, RING2 and IBR domains of parkin result in an almost complete loss of ubiquitin conjugating enzyme H7 (UbcH7)-binding activity, which indicates that all three domains are functionally important in recruiting specific E2 ubiquitin conjugating enzymes (Tanaka *et al.*, 2001). The RING1 and RING2 domains are thought to collaborate to trap UbcH7 (Figure 2-6) (Tanaka *et al.*, 2001). Amino acid substitutions in the RING1 domain change the subcellular localization of parkin and enhance cytoplasmic and nuclear inclusions (Cookson *et al.*, 2003). In addition, the amino acid substitutions C289G and C418R, which replace conserved cysteine residues in the RING domains, significantly decrease the solubility of parkin in cells (Gu *et al.*, 2003). Ubiquitination generally occurs near the amino-terminus of proteins and ubiquitin monomers are attached to lysine residues (Pickart, 2001). Several lysine residues are absolutely conserved, including one in the UBL and two in the UPD, and these may be targets for ubiquitination. The existence of orthologues of mammalian *parkin* in invertebrates but not plants nor fungi (Marin and Ferrus, 2002) suggests an animal specific function for *parkin* activity. The highly conserved protein domains and sub-domains suggest the probable conservation of each domain's function, and given the high degree of similarity we suggest that the function of the *Drosophila* parkin protein is similar to that of the human parkin protein.

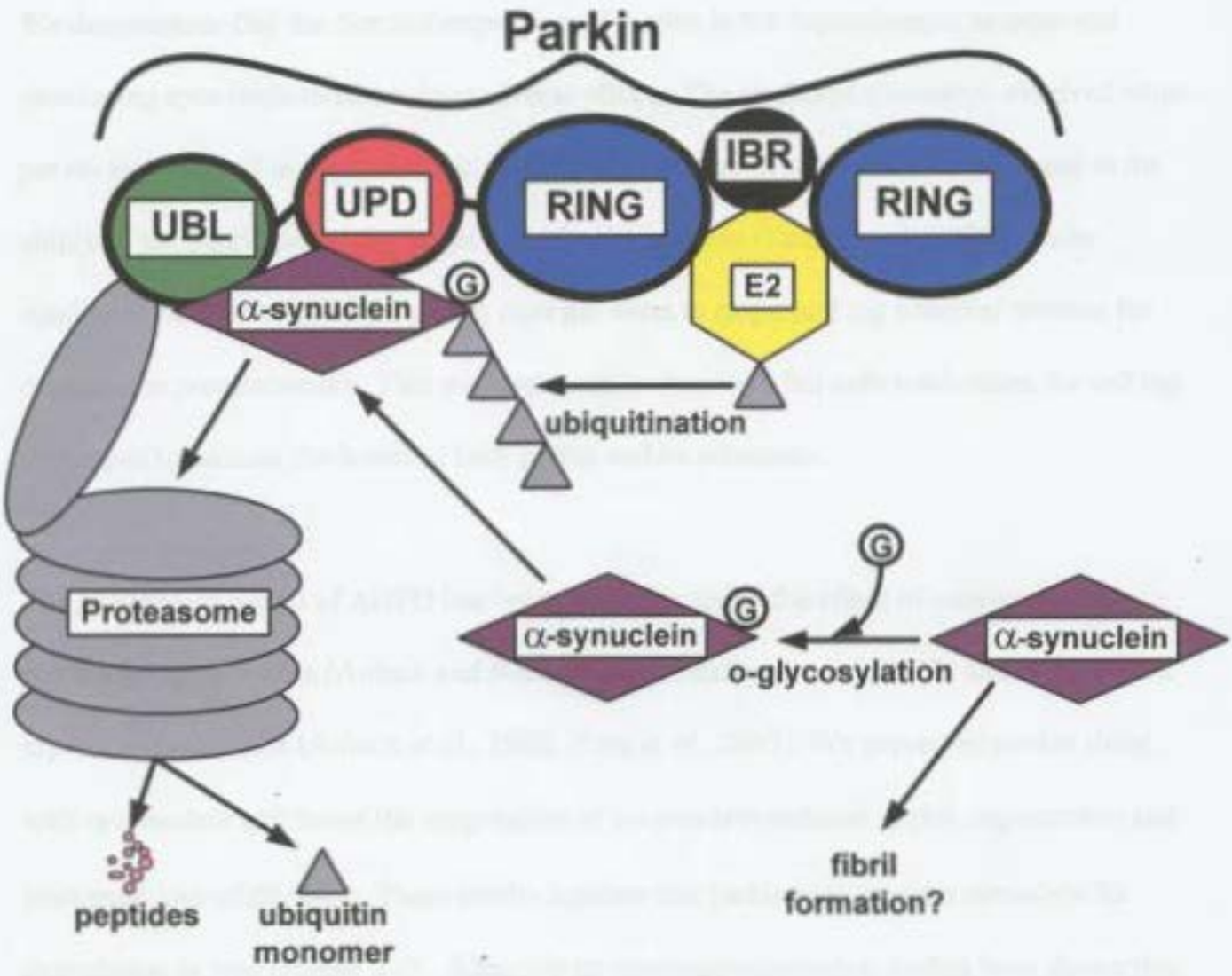

Figure 2-6: Model of parkin-directed ubiquitination of α-synuclein.
The parkin protein consists of two functionally distinct regions. The UBL/UPD region binds target proteins such as glycosylated α-synuclein. The RING-box (RING1-IBR-RING2) region recruits specific E2 ubiquitin conjugating enzymes, which add ubiquitin monomers to the target protein. In addition to substrate binding the UBL domain interacts with the proteasome. Ubiquitin tagged α-synuclein is directed to the proteasome and degraded into polypeptides and ubiquitin monomers. UBL – Ubiquitin-like Domain, UPD – Unique Parkin Domain, RING1 or 2 – Really Interesting New Gene finger 1 or 2 domain, IBR – In-Between Ring domain.

We demonstrate that the directed expression of *parkin* in the dopaminergic neurons and developing eyes leads to no obvious adverse effects. The unaltered phenotype observed when *parkin* is expressed in dopaminergic neurons is likely due to substrate specificity and to the ability of the parkin protein to target itself for degradation (Zhang *et al.*, 2000). Under conditions of over-expression, parkin does not seem to target and tag essential proteins for degradation promiscuously. This may represent an excellent fail-safe mechanism the cell has developed to balance the levels of both parkin and its substrates.

The *Drosophila* model of ADPD has been used to examine the effect of various pharmacological agents (Auluck and Bonini, 2002; Pendleton *et al.*, 2002) and other genetic aspects of the disease (Auluck *et al.*, 2002; Yang *et al.*, 2003). We expressed *parkin* along with *α-synuclein* and found the suppression of *α-synuclein*-induced retinal degeneration and premature loss of climbing. These results indicate that parkin may target α-synuclein for degradation *in vivo* (Figure 2-6). Although co-immunoprecipitation studies have shown that parkin does not interact with or ubiquitinate non-modified α-synuclein (Chung *et al.*, 2001), parkin will ubiquitinate O-glycosylated α-synuclein (Shimura *et al.*, 2001). Since we show suppression of the *α-synuclein*-induced phenotype, we believe that ectopically expressed *α-synuclein* is modified in *Drosophila*, enabling its ubiquitination by parkin. A model of the modification of α-synuclein and subsequent ubiquitination by parkin is presented in Figure 2-6.

In order to select rational potential therapeutic agents, the molecular mechanisms behind disease progression must be characterized. Gene function studies with homologues of disease-

causing genes in model organisms have been made practical through the advent of genome projects. Over-expression of *parkin* has no apparent adverse consequences and it suppresses the *α-synuclein*-induced PD symptoms in *Drosophila*. If this relationship is conserved in humans, we suggest that up-regulation of *parkin* should be a viable treatment for PD, and the selection of therapeutic strategies should be directed towards this end.

2.5 Conclusion

Our experiments demonstrate that the directed expression of the *parkin* gene counteracts the PD-like symptoms in the *α-synuclein*-induced *Drosophila* model of PD. Manipulation of the ubiquitin/proteasome degradation pathway in such a specific manner apparently remedies the toxic accumulation of α-synuclein. This study demonstrates the success of selective targeting of toxic proteins for degradation as an approach to address neurodegenerative conditions such as Parkinson's disease. The development of therapies that regulate *parkin* expression or parkin protein activity may be crucial in the treatment of PD.

2.6 Additional data for Chapter 2

2.6.1 Testing of transgenic response to Gal4

Generation of the stable transgenic lines that allow the conditional expression of *parkin* have been described previously (Haywood and Staveley, 2004). Expression of *parkin* was directed to the developing eye using the *GMR-Gal4* transgene (Freeman, 1996). *In situ* hybridization of *parkin* was performed on the eye discs of *GMR-Gal4 /+; UAS-parkin /+* and control *GMR-Gal4 /+; + /+* third instar larvae to determine transgenic expression (Figure 2-7). The blue

vertical stripe in the eye disc shows expression of the transgene (Figure 2-7B, black arrow), consistent with the expression of *GMR-Gal4* in a wave behind the morphogenetic furrow (Freeman, 1996). This demonstrates that the *parkin* transgenes are transcribed in response to Gal4.

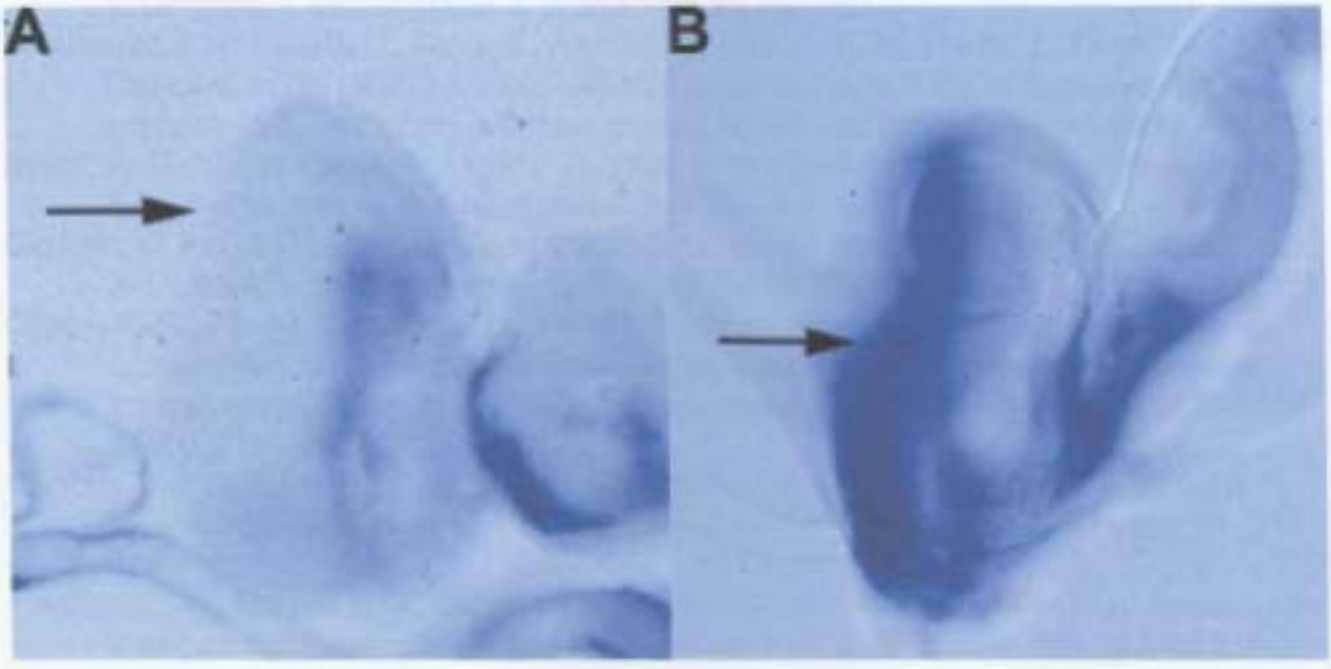

Figure 2-7: Transgenic *Drosophila melanogaster* express *parkin*.
Third instar larval eye imaginal discs were subjected to *in situ* hybridisation with a probe to *parkin*. A, w^{1118} control eye imaginal disc; B, *GMR-Gal4/+;UAS-parkin* $^{2.1}$/+ eye imaginal disc expressing *parkin*.

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Chapter 3: Parkin reduces mutant α-synuclein-induced degeneration in a fly model of Parkinson's

A version of this chapter is in press in Genome (Haywood and Staveley, 2006)

3.1 Introduction

The neurodegenerative disorder Parkinson's disease (PD) is characterized by the progressive loss of motor control, difficulty initiating and sustaining movements and the selective loss of dopaminergic neurons from the substantia nigra region of the brain (Dawson, 2000; Parkinson, 1817; Spacey and Wood, 1999). By the time the disease is diagnosed, generally 80% of these neurons are lost as between 50% and 60% of the nigral neurons can be lost with no obvious clinical consequence (Lansbury and Brice, 2002). Although the majority of PD cases are apparently sporadic, approximately 5 to 15% have been determined to have an inherited basis (de Silva *et al.*, 2000; Mizuno *et al.*, 2001). Recently, mutations in a number of genes have been identified as causes of PD, and many of these genes are associated with the ubiquitin/proteasome protein degradation pathway.

One of the first identified genetic causes of PD was mutations in *α-synuclein* (Polymeropoulos *et al.*, 1996; Polymeropoulos *et al.*, 1997). The α–synuclein protein is found at the pre-synaptic region of neurons (Clayton and George, 1999; Jakes *et al.*, 1994; Kahle *et al.*, 2000) and appears to be involved in the biosynthesis of dopamine (Baptista *et al.*, 2003; Perez *et al.*, 2002). Three independent non-synonymous point mutations in *α-synuclein,* have been identified as causes of PD (Kruger *et al.*, 1998; Polymeropoulos *et al.*, 1996; Polymeropoulos *et al.*, 1997; Zarranz *et al.*, 2004). Alteration of the primary structure of the α-synuclein protein may enhance oligomerization and fibril formation (Conway *et al.*, 1998). Whether these fibrils are a cause of toxicity or act in a mechanism to sequester toxic oligomers of α-synuclein is an unresolved question. As triplication of the *α-synuclein* locus has been shown

to lead to the development of the disease (Singleton *et al.*, 2003), both the composition and the dosage of α-synuclein appear to play roles in the development of PD.

A number of point mutations and deletions of the *parkin* gene cause Autosomal Recessive Juvenile Parkinson's disease (ARJP) (Kitada *et al.*, 1998; Nisipeanu *et al.*, 1999). The *parkin* gene encodes a 465 amino acid protein (Kitada *et al.*, 1998), which functions as one of a number of E3 ubiquitin protein ligases (Shimura *et al.*, 2000). Ubiquitin protein ligases mediate ubiquitination, the sequential attachment of a number of ubiquitin monomers, of damaged, misfolded and short-lived proteins, which are subsequently targeted to the proteasome for degradation (Hershko and Ciechanover, 1998; Pickart, 2001). *In vitro* ubiquitination assays demonstrate that the parkin protein can ubiquitinate a glycosylated form of α–synuclein (Shimura *et al.*, 2001). Elevated expression of *parkin* protects neuronal explants from the toxicity associated with expression of mutant *α–synuclein* (Petrucelli *et al.*, 2002). The loss of *parkin* may lead to an accumulation of its protein substrates and endoplasmic reticulum stress (Imai *et al.*, 2000; Imai *et al.*, 2001), which then may trigger the activation of cellular suicide mechanisms (Rao *et al.*, 2002). The role of parkin appears to be the protection of cells from the toxic effects of inappropriate protein behaviour.

The conditional expression of human *α–synuclein* in transgenic *Drosophila melanogaster* provides an excellent model of PD (Feany and Bender, 2000). Pan-neuronal expression of both wild type and mutant *α-synuclein* leads to premature loss of climbing ability, a feature that control flies retain into old age (Feany and Bender, 2000). In addition, the expression of *α–synuclein* in the developing eye results in precocious degeneration of the retina. Expression

of *Drosophila melanogaster parkin* can suppress the PD-like phenotypes caused by directed expression of wild type *α-synuclein* (Haywood and Staveley, 2004). These features recapitulate the main behavioural and pathological phenotypes of PD and provide an excellent model system to study the biological basis of the disease.

The A30P mutant form of *α-synuclein*, that substitutes the alanine at position 30 with a proline, causes Autosomal Dominant PD in humans (Kruger *et al.*, 1998). In *Drosophila melanogaster*, expression of *A30Pα-synuclein* in a pan-neuronal fashion has a deleterious effect on climbing ability without affecting lifespan (Feany and Bender, 2000). Recently, we have demonstrated that co-expression of *parkin* with wild type *α-synuclein* counteracts the Parkinson's-like effects of *α-synuclein* expression (Haywood and Staveley, 2004). From the literature it is unclear as to whether alterations in α-synuclein inhibit its ability to be targeted for degradation by parkin *in vivo*. Should the A30P amino acid substitution prevent parkin from mediating α-synuclein ubiquitination then the loss of climbing ability and retinal degeneration caused by altered α-synuclein will not be inhibited by co-expression of *parkin*. To determine if *parkin* has the ability to suppress the deleterious effects of mutant *α-synuclein*, we have co-expressed *parkin* with *A30Pα-synuclein* and compared the flies climbing ability and retinal degeneration with flies that express mutant *α-synuclein* alone. We demonstrate that *parkin* can counteract the effects of mutant *α–synuclein*-induced retinal degeneration and improve the flies climbing activity. This is the first demonstration that a directed increase in *parkin* expression can counteract the effects of mutant *α-synuclein* in an experimental organism.

3.2 Methods

3.2.1 Fly stocks and culture

Dr. M. Feany (Harvard Medical School) generously provided the *UAS-A30Pα–synuclein* flies (Feany and Bender, 2000) and, similarly, Dr. J. Hirsh (University of Virginia) provided the *Ddc-Gal4*$^{4.36}$ stock (Li *et al.*, 2000). The *GMR-Gal4* flies (Freeman, 1996) were obtained from the Bloomington Drosophila Stock Center of Indiana University at Bloomington. The two lines, *UAS-parkin*$^{1.1}$*/TM3* and *UAS-parkin*$^{2.1}$, were independently derived through standard *Drosophila* transgenic techniques as previously reported (Haywood and Staveley, 2004). These lines were used to generate double transgenic lines of the genotypes *UAS-A30Pα–synuclein/CyO;UAS-parkin*$^{1.1}$*/TM3* and *UAS-A30Pα–synuclein/CyO;UAS-parkin*$^{2.1}$*/TM3* using standard techniques. To drive expression of the transgenes, *Ddc-Gal4*$^{4.36}$ (for expression in the dopaminergic neurons) or *GMR-Gal4* (for expression in the eye) homozygous females of these lines were crossed to *UAS-A30Pα–synuclein* males with or without *UAS-parkin*$^{1.1}$ or *UAS-parkin*$^{2.1}$. All flies were cultured on standard cornmeal/yeast/molasses/agar media at 25°C.

3.2.2 Climbing ability assay

Flies were assayed for their ability to climb as by a standard method (Feany and Bender, 2000). Every five days, eighty male flies from a cohort were assayed for their ability to climb six centimetres in eighteen seconds in a sterile plastic vial. Twenty trials were carried out for each time point. Flies were tested for up to ninety days. This experiment was repeated and the results compiled and statistically analysed through Prism 4.02 software. Initially the data

was graphed with the standard errors of the mean and subjected to a one-way ANOVA, this was followed up by linear regression curve fit analysis with 95% confidence intervals.

3.2.3 Aging analysis

Adult males were aged in small groups ($n \leq 20$ individuals) in vials upon standard media at 25°C and scored for viability every two to three days as described previously (Staveley *et al.*, 1990). The total numbers of individuals aged were: w^{1118}; *Ddc-Gal4*$^{4.36}$/+ = 180, *UAS-A30Pα–synuclein*/+; *Ddc-Gal4*$^{4.36}$/+ = 218, *UAS-A30Pα–synuclein*/+; *UAS-parkin*$^{1.1}$/*Ddc-Gal4*$^{4.36}$ = 182, *UAS-A30Pα–synuclein*/+; *UAS-parkin*$^{2.1}$ /*Ddc-Gal4*$^{4.36}$ = 245. The data was subjected to a log rank test and the mean life spans were also compared using Prism 4.02 software.

3.2.4 Histological examination of adult *Drosophila melanogaster* retinas

Nine adult flies of each genotype were aged (one or thirty days after eclosion), fixed in Karnovsky's fixative and embedded in epon as previously described (Feany and Bender, 2000). Tangential retinal sections were prepared at a thickness of 0.5 μm and stained with toluidine blue, examined by light microscopy and photographed at magnification of 800 times. Eight eye slices from separate 30-day-old flies were analysed by counting the number of ommatidia in that slice. These numbers were collated and an average percentage of disrupted ommatidia calculated and statistically analysed through Prism 4.02 software.

3.3 Results

3.3.1 Directed expression of *parkin* increases the climbing ability of flies that express mutant *α–synuclein*

Aging assays were carried out in tandem with the climbing assays described above in order to account for changes in climbing ability as a result of premature senescence. Comparison of survival curves indicates that there is no difference between the longevity of flies that express *A30Pα-synuclein* with and without the *UAS-parkin*$^{1.1}$ transgene (Figure 3-1A). A Logrank test shows the two curves are not significantly different. The survival curve of flies that express *A30Pα-synuclein* and *UAS-parkin*$^{2.1}$ show a slight and significant decrease in median survival age compared with flies that express *A30Pα-synuclein* alone (Figure 3-1A).

Flies that express mutant *α–synuclein* with and without *parkin* in their dopaminergic neurons were assayed for climbing ability. Flies that express both *A30Pα-synuclein* and *parkin* transgenes, specifically in the dopaminergic neurons under the control of *Gal4,* show an extention of climbing ability at later ages compared with flies that express mutant *α-synuclein* alone (Table 3-1 and Figure 3-1B). This suggests that *parkin* can act to prevent any deleterious effects mutant *α–synuclein* expression may have and certainly acts to improve the climbing ability of these flies over their lifespan. On the basis of the aging assay, apparently the improved climbing ability in later life is not due to increased mean lifespan.

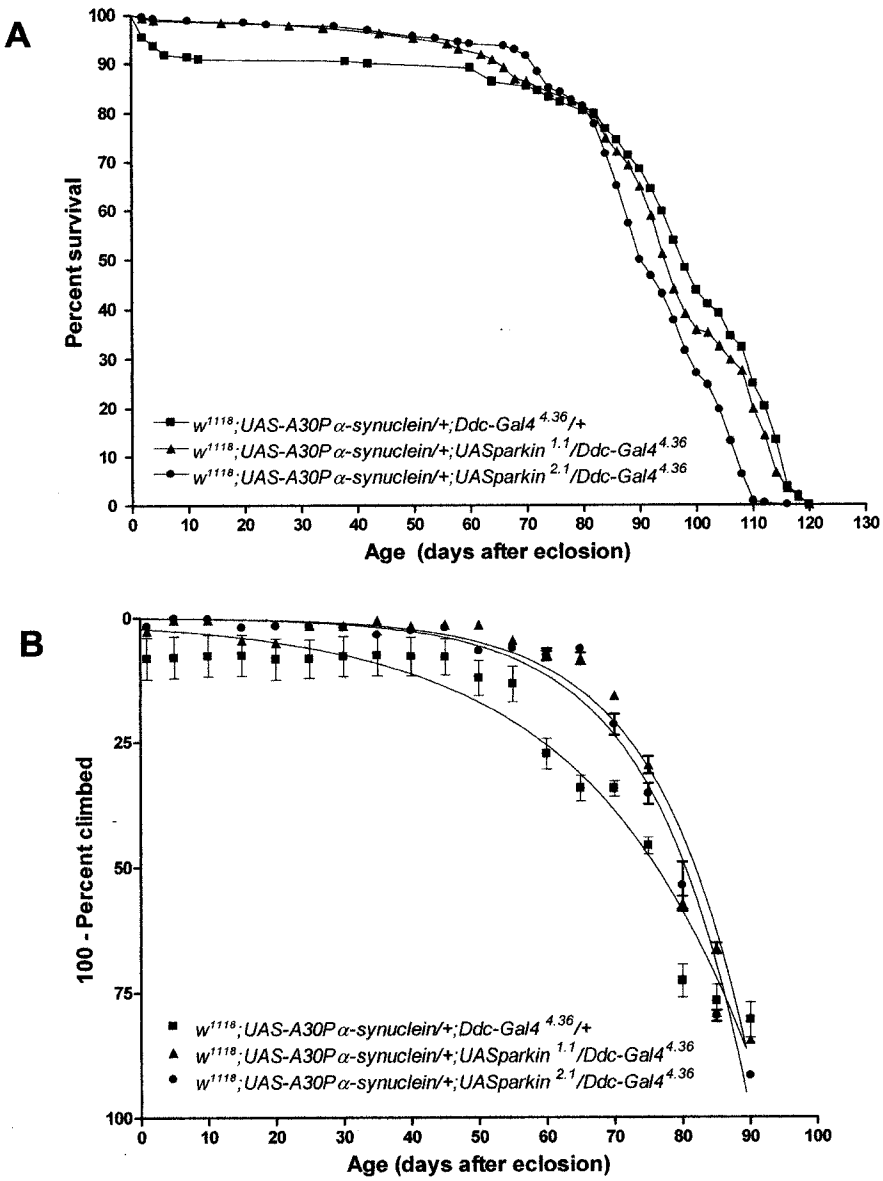

Figure 3-1: Expression of *parkin* increases climbing ability of flies expressing *α-synuclein*
A - The life spans of flies that express *A30Pα–synuclein* with and without *parkin* are shown. Genotypes are w^{1118};*UAS-A30Pα–synuclein/+;Ddc-Gal4*$^{4.36}$/+ (square); w^{1118};*UAS-A30Pα–synuclein/+;UAS-parkin*$^{1.1}$*/Ddc-Gal4*$^{4.36}$ (triangle); w^{1118};*UAS-A30Pα–synuclein/+;UAS-parkin*$^{2.1}$*/Ddc-Gal4*$^{4.36}$ (circle). Logrank test comparison of flies that express mutant *α-synuclein* with and without *parkin*$^{1.1}$ are not significantly different, p = 0.1106. However comparison flies that express mutant *α-synuclein* with and without *parkin*$^{2.1}$ are significantly different, p = 0.0001. B – Aged flies that express *parkin* and mutant *α–synuclein* climb significantly better than flies that express mutant α–*synuclein.* The percentage of flies that climbed successfully was subtracted from 100. The genotypes are marked the same as in A. The error bars show the standard error of the mean of twenty trials at each point. Note the error bars are mostly within the symbols.

Table 3-1: Comparison of the non-linear fitted curves for climbing ability showing slopes (K), standard error (SE), and confidence intervals (CI).
The slope of the fitted curve for the flies that express mutant *α-synuclein* with and without parkin are 0.05 and 0.08 or 0.07 and do not overlap within a 95% confidence interval.

Genotype	Slope (K)	Standard error (SE)	95% Confidence intervals (CI)
UAS-A30P α-synuclein/+; Ddc-Gal4/+	0.05	0.0009	0.050 to 0.054
UAS-A30P α-synuclein/+; Ddc-Gal4/UAS-parkin$^{1.1}$	0.08	0.0012	0.074 to 0.078
UAS-A30P α-synuclein/+; Ddc-Gal4/ UAS-parkin$^{2.1}$	0.07	0.0016	0.070 to 0.076

3.3.2 Directed expression of *parkin* suppresses mutant α–synuclein-induced degeneration of the ommatidial array

Ectopic expression of *parkin* prevents premature wild type α–synuclein-induced degeneration in the *Drosophila melanogaster* eye (Haywood and Staveley, 2004). To determine if *parkin* has a similar ability to suppress mutant α-synuclein-induced premature degeneration, *A30Pα-synuclein* and *parkin* were co-expressed in the *Drosophila* eye. Cross-sections of the retinas of one-day-old flies that express *A30Pα-synuclein* or both *mutant α–synuclein* and *parkin* appear intact and normal (Figure 3-2A and B). As previously described, the retinas of thirty-day-old flies that express *mutant α–synuclein* show signs of premature degeneration (Feany and Bender, 2000), such as disintegration of the outer ring of the ommatidia and distortion of the normally close arrangement of rhabdomeres (Figure 3-2C, black arrows). On average approximately 40 percent of ommatidia had a defect including degeneration of the outer ring of the ommatidia or separation of the normally close arrangement of rhabdomeres (Figure 3-2E). In contrast thirty-day-old flies that express both *A30Pα-synuclein* and *parkin* appear to maintain an intact ommatidial array (Figure 3-2D), with only 5% of ommatidia having any defect (Figure 3-2E). Retinal deterioration can be observed by examining an optical effect termed the pseudo-pupil, which is lost in aged flies that express mutant *α–synuclein* (Feany and Bender, 2000). When 20-day-old flies that co-express mutant *α–synuclein* and *parkin* were examined, there appeared to be retention of this optical effect compared with flies that express mutant *α–synuclein* alone (data not shown). Overall, expression of *parkin* suppresses degeneration of the ommatidial array caused by the expression of mutant *α–synuclein.*

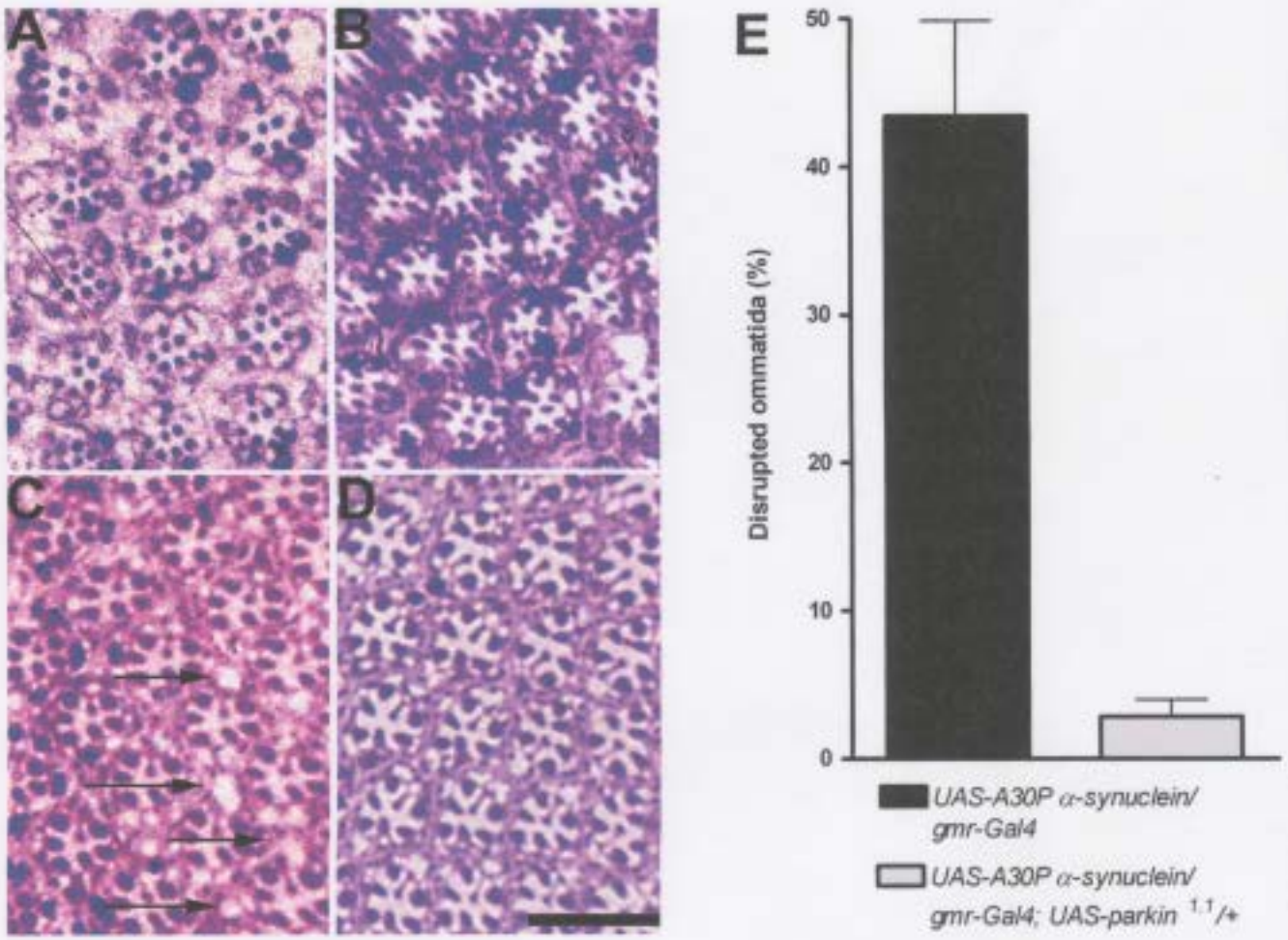

Figure 3-2: Expression of *parkin* suppresses *α–synuclein*-induced retinal degeneration
A and B are 0.5 μm tangential sections from one-day-old flies and C and D are 0.5 μm tangential sections from thirty-day-old flies. The genotypes are (A and C) w^{1118};*UAS-A30Pα–synuclein/GMR-Gal4*, (B and D) w^{1118};*UAS-A30Pα–synuclein/GMR-Gal4*;*UAS-parkin*$^{1.1}$/+. Black arrows indicate degeneration of outer edges of ommatidia. Scale bar is 15 μm. E - The distorted ommatidia in a single section from eight separate 30-day-old fly eyes were counted and displayed as a percentage of total ommatidia.

3.4 Discussion

Our experiments demonstrate that the directed expression of *parkin* in the developing eye negates the retinal defects resulting from mutant *α-synuclein* expression. In addition, we demonstrate that increased *parkin* expression in the dopaminergic neurons extend the climbing ability of aged flies that express mutant *α–synuclein.* This suggests that parkin can suppress the degeneration resulting from the mutant α-synuclein expression in spite of the amino acid substitution present in the mutant form of α-synuclein that is thought to lead to a conformational change in the protein (Conway *et al.*, 1998). While the exact mechanism of phenotype suppression is not clear these result indicate that mutant α-synuclein is likely a target of parkin's ubiquitin ligase activity.

Co-immunoprecipitation studies have suggested that the parkin protein does not interact with or ubiquitinate unmodified α–synuclein (Chung *et al.*, 2001) but will ubiquitinate O-glycosylated α–synuclein (Shimura *et al.*, 2001). We have established the suppression of mutant *α–synuclein*-induced retinal degeneration by the ectopic expression of *parkin.* Therefore, we believe that mutant α–synuclein protein is modified in *Drosophila melanogaster* in a manner that will enable it to be ubiquitinated by the parkin ubiquitin protein ligase then targeted to the proteasome for degradation.

Transgenic *Drosophila melanogaster* that express either the wild-type or mutant form of *α-synuclein* in their central nervous systems, via the pan-neural *elav-Gal4* transgene, have shown an age-dependent reduction in climbing ability when compared with control flies (Feany and

Bender, 2000). Notably flies that express mutant *α-synuclein* under the control of *elav-Gal4* show a greater reduction in climbing ability. Although flies that express wild-type *α-synuclein* in their dopaminergic neurons show a marked premature loss of climbing ability (Haywood and Staveley, 2004), expression of mutant *α-synuclein* results in only a slight premature loss of the ability to climb (Auluck *et al.*, 2002). Nevertheless, over-expression by both of the *parkin* transgenes has the effect of extending the climbing ability of flies that express *A30Pα-synuclein* when compared to the controls. Thus the premature loss of climbing ability arising from a mutant form of *α-synuclein* that is known to cause PD in humans is prevented by the directed expression of *parkin*.

Over-expression of *parkin* suppresses the PD-like symptoms induced in *Drosophila melanogaster* by wild-type (Haywood and Staveley, 2004) and mutant *α-synuclein* with no apparent adverse consequences. We suggest that the manipulation of the ubiquitin/proteasome degradation pathway in such a specific manner acts to remedy the toxicity of the accumulation of α–synuclein. Activation of *parkin* may be a viable treatment for PD caused by increased levels or mutant forms of *α-synuclein* and we suggest that the selection of therapeutic strategies should be directed towards this end.

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Chapter 4: Analysis of *parkin* expression in the developing adult eye and wing

This chapter has not been published and is supplemental to Chapters 2 and 3.

4.1 Introduction

Autosomal Recessive Juvenile Parkinson's (ARJP) disease arises from mutations in *parkin* (Kitada *et al.*, 1998). The parkin protein functions as an E3 ubiquitin protein ligase that acts to mediate the ubiquitination of a number of target proteins (Chung *et al.*, 2001; Imai *et al.*, 2001; Shimura *et al.*, 2000; Zhang *et al.*, 2000), to be subsequently degraded by the proteasome. The mutations in the *parkin* gene associated with ARJP are believed to lead to either a non-functioning protein or complete lack of protein. The predicted consequence of little or no parkin protein ubiquitin ligase activity should be the accumulation of the proteins that parkin that would normally target for proteasomal degradation. In turn, the abnormal elevated levels of these proteins may cause an ER stress-induced cell death response (Imai *et al.*, 2000; Imai *et al.*, 2001; Rao *et al.*, 2002). The role of *parkin* is clearly important in neuronal survival.

The *Drosophila parkin* mutant model of PD exhibits a premature loss of climbing and flying ability (Greene *et al.*, 2003). These mutants display a dropped-wing phenotype that is the result of premature apoptotic muscle degeneration induced by mitochondrial dysfunction. Other features of these flies are decreased mass and cell size along with decreased longevity. These phenotypes are not typically observed in humans (Pesah *et al.*, 2004). In addition, the male *parkin* mutant flies are sterile and surprisingly there is a distinct lack of dopaminergic degeneration in both male and female flies (Greene *et al.*, 2003; Pesah *et al.*, 2004). As the expression of human *parkin* in *Drosophila* can suppress these phenotypes, the similarity of the human and *Drosophila* form of *parkin* is evident (Greene *et al.*, 2003). The non-neuronal phenotypic consequences of parkin loss appear

to be much more severe in flies than those observed in human PD, which may suggest that *parkin* plays a greater role in non-neuronal *Drosophila*. To examine the effect of *parkin* expression at different developmental stages and tissues expression of *parkin* was directed to the developing eye and wings.

4.2 Materials and Methods

4.2.1 Fly stocks and culture

The *GMR-Gal4* flies (Freeman, 1996), *eyeless-Gal4*$^{3-8}$ (w[*];{w[+m*]=Gal4-ey.H}3-8) and *apterous-Gal4* (y^1 w^{1118}; P{GawB}ap^{md544}/CyO) (Calleja *et al.*, 1996) flies were obtained from the Bloomington Drosophila Stock Center at the University of Indiana, Bloomington. The *C5-Gal4* flies were a kind gift from G. Boulianne, University of Toronto (Gustafson and Boulianne, 1996). The *UAS-parkin*$^{2.2}$ transgenic flies were generated by standard injection techniques into w^{1118} embryos of the pUAST-*parkin* transgene (a *Bgl*II/*Xho*I fragment containing the *parkin* cDNA (SD01679), sub-cloned into the *pUAST* vector) such that the full-length *parkin* cDNA is located on the second chromosome. To drive expression of the transgene, *ap-Gal4* and *C5-Gal4* (for expression in the wing) and *ey-Gal4* and *GMR-Gal4* (for expression in the eye) females were crossed to w^{1118} (control), *UAS-parkin*$^{2.2}$ males. All flies were cultured on standard cornmeal/yeast/molasses/agar media at 25°C.

4.2.2 Wing mounts and wing length analysis

Flies of the genotypes 1) w^{1118};*C5-Gal4*; 2) w^{1118};*UAS-parkin*$^{2.2}$/+;*C5-Gal4*/+; 3) w^{1118};*ap-Gal4,* and 4) w^{1118};*UAS-parkin*$^{2.2}$*/ap-Gal4* were aged for at least one day but not more than five days, collected and then quick-frozen in an ethanol bath at –70°C. Flies were dissected in 95% ethanol so that the whole dorsal region was intact (wings and upper thorax). These were incubated in 1M NaOH for 20 minutes to remove excess tissue and then washed in 50:50 glycerol:ethanol and mounted in this medium. The tissue was arranged to look at the wings from an overhead or dorsal view and covered with a cover slip and allowed to dry for several hours before the cover slips were sealed with clear nail varnish. The left wing from three males and three females of each genotype were examined by light microscopy and photographed at 10X magnification. The photograph negatives of the wings were scanned and the wing lengths measured from the wing tip to the intersection of wing veins L4 and L5 using Image J software. Wing lengths differences were analysed statistically by a two way ANOVA using Prism 4.02 software.

4.2.3 Scanning electron microscopy of the *Drosophila* eye

Flies of each genotype 1) w^{1118};*GMR-Gal4*, 2) w^{1118};*UAS-parkin*$^{2.2}$*/GMR-Gal4*, 3) w^{1118};*ey-Gal4*/+, and 4) w^{1118};*UAS-parkin*$^{2.2}$*/ey-Gal4* were collected within 24 hours of eclosion, aged for one day and frozen in a -70°C ethanol bath. Whole flies were mounted, desiccated overnight, coated in gold and then photographed at 150X magnification with a Hitachi S-570 SEM as per standard methods. For each genotype at least six male flies were observed.

4.3 Results

The *Drosophila* eye is a highly regular structure, composed of some 800 individual units, such that any disruption of the structure is markedly apparent. As previously shown, expression of *parkin* in the developing eye disc, via the *GMR-Gal4* transgene, does not alter the normal phenotype of either male or female adult eye (Figure 4-1) (Haywood and Staveley, 2004). This result is not unexpected as parkin functions as a ubiquitin ligase one would expect that it would only mediate the ubiquitination of its specific target proteins and not mediate the promiscuous ubiquitination of proteins simply because those protein are in excess. Actually, parkin can target itself for ubiquitination in a self-regulating loop to prevent too much of the protein from being in the cell (Imai *et al.*, 2000; Zhang *et al.*, 2000). The expression of *parkin* under the control of the *ey-Gal4* transgene, shows no alteration of the normal architecture and configuration of the adult eye (Figure 4-2). However both male and female flies that express *parkin* have eyes that appear slightly smaller than controls (Figure 4-2).

Expression of parkin in the developing eye has no effect on the gross morphology of the eye nor does there appear to be any effect on climbing or longevity when *parkin* is ectopically expressed in the dopaminergic neurons (Haywood and Staveley, 2004). To assess the effect of ectopic expression of *parkin* in other tissue types *parkin* expression was directed to the developing wing under the control of the *C5-Gal4* transgene. No variation in the normal architecture of the adult wing was observed with *parkin*

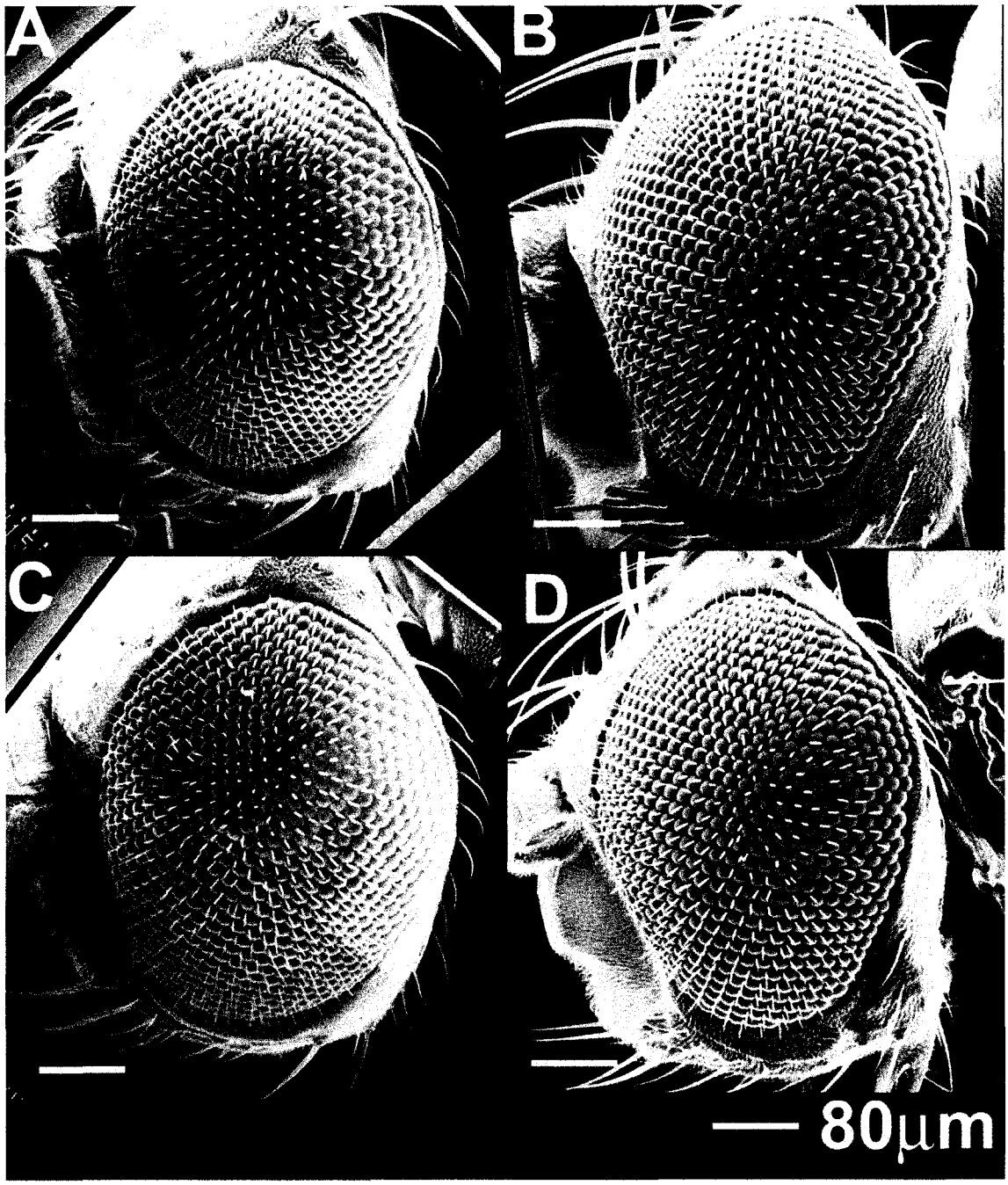


Figure 4-1: Expression of *parkin* in the morphogenetic furrows does not affect the adult eye.
Scanning electron microscopy of one day old fly eyes that express *parkin* appear the same as control fly eyes. Panels A and B are male fly eyes and panels C and D are female fly eyes. The genotypes are (A, C) w^{1118};*GMR-Gal4/+*, (B, D) w^{1118};*UAS-parkin*$^{2.2}$*/GMR-Gal4*.

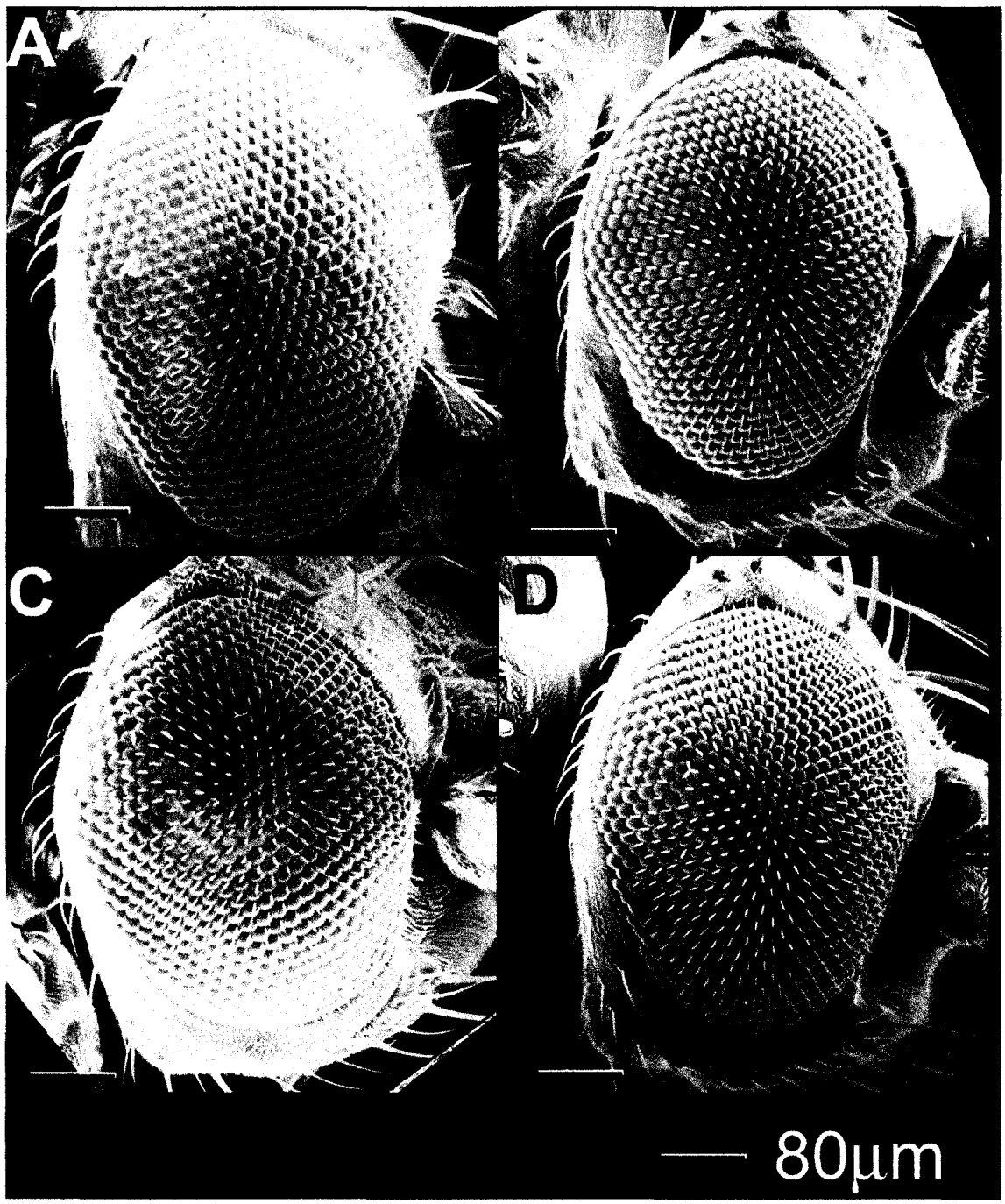
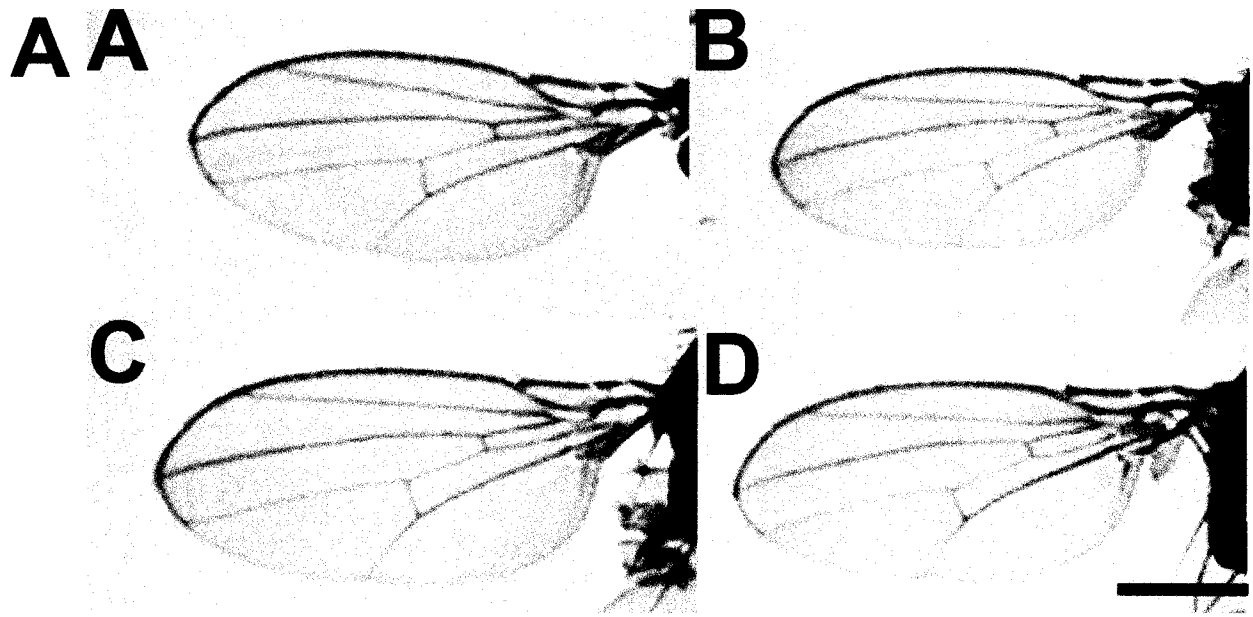


Figure 4-2: Expression of *parkin* early in the developing eye results in slightly smaller adult eyes.
Scanning electron microscopy of adult male *Drosophila* eyes that express *parkin* appear slightly smaller than control. Panels A,B are male fly eyes and panels C, D are female fly eyes. The genotypes are (A, C) w^{1118};*ey-Gal4*, (B, D) w^{1118};*UAS-parkin*$^{2.2}$/*ey-Gal4*.

expression (Figure 4-3 panel A), nor was there a change in the average wing length of male or female flies (Figure 4-3 panel B, Table 4-1). Conversely, when *parkin* was expressed in the presumptive dorsal region of the wing imaginal disc via the *ap-Gal4* transgene, a reduction in the wing size was observed for both the male and female flies that was not accompanied by gross morphological variations in the layout and structure of the wing (Figure 4-4 panel A). A 17% reduction in the wing length of the adult male flies and a 7% reduction in the wing length of the female flies was measured (Table 4-1, Figure 4-4 panel B).

4.4 Discussion

The directed expression of *parkin* in the differentiating eye disc and the dopamine producing neurons has been shown to have no obvious deleterious effects (Haywood and Staveley, 2004). However, differences in wing length when *parkin* is expressed in the dorsal wing disc via the *ap-Gal4* transgene cannot be discounted. Other than this there is no evidence to suggest that the expression of *parkin* results in any other adverse effects. The phenomenon revealed by the genetic combination of *ap-Gal4* and *UAS-parkin* may provide valuable insight in the biological role of parkin. The *ap-Gal4/UAS-parkin* phenotype can also be exploited in order to genetically screen for modifiers of the *parkin* gene.



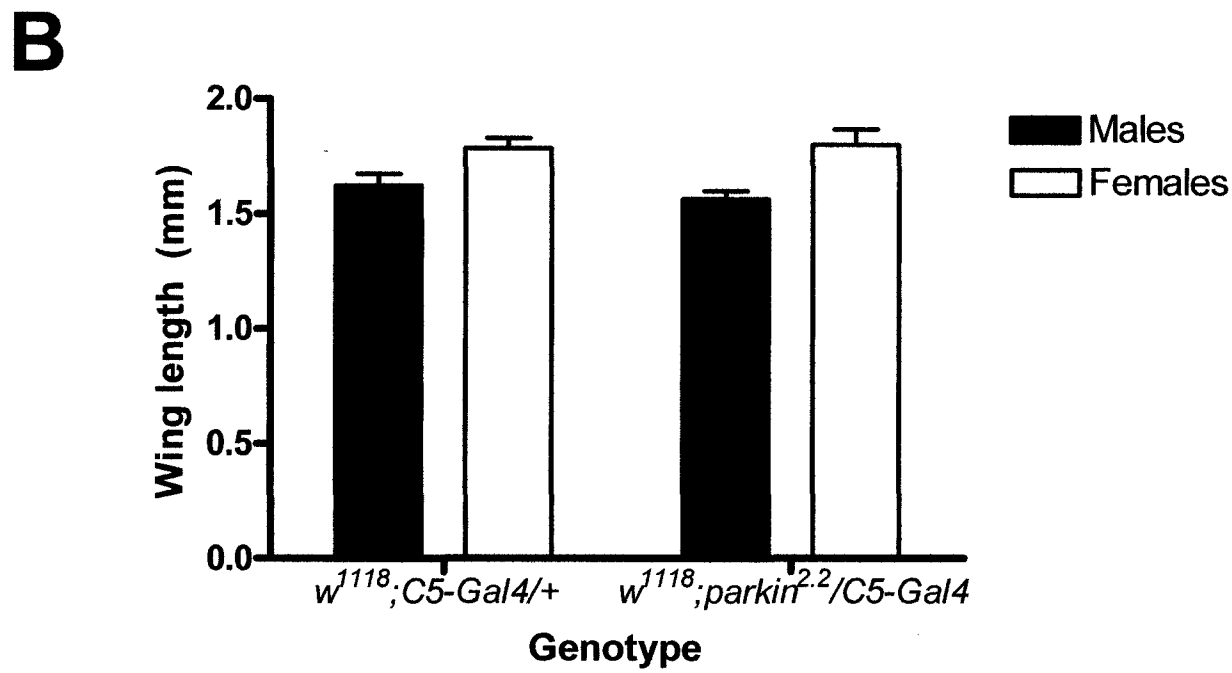


Figure 4-3: Expression of parkin in the developing wing does not alter the overall structure of the wing or wing length.
Panel A: Left wings of two-day-old adult *Drosophila* were removed, mounted and photographed. A, B are male and C, D are female. The genotypes are (A, C) w^{1118};*C5-Gal4*; (B, D) w^{1118};*UAS-parkin*$^{2.2}$*/C5-Gal4*. Scale bar indicates 0.5 mm. Panel B: The average wing length of three wings of each genotype and gender shows there is no significant difference in the wing lengths.

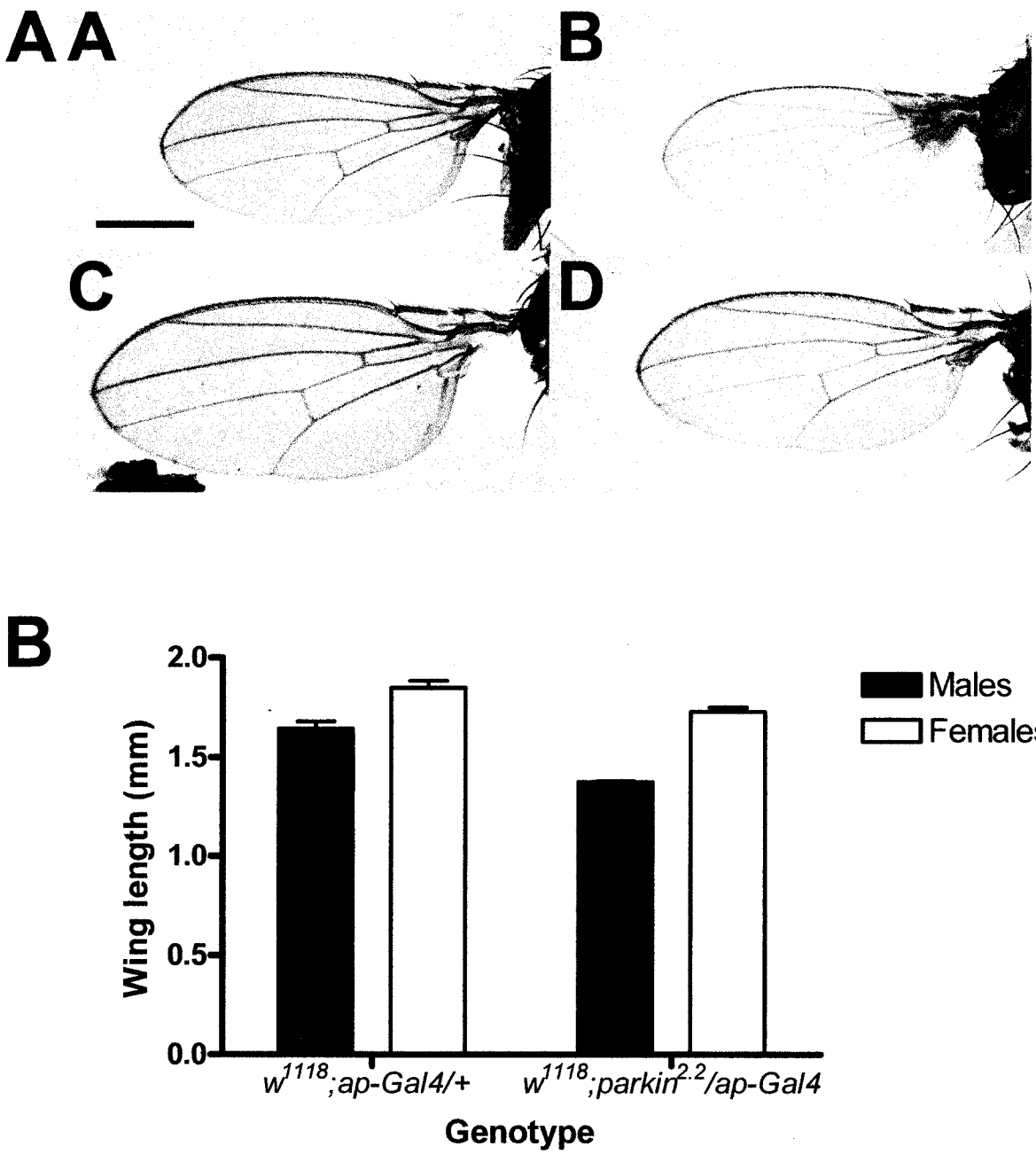


Figure 4-4: Expression of *parkin* in the dorsal wing margin reduces the size of the wing.

Panel A: Left wings of two-day-old adult *Drosophila* were removed, mounted and photographed. A, B are male and C, D are female. The genotypes are (A, C) w^{1118};*ap-Gal4*; (B, D) w^{1118}; *UAS-parkin*$^{2.2}$*/ap-Gal4*. Scale bar indicates 0.5 mm. Panel B: The average wing length of three wings of each genotype and gender shows there is a significant difference in the wing lengths of flies that express *parkin* compared with control.

Table 4-1: Wing length of flies that express *parkin* in the developing wing pouch via the *C5-Gal4* wing transgene

Genotype	Males			Females		
	Length	SEM	N	Length	SEM	N
w^{1118};*C5-Gal4/+*	1.623	0.053	3	1.785	0.045	3
w^{1118};$parkin^{2.2}$/*C5-Gal4*	1.565	0.034	3	1.801	0.066	3

Table 4-2: Wing length of flies that express *parkin* in the presumptive dorsal region of the wing imaginal disc via the *ap-Gal4* transgene.

Genotype	Males			Females		
	Length	SEM	N	Length	SEM	N
w^{1118};*ap-Gal4/+*	1.645	0.035	3	1.848	0.038	3
w^{1118};$parkin^{2.2}$/*ap-Gal4*	1.377	0.004	3	1.730	0.023	3

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Chapter 5: A method to generate truncated *parkin* transgene

This chapter has not been published and is supplemental to Chapters 2 and 3.

5.1 Introduction

In order to understand the function of *parkin* and its target proteins, an alternative genetic model of PD is desirable than the ones currently available. The parkin protein can be divided into two main domains; the Ubiquitin-like Domain/Unique Parkin Domain (UBL/UPD) region, which binds target proteins, and the RING-box (RING1-IBR-RING2) region, which recruits specific E2 ubiquitin conjugating enzymes that act to attach ubiquitin monomers to the target protein (Tanaka *et al.*, 2001). Expression of a truncated form of *parkin* that includes only the E2 binding domain could act in a dominant negative fashion by binding the specific E2 enzymes that full-length parkin would normally bind, which would prevent normal parkin from mediating the attachment of ubiquitin molecules to proteins that need to be destroyed. This in turn could lead to a build of toxic proteins. Over-expression of *ringbox (RB) parkin* will be used to determine if such inhibitory effects exist and if truncation mutations in *parkin* lead to stress induced apoptosis.

5.2 Materials and Methods

5.2.1 Generation of plasmid with a truncated form of *parkin*

Utilizing the *parkin* cDNA (SD01679) (Haywood and Staveley, 2004; Stapleton *et al.*, 2002) from Research Genetics as a template the RING-BOX region of *parkin* was generated by PCR. The primers were designed to generate a *Bgl*II restriction enzyme site at the 5' end and to adjust the sequence to generate a *Drosophila* Kozak consensus

translation initiation sequence (C/A)AA(A/C)ATG) (Cavener, 1987). The sequence selected for redesign is located just prior to the segment of the gene encoding the RING BOX. This particular sequence was:

GAA AGT CTG GAG GTG GCC TGC GTG GAC and the changes made were:

GAA [redacted]T CT[redacted]A[redacted]T[redacted] GCC TGC GTG GAC to generate the *dparkCtermBglII* primer that included a stretch of 12 nucleotides of perfect match towards the 3' end of the primer, to facilitate specific annealing. The commonly used *PM001* primer (CGT TAG AAC GCG GCT ACA AT) was selected for the 3' end, which encompasses the remainder of the multiple cloning site of the vector for flexibilty of cloning. The PCR cycle was designed to create maximal product with little non-specific product, several reactions were set up with varying salt and template concentrations. The best result was obtained with a final concentration of 1x PCR buffer, 2.75 mM $MgCl_2$, 0.1 mM dGTP, 0.1 mM dTTP, 0.1 mM dATP, 0.1 mM dCTP, 0.1 μM *PM001* primer, 0.1 μM *dparkCtermBglII* primer, 10 units of *Taq* DNA polymerase, 0.01 μg of template and the cycle parameters of 1x 95°C for 3 minutes, then 20 x 95°C for 1 minute, 50°C for 30 seconds, 72°C for 1.5 minutes followed by a final extension time of 4 minutes at 72°C before being cooled to 5°C. The resulting PCR reactions were run on a 1% agarose gel with a standard 1 kb ladder to confirm that the resulting product was of the expected size. The single resulting band at 900 base pairs was cut from the gel and the DNA extracted using the Qiagen Gel Extraction kit. A 1 μl aliquot of the extracted sample was electrophoresed on a 1% agarose gel to confirm the product was retrieved. The remaining sample was digested with *Bgl*II/*Xho*I, subjected to a phenol/chloroform extraction to remove proteins and ligated into a *Bgl*II/*Xho*I cut pUAST vector with DNA

ligase enzyme in a slow melt bath (cup of ice left at room temperature) overnight. This ligated vector was transformed into *E. coli* and small-scale preparations checked for correct insert orientation before a large-scale preparation was performed. The insert will then be sequenced to check if the correct portion of the *parkin* gene has been amplified and cloned.

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Chapter 6: Dopa decarboxylase *(Ddc)-Gal4* dramatically reduces life span

A version of this chapter has been published in Drosophila Information Service (Haywood *et al.*, 2002).

6.1 Introduction

The UAS/Gal4 ectopic expression system (Brand and Perrimon, 1993; Phelps and Brand, 1998) has become an extremely useful approach for the study of specific genes in *Drosophila melanogaster*. This expression system relies upon Gal4 to bind the upstream activation sequence (UAS) in order to activate transcription of the target gene. A variety of transgenic *Drosophila* lines are readily available that express *Gal4* in specific tissues or cell types. Our laboratory is interested in models of neurodegenerative diseases and we have initiated work with the *Dopa decarboxylase (Ddc)-Gal4* transgenic expression lines to model Parkinson's disease in *Drosophila* by expressing genes in dopaminergic neurons.

The first *Drosophila melanogaster* model of Parkinson's disease was developed by the generation of transgenic lines bearing wild-type and mutant forms of the human *α-synuclein* gene cloned downstream of the UAS yeast promoter (Feany and Bender, 2000). There is no apparent *Drosophila* homologue of *α-synuclein* but expression of the human α-synuclein protein in the *Drosophila* nervous system recapitulated some features of Parkinson's disease. Expression of α-synuclein in the dopaminergic neurons (Feany and Bender, 2000) was driven by a transgene comprised of the *Ddc* gene promoter cloned upstream of *Gal4*. Originally, this transgene was developed to examine a *Drosophila* model of cocaine addiction (Li *et al.*, 2000). The Parkinsonian flies, apparently normal at a young age, demonstrated a premature loss of locomotor (climbing) ability, loss of dopaminergic neurons and accumulation of α-synuclein-containing inclusions. In

addition, *GMR-Gal4* driven expression in the developing eye resulted in age-dependent retinal neurodegeneration. Subsequent treatment of the transgenic α-synuclein expressing flies with a number of pharmacological agents such as the dopamine precursor L-DOPA, dopamine receptor agonists (bromocriptine, pergolide and SK&F38393) and the anticholinergic atropine, all restored or partially restored the age-dependent loss ofclimbing ability (Pendleton *et al.*, 2002). Further, this model has been used to examine the suppression of the α-synuclein toxicity by the molecular chaperone, HSP70 (Auluck *et al.*, 2002).

A good understanding of the effects of *Ddc-Gal4* expression in *Drosophila melanogaster* is essential to properly interpreting this model system. As a prelude to our exploration of models of Parkinson's disease in *Drosophila*, we began to investigate the biological properties of the *Ddc-Gal4* driver lines. We have shown that *Ddc-Gal4* causes reduced viability and is therefore not inactive in *D. melanogaster*.

6.2 Materials and Methods

6.2.1 Fly stocks and culture

Ddc-Gal4$^{4.3D}$ and *Ddc-Gal4*$^{4.36}$ flies (Li *et al.*, 2000) were obtained from Jay Hirsh at the Department of Biology, University of Virginia and w^{1118} flies were obtained from Dr. Howard Lipshitz at the Hospital for Sick Children in Toronto. To obtain heterozygotes, *Ddc-Gal4* homozygous males were crossed to w^{1118} females. All flies were cultured on standard cornmeal/yeast/molasses/agar media at 25°C.

6.2.2 Aging analysis

Adult males were aged in small groups upon standard media at 25°C and scored for viability every two to three days as described previously (Staveley *et al.*, 1990). The number of individuals aged were as follows: *Ddc-Gal4*$^{4.3D}$ homozygotes n = 102; *Ddc-Gal4*$^{4.36}$ homozygotes n = 133; *Ddc-Gal4*$^{4.3D}$ heterozygotes n = 280; *Ddc-Gal4*$^{4.36}$ heterozygotes n = 119; w^{1118} n = 83).

6.2.3 Locomotion assay

The flies were assayed for their ability to climb in a manner similar to that described by Feany and Bender (2000). Every 4 to 5 days, 10 male flies of a cohort of aged flies were assayed for their ability to climb to the top of a vial within a period of 18 seconds. Twenty trials were carried out for each time point.

6.3 Results and Discussion

An investigation of the baseline biological consequences of *Gal4* expression as directed by the *Ddc* promoter is essential to our studies of Parkinson's disease models in *Drosophila*. We began by crossing the *Ddc-Gal4*$^{4.3D}$ and *Ddc-Gal4*$^{4.36}$ driver lines to w^{1118} to conduct climbing assays. It quickly became apparent that the stocks of both *Ddc-Gal4* insertion lines required extra care to maintain and that the flies were apparently short-lived in both cases. As a result we decided to conduct longevity trials.

Homozygotes of both insertions of the *Ddc-Gal4* transgene display a greatly reduced life span (Figure 6-1). For example only 43% and 44% of homozygous males for *Ddc-Gal4*$^{4.3D}$ and *Ddc-Gal4*$^{4.36}$ respectively were alive by Day 6 after eclosion. While 50% of *Ddc-Gal4*$^{4.3D}$ male heterozygotes survive past the age of 88 days and *Ddc-Gal4*$^{4.36}$ male heterozygotes past 70 days of age. We assigned w^{1118} as the control strain in these experiments, 50% of which survived between 58 and 60 days. The shorter median life span of the control may reflect an insufficiency of the w^{1118} stock rather than an increase in viability of the heterozygotes. It is important to note that the very similar longevity profiles of the two independent insertions of *Ddc-Gal4* suggest that the reduction in life span is due to the expression of the transgene and not the site of insertion.

The reduced viability of the *Ddc-Gal4* homozygotes forced us to examine the locomotor activity of the *Ddc-Gal4* heterozygotes (Figure 6-2). However, the *Ddc-Gal4*$^{4.3D}$ and *Ddc-Gal4*$^{4.36}$ heterozygotes retain their ability to climb with age in a manner similar to the w^{1118} controls. Due to greatly reduced viability, the locomotion of homozygotes was not measured.

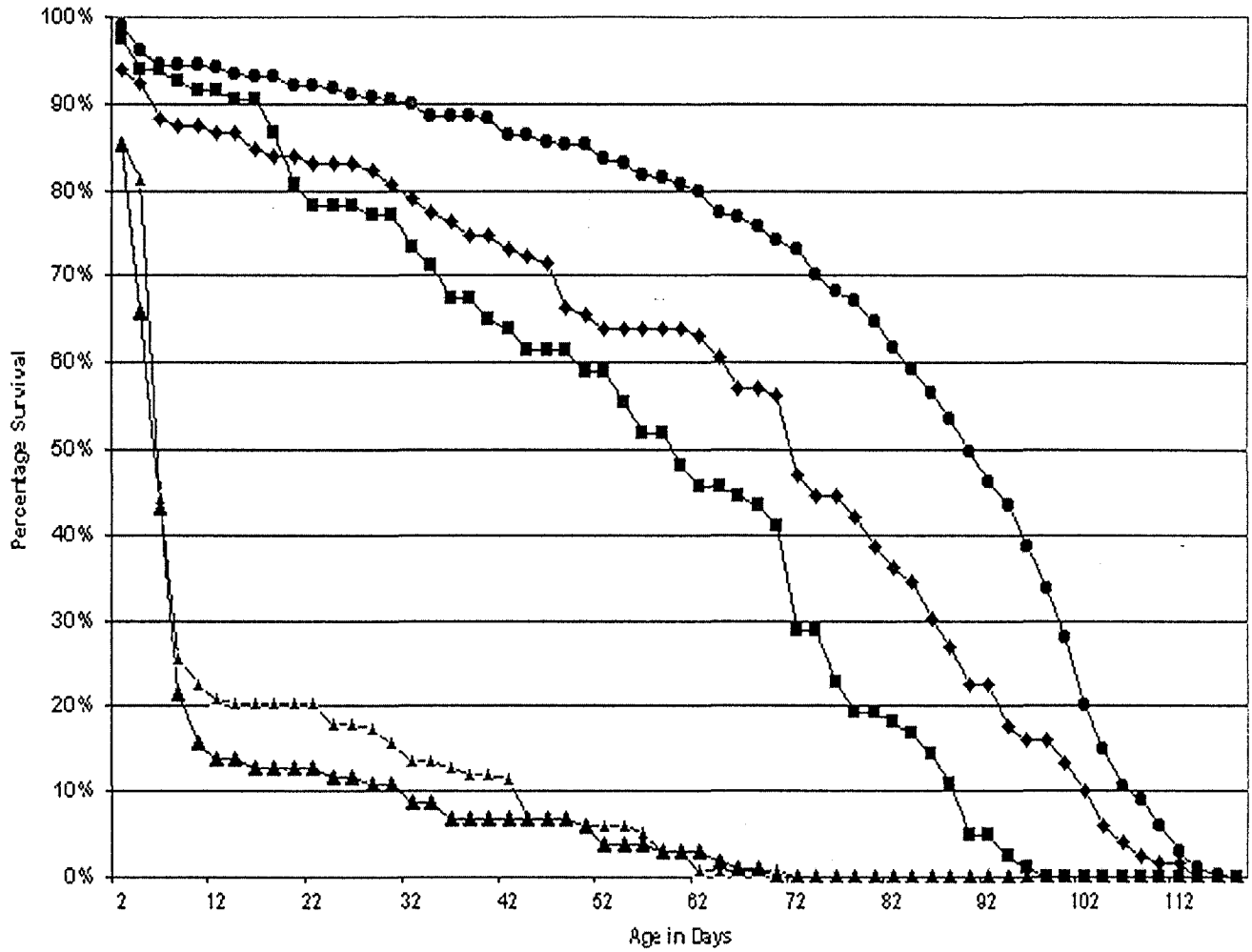

Figure 6-1: *Ddc-Gal4* reduces life span.
Male homozygotes of the independent insertions of the *Ddc-Gal4* transgene, *Ddc-Gal4* $^{4.3D}$ (large triangles) and *Ddc-Gal4* $^{4.36}$ (small triangles) both display a greatly reduced life span. The heterozygotes of *Ddc-Gal4* $^{4.3D}$ (solid circles) and *Ddc-Gal4* $^{4.36}$ (solid diamonds), however display a normal, if not extended life span when compared to the control w^{1118} (solid squares) individuals.

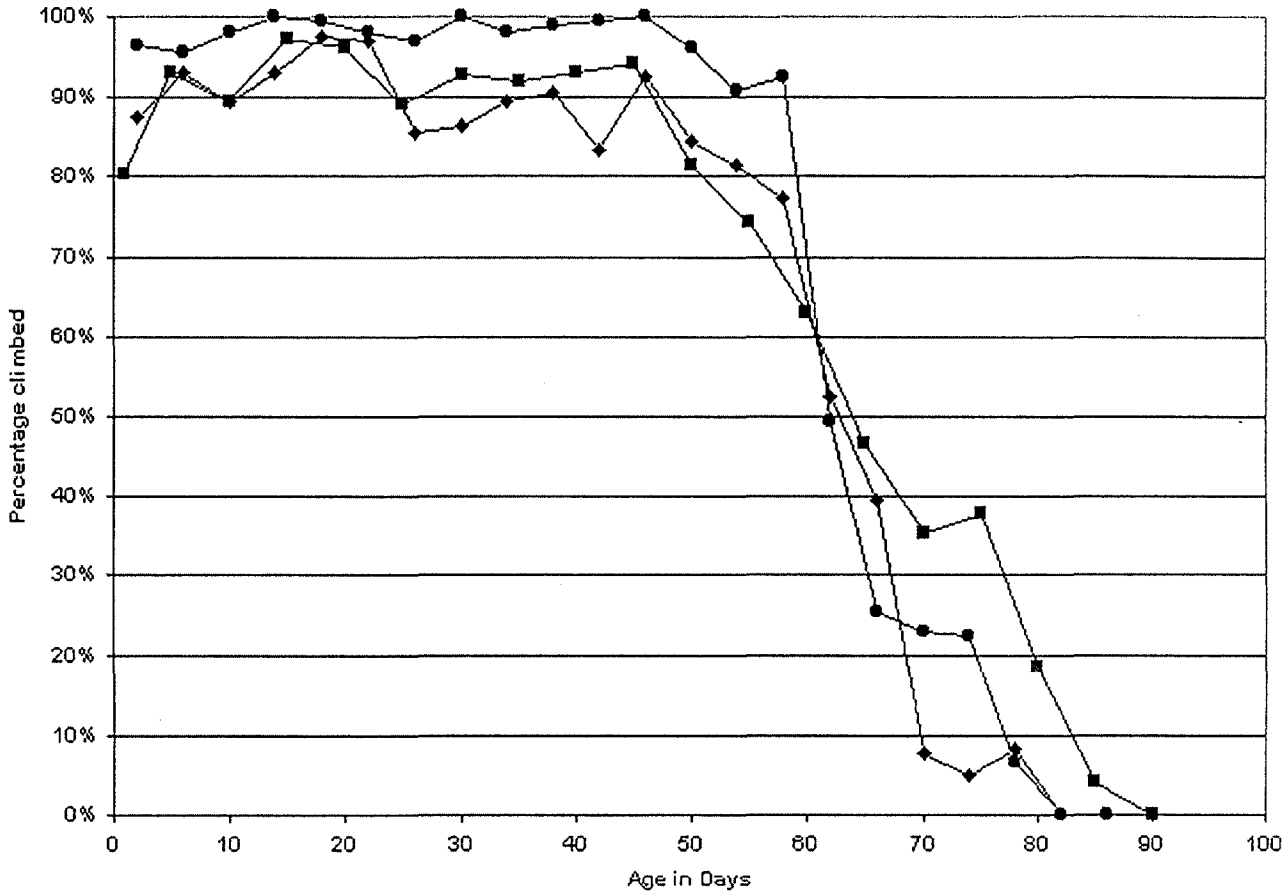

Figure 6-2: *Ddc-Gal4* heterozygotes have normal locomotor (climbing) activity.
The heterozygotes of *Ddc-Gal4*$^{4.3D}$ (solid circles) and *Ddc-Gal4*$^{4.36}$ (solid diamonds) display a normal level of climbing ability when compared to the control w^{1118} (solid squares) flies. The locomotion of homozygotes was not measured due to poor viability.

The UAS/Gal4 ectopic expression system has made it possible to express genes and test the effects of overexpression in a variety of tissues. The *Ddc-Gal4* driver has been used for expression of transgenes in the dopaminergic neurons including expressing the *α-synuclein* gene to model Parkinson's disease (Feany and Bender, 2000). Although no UAS (cggagtactgtcctcc) promoter sequences are naturally found in *D. melanogaster* (Berkeley Drosophila Genome Project, pers. comm.), our laboratory has demonstrated that expression of *Gal4* in the eye with the *GMR-Gal4* transgene leads to increased levels of apoptosis and morphological defects (Kramer and Staveley, 2003). Although the mechanism by which Gal4 induces cell death is unclear, death of the dopaminergic neurons could certainly result in premature lethality in these flies.

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Chapter 7: Analysis of apoptosis in *Drosophila melanogaster* with multiple inserts of the *Ddc-Gal4* transgene

This chapter is supplemental to Chapter 6 and is part of a paper in preparation.

7.1 Introduction

The *UAS/Gal4* ectopic expression system (Brand and Perrimon, 1993; Phelps and Brand, 1998) is widely used in the study of the effects of directed ectopic expression of specific genes in *Drosophila melanogaster*. *Gal4* expression is believed to be benign as there are no upstream activation sequence (UAS) sites or sequences in the *Drosophila* genome. Contrary to this belief expression of *Gal4* appears to have negative consequences particularly when multiple copies of the transgene are present (Kramer and Staveley, 2003, Sheppard, Haywood, Saunders and Staveley, in preparation). Expression of *Gal4* in the dopaminergic neurons leads to a shorter lifespan (Haywood *et al.*, 2002) and expression of *Gal4* in the developing eye causes a rough eye phenotype and appears to lead to excessive cell death (Kramer and Staveley, 2003).

As I use the *Ddc-Gal4 Drosophila* line, which expresses *Gal4* in the dopaminergic neurons, a further understanding of the effects of *Ddc-Gal4* expression was required in order to accurately interpret data generated from the progeny of these flies. Here, I specifically looked in the third instar larval brain to see where *Gal4* is expressed through directed expression of Green Fluorescent Protein (GFP). Third instar brains of flies that have multiple copies of the *Ddc-Gal4* transgene were then analysed for their level of apoptosis using the vital dye Acridine Orange.

7.2 Materials and Methods

7.2.1 Fly stocks and culture

UAS-GFP lines were obtained from the Bloomington Stock Center. *Ddc-Gal4* $^{4.3D}$ and *Ddc-Gal4* $^{4.36}$ flies (Li *et al.*, 2000) were obtained from Dr.Jay Hirsh at the Department of Biology, University of Virginia and w^{1118} line was obtained from Dr. Howard Lipshitz at the Hospital for Sick Children in Toronto. *Ddc-Gal4*$^{4.36}$ and *Ddc-Gal4*$^{4.3D}$ transgenic lines were combined using standard genetic techniques as follows. They were stably balanced onto a multiple balanced line w^{1118}; *L/CyO; Ki ftz/TM3, Sb e* or w^{1118}; *L/CyO; Ki ftz*^{20}*/TM6B, Tb Hu e* by an initial cross and a then backcross to obtain lines that were: w^{1118}; *L/CyO; Ddc-Gal4*^{36} */TM6B* and w^{1118}; *Ddc-Gal4*^{3D} */CyO; Ki ftz/TM6B.* These were then crossed together to obtain a stable line that had at least one insert of *Ddc-Gal4* transgene on both the second and third chromosome. Initially these lines were very weak and were difficult to maintain however over time they became stronger and more viable, possibly due to selection of beneficial quantitative modifiers. All flies were cultured on standard cornmeal/yeast/molasses/agar media at 25°C. To obtain the various genotype combinations in the adults, specific parents were crossed such that the chromosome could be followed to ensure that the offspring had the correct genotype. For larval analysis a separate mating scheme was developed to ensure that 100% of offspring had the appropriate genotype for subsequent brain analysis. These mating schemes are shown in Table 7.1.

Table 7-1: Mating scheme to obtain larval *Drosophila* with multiple copies of the *Ddc-Gal4* transgene

Male / Female	w^{1118};+/+;+/+	w^{1118}; *Ddc-Gal4/Ddc-Gal4;* +/+
w^{1118}; +/+;+/+	w^{1118}; +/+;+/+	
w^{1118}; *Ddc-Gal4/Ddc-Gal4; Ddc-Gal4/Ddc-Gal4*		w^{1118}; *Ddc-Gal4/Ddc-Gal4; Ddc-Gal4/+*

7.2.2 Acridine Orange staining of *Drosophila* larval brains

This protocol was adapted from Bonini (2000). Third instar larvae that were crawling up the sides of vials were dissected in phosphate buffered saline (PBS) with 0.1% Tween20 (PBT) to prevent the carcasses from sticking to the plastic of the 1.5 ml microfuge tubes and pipette tips. The carcasses were incubated in 0.5μg/ml Acridine Orange solution for 5 minutes. The carcasses were rinsed in PBT and further dissected before being wet-mounted in PBS (Bonini, 2000). The brains were visualized by fluorescent optics and photographed.

7.2.3 Visualisation of GFP in *Drosophila* larval brains

Ddc-Gal4^{36} homozygous females were crossed to *UAS-GFP* males and raised at 25°C on standard cornmeal/yeast/agar. Crawling third instar larvae were dissected in PBT and wet mounted in PBS. The brains were visualized by fluorescent optics and photographed.

7.3 Results and Discussion

The double *Ddc-Gal4* lines produce progeny that had 2, 3 or 4 copies of the insert, notably homozygotes were viable albeit weak. Flies homozygous for the *Ddc-Gal4* transgene on the second chromosome that had at least one more copy of the transgene on the third chromosome were sterile, which combined with the fact that I did not have a larval marker for the second chromosome made it impossible to obtain larva with four copies of *Ddc-Gal4* transgene. Other combinations were attempted unsuccessfully.

To determine the expression pattern of *Gal4* by the *Ddc-Gal4* transgenes GFP expression was directed under the control of the UAS via the *Ddc-Gal4*^{36} transgene and third instar larval brains were dissected and examined. The predominant staining pattern is observed in the base of the larval brain in two distinct spots on each side of the larval brain (Figure 7-1A and B). Based on an atlas of the *Drosophila* brain, this region appears to be the noduli, which are two ball-like neuropils that receive connections from protocerebral bridge neurons that, en route, provide collaterals to the staves of the fore brain (Hansen, 1995-2000). There is further staining in the central nervous system (CNS) in distinct places that appear to be the cell bodies of serotonergic nerves (Figure 7-1A). This staining was not in the expected regions of the brain that are annotated in Figure 7-1C.

Previous studies have shown that overexpression of *Gal4* can cause apoptosis and developmental defects when expressed in the eye imaginal disc (Kramer and Staveley, 2003, Kramer, Haywood, Sheppard and Staveley submitted). To determine if overexpression of *Gal4* in the dopaminergic neurons causes apoptosis we expressed *Gal4* through three copies of the *Ddc-Gal4* transgene and examined the brains of crawling third instar larvae for apoptosis. Apoptotic cells were observed ubiquitously in the lobes of both the control and triple insert *Ddc-Gal4* flies (Figure 7-2). However excessive apoptosis was observed in the two brain lobes of the triple insert *Ddc-Gal4* flies compared with the control fly brain with no *Ddc-Gal4* transgene (Figure 7-2).

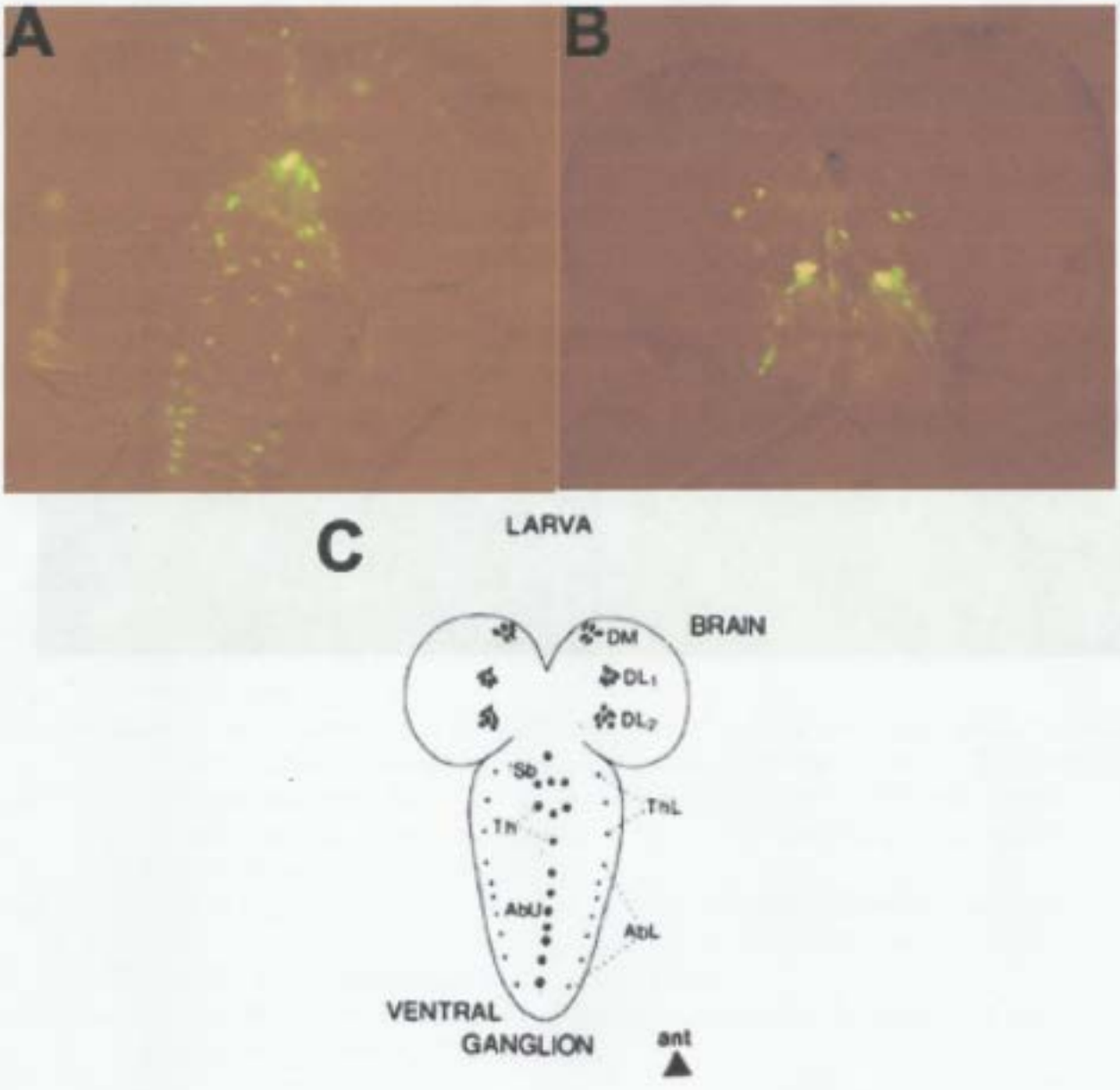

Figure 7-1: Dopaminergic specific expression of GFP shows staining in the dopaminergic neurons and seroteroneurgic neurons.
The brain and central nervous system (CNS) of w^{1118};*UAS-GFP*/+; *Ddc-Gal4*^{36}/+ third instar larvae visualized by fluorescent optics show staining in distinct regions of the brain and CNS. Both figures A and B are the same genotype but from a different angle. C - a schematic diagram of the dopaminergic neurons in a larval brain. Six clusters in the brain and several in the ventral ganglion. DM - dorsoposterior region of the superior protocerebrum near midline, DL1 – Dorsal region fo brain lobes, more latereal and more posterior than DM, DL2 – Posteromedial region of brain lobes, Sb – Subesophageal ganglion, posterior. Th – Prothoracic segment, medials, ThL – Thoracic segments, laterals, AbU – Abdominal segments, medials, AbL – abdominal segments, laterals.

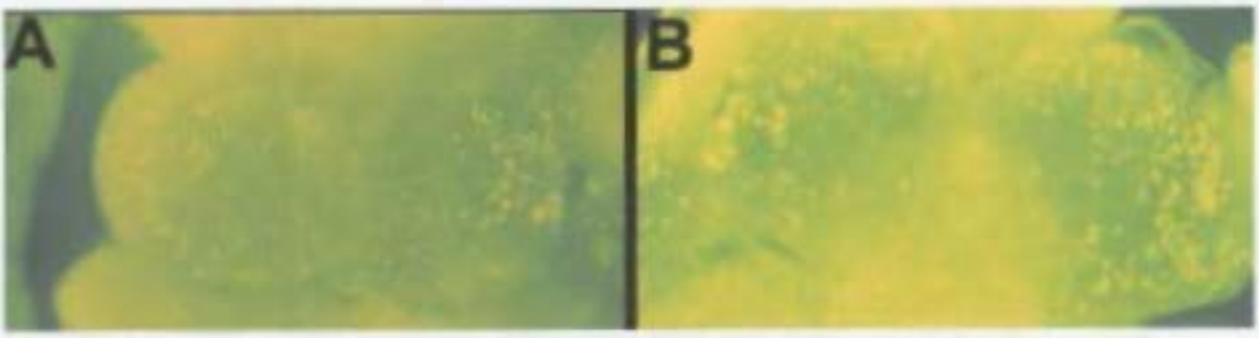

Figure 7-2: Expression from multiple copies of a transgene of *Gal4* in dopaminergic neurons shows increased apoptosis in the brain lobes.
The brain and CNS was dissected, incubated in Acridine Orange and visualized by fluorescent optics. An increased number of Acridine Orange reactive cells, which stain cells undergoing apoptosis are in panel B. The genotypes are A – w^{1118}, B- w^{1118}; *Ddc-Gal4/Ddc-Gal4; Ddc-Gal4/+*.

To reconcile the ubiquitous staining for apoptotic cells we speculate that the expression of Gal4 in the dopaminergic neurons has already caused the death of these neurons and downstream of their demise other cells, that they would normally signal to keep alive, are dying.

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Chapter 8: Gal4-induced cell death is suppressed by *parkin*

A version of this chapter has been submitted to BMC Biology (Haywood, Kramer, Sheppard and Staveley, 2006).

8.1 Introduction

Drosophila melanogaster have emerged as one of the most successful models for the analysis of human disease genes in neurological disorders (Bier, 2005). The UAS/Gal4 ectopic expression system is widely used in *D. melanogaster* to carry out studies of gene function and regulation (Brand and Perrimon, 1993; Phelps and Brand, 1998). This bipartite expression system utilizes the yeast transcription factor Gal4 and its target sequence, UAS (Upstream Activation Sequence), to which Gal4 binds in order to activate gene transcription. *Gal4* can be expressed under the control of *Drosophila*-specific promoters with little effect on the organism. However, in certain tissues, expression of *Gal4* can have adverse effects (Kramer and Staveley, 2003). For example, expression of *Gal4* in the developing eye using the *glass multiple reporter* (*GMR*)-*Gal4* transgene leads to a disorganised ommatidial array in the adult. This phenotype had been previously described (Freeman, 1996; Helms *et al.*, 1999; Hiesinger *et al.*, 1999; White and Jarman, 2000), however, no biological basis had been determined. As neurodegenerative diseases are often characterised by the accumulation of toxic proteins (Taylor *et al.*, 2002), the ectopic expression of *Gal4* could provide a system to examine components that could act to counter toxic protein accumulation.

PD is a highly prevalent neurodegenerative disease with symptoms that include stationary muscle tremors, difficulty initiating movement and muscle rigidity (Spacey and Wood, 1999). Loss of dopamine and dopamine-producing neurons is at the root of these symptoms. The loss of dopaminergic neurons, likely apoptotic in nature, may be activated through the accumulation of toxic proteins leading to stress of the endoplasmic

reticulum (ER) (Takahashi *et al.*, 2003). Inherited forms of PD account for approximately 5-10% of all PD patients (Mizuno *et al.*, 2001) and many of these genes are linked to the ubiquitin/proteasome protein degradation system (UPS) (Betarbet *et al.*, 2005). The accumulation of toxic proteins due to the failure of the ubiquitin-dependent process of protein degradation has been proposed as a major factor in the destruction of neurons in sporadic and familial PD (Cookson, 2005; McNaught and Jenner, 2001; McNaught *et al.*, 2001). Mutations in the *parkin* gene are the leading cause of early-onset PD accounting for 49% of familial and 19% of sporadic cases with a mean age of onset less than 45 years old (Kruger, 2004; Lucking *et al.*, 2000). The parkin protein functions as an E3 ubiquitin ligase (Shimura *et al.*, 2000), which mediates ubiquitination of specific target proteins (Chung *et al.*, 2001; Imai *et al.*, 2001; Shimura *et al.*, 2000; Zhang *et al.*, 2000). Ubiquitin-tagged proteins are subsequently digested by the proteasome. Recently, we have shown that *parkin* expression in *Drosophila* prevents the toxic effects of both wild type and mutant human α-synuclein (Haywood and Staveley, 2004; Haywood and Staveley, 2006). The role of toxic protein accumulation in PD may become clear as more targets of parkin ubiquitination are identified.

To address concerns about the effects of *Gal4* expression, I showed that the rough eye phenotype caused by *GMR-Gal4* is largely a result of Gal4-induced apoptosis during development and can be suppressed by inhibition of caspase activity. Furthermore, I addressed the possibility that *parkin* expression could suppress the consequences of high levels of Gal4, in the developing eye. I found that parkin can suppress developmental defects and apoptosis caused by Gal4. The ability of *parkin* to counter the toxicity of

exogenous and endogenous proteins may provide great insight into our understanding of neurodegenerative disease.

8.2 Materials and Methods

8.2.1 Fly stocks and culture

The *GMR-Gal4* (Freeman, 1996) and *UAS-p35* flies were obtained from the Bloomington Drosophila Stock Center, Indiana University at Bloomington. The *GMR-DIAP2* fly line was obtained from Dr. John Nambu (Wing *et al.*, 1998). *UAS-parkin*$^{2.1}$ flies were generated as previously described (Haywood and Staveley, 2004). Dr. Howard Lipshitz provided the w^{1118} flies. Stable double transgenic lines *GMR-Gal4/GMR-Gal4*; *UAS-parkin*$^{2.1}$*/UAS-parkin*$^{2.1}$ and *GMR-Gal4/GMR-Gal4;GMR-DIAP2/+* genotypes were generated using standard techniques. All flies were cultured on standard cornmeal/yeast/molasses/agar media at 25°C.

8.2.2 Scanning electron microscopy of the *Drosophila* eye

Flies were collected and aged for two days after eclosion before being frozen at -70°C. Whole flies were mounted, desiccated overnight and coated in gold before photography at 150X magnification with a Hitachi S-570 scanning electron microscope as previously described (Kramer and Staveley, 2003). For each condition at least six flies were analysed.

8.2.3 Histological examination of *Drosophila* adult retinas

Nine adult flies of each genotype were collected two days after eclosion, fixed in Karnovsky's fixative and embedded in epon as described in Feany and Bender (2000). Tangential retinal sections were prepared at a thickness of 0.5 μm and stained with toludine blue, examined by light microscopy and photographed.

8.2.4 Acridine Orange staining of imaginal discs

Crawling third instar larvae were dissected in phosphate buffered saline (PBS) pH 7.5 and the carcass, with attached discs, was incubated in 0.5 μg/ml Acridine Orange solution for five minutes according to standard methods (Bonini, 2000). The carcass was then rinsed in PBS; dissection was completed and the imaginal discs wet-mounted in PBS. The imaginal discs were viewed by fluorescent optics using a Nikon Eclipse fluorescent microscope and photographed immediately using a Nikon 35 mm camera attached to the microscope.

8.3 Results

8.3.1 High levels of *Gal4* expression cause apoptosis

The *GMR-Gal4* transgene produces a high level of Gal4 in the eye imaginal discs in cells posterior to the morphogenetic furrow (Freeman, 1996). This expression causes pronounced developmental defects in the adult eye (Figure 8-1B) when compared to

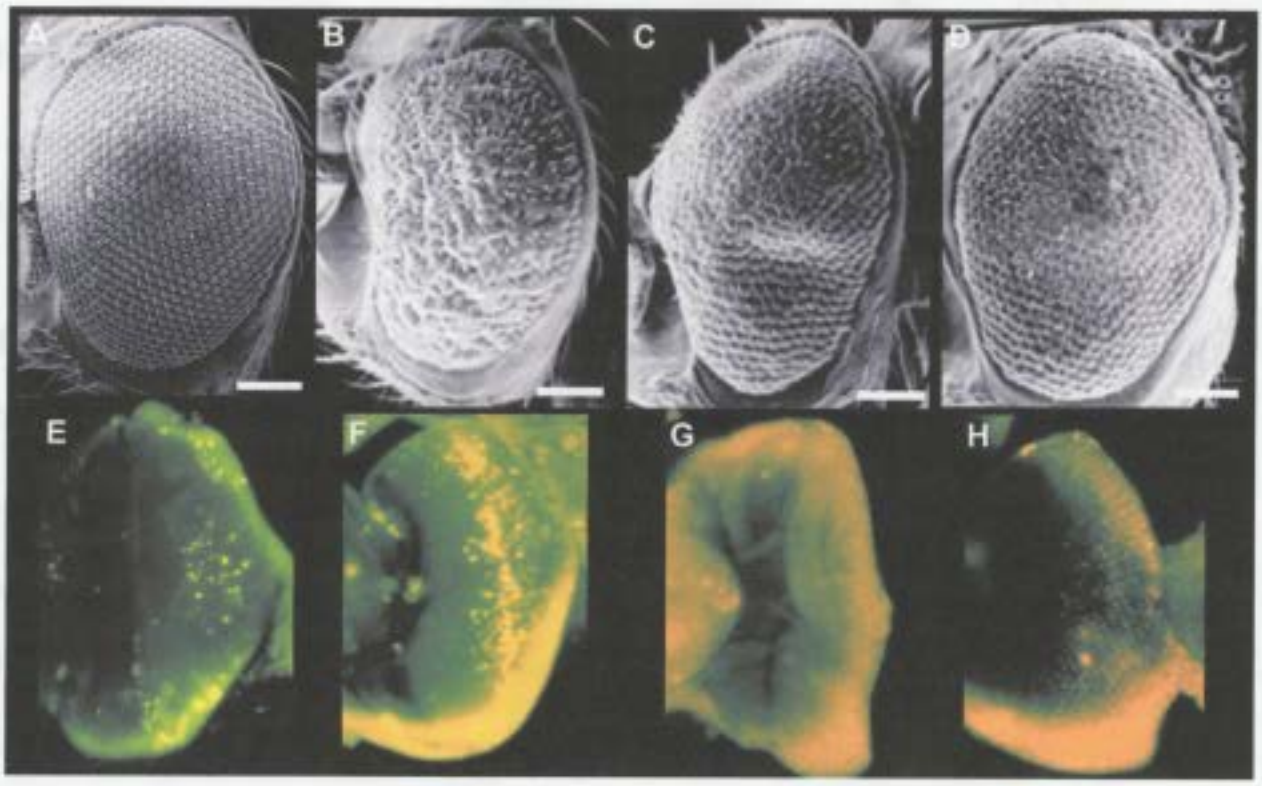

Figure 8-1: Expression of *p35* inhibits developmental defects and apoptosis in *GMR-Gal4* homozygotes.
SEM (A-D) reveals the rough eye phenotype seen in *GMR-Gal4* homozygotes (B) as compared to a control (A). The rough eye phenotype is inhibited in the presence of one (C) or two (D) copies of the *UAS-p35* transgene. Acridine Orange staining reveals apoptotic cells in the eye imaginal discs of third instar larvae. The amount of apoptosis is greater in *GMR-Gal4* homozygotes (F) than in control larvae (E). The presence of one (G) or two (H) copies of *UAS-p35* essentially eliminates all apoptosis from the eye imaginal discs. Genotypes are w^{1118} (A, E), *w;GMR-Gal4/GMR-Gal4* (B, F), *w;GMR-Gal4/GMR-Gal4;UAS-p35/+* (C, G), *w;GMR-Gal4/GMR-Gal4;UAS-p35/UAS-p35* (D, H). The scale bar is 88 μm.

control flies (Figure 8-1A) that do not contain the *GMR-Gal4* transgene. Flies that are homozygous for the *GMR-Gal4* transgene also show a high level of apoptosis in the eye imaginal disc in the area posterior to the morphogenetic furrow (Figure 8-1F) (Kramer and Staveley, 2003). In contrast, control flies show some, but much less, apoptosis in the eye imaginal discs (Figure 8-1E). This suggests that the presence of Gal4 in the *GMR-Gal4* homozygotes induces excessive apoptosis during development.

To further examine this possibility, we co-expressed the caspase inhibitor *p35* along with *Gal4* in the eye imaginal discs. Flies that are homozygous for the *GMR-Gal4* transgene and either one (Figure 8-1G) or two (Figure 8-1H) copies of the *UAS-p35* transgene show a nearly complete elimination of apoptosis during eye development. The disorganization of the ommatidial array observed with *GMR-Gal4* homozygotes (Figure 8-1B) is reduced in the presence of one (Figure 8-1C) and two (Figure 8-1D) copies of *UAS-p35*. This strongly suggests that Gal4 can cause caspase-mediated apoptosis in the developing eye to result in developmental defects.

8.3.2 Expression of *parkin* prevents Gal4-induced ommatidial disarray

As the expression of *Gal4* acts to cause cell death, bought on by the accumulation of toxic proteins, we tested if expression of *parkin* could prevent Gal4-induced phenotypes in the developing eye. Co-expression of *parkin* almost completely ameliorates the Gal4-induced rough eye ultra-structure (Figure 8-2C compared to B). While the overall size of the eye is still slightly smaller than the control, the structure is very similar to that of the control flies eye (Figure 8-2A).

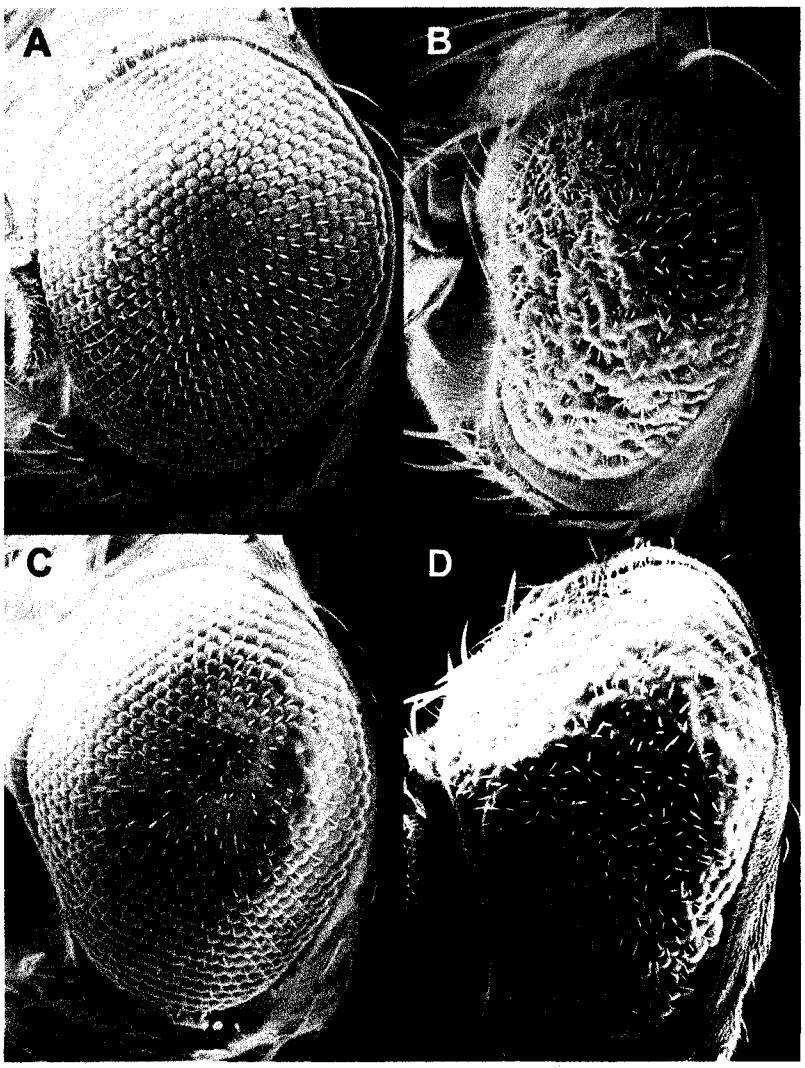

Figure 8-2: Expression of *parkin* prevents Gal4-induced ultra-structure disarray
Scanning electron micrograph of adult male flies show the rough eye phenotype caused by *Gal4* expression is suppressed in a dose-dependant fashion by expression of *parkin*. The genotypes are A - w^{1118}; B - *GMR-Gal4/GMR-Gal4*; C - *GMR-Gal4/GMR-Gal4*; *UAS-parkin*$^{2.1}$*/UAS-parkin*$^{2.1}$ and D - *GMR-Gal4/GMR-Gal4;GMR-DIAP2/TM3*. The scale bar is 88 μm.

8.3.3 Expression of *DIAP2* does not prevent Gal4-induced defects

As parkin appears to prevent the disrupted ommatidial array caused by Gal4 and it is known to function as an ubiquitin ligase, we tested if the anti-apoptotic ubiquitin ligase, *Drosophila* inhibitor of apoptosis 2 (DIAP2) could repress the *Gal4*-induced rough eye phenotype. The *GMR-DIAP2* transgene was used to express the anti-apoptotic E3 ubiquitin ligase directly in the developing eye along with *Gal4*. Analysis of these eyes showed no variation of phenotype compared with the *Drosophila* eyes that expressed *Gal4* alone (Figure 8-2D), which indicates that DIAP2 has no effect on Gal4 induced developmental defects. To test the functionality of *GMR-DIAP2*, we determined that this transgene retained the ability to suppress the rough eye and teardrop shape that results in flies that express *GMR-rpr* (White *et al.*, 1996; Wing *et al.*, 1998) (data not shown). The ability to prevent phenotypes produced by excessive Gal4 is specific to the parkin E3 ubiquitin ligase.

8.3.4 Parkin mildly suppresses Gal4-induced disorganisation of the ommatidial array

Tangential sections of adult *Drosophila GMR-Gal4* eyes were found to have a vastly different structure compared to control flies (Figure 8-3B and A). Overall, the typical crystalline array of ommatidia was not present and large gaps and areas of indistinct tissue were present. On closer examination, the normal circular structure of each ommatidial region with seven of the eight photoreceptors visible within each circle was completely disrupted (Figure 8-3E vs D). Co-expression of *parkin* with *Gal4* appears to mildly suppress the disorganisation of the ommatidial array (Figure 8-3C and F).

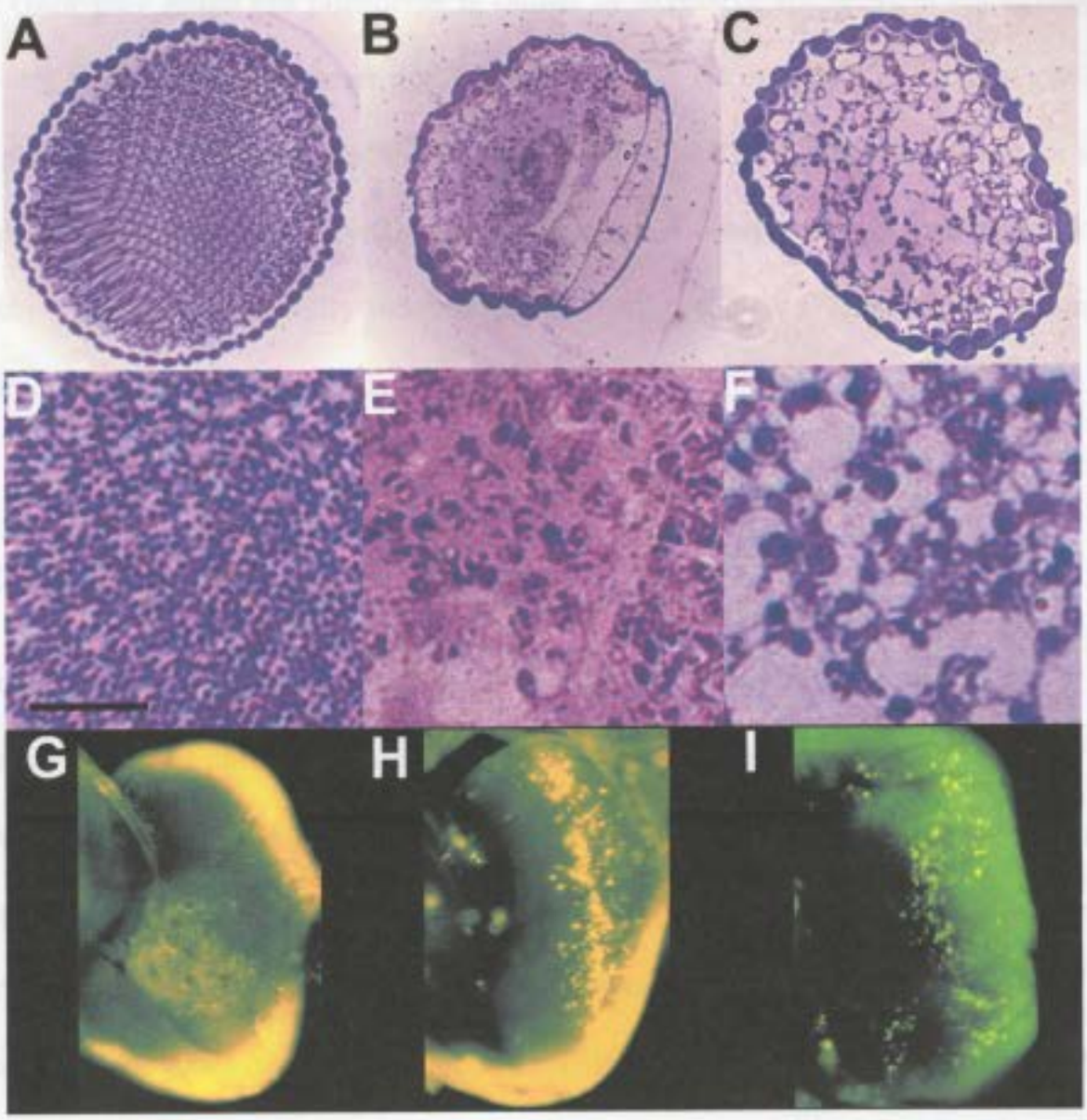

Figure 8-3: Expression of *parkin* prevents *Gal4*-induced apoptosis in the developing eye imaginal disc but only mildly suppresses retinal disarray
Light microscopy of toludine blue stained 0.5 μm sections of epon embedded one-day-old adult female *Drosophila* retinas. Whole slices are shown (A-C) with an enlarged image of a portion of the slice below (D-F). The size marker is 80 μm for D-F. Acridine Orange staining of 3rd instar larval eye imaginal discs (G-I) showing the level of apoptosis caused by *Gal4* is diminished in the eye imaginal disc in a dose dependant fashion by *parkin*. The genotypes are w^{1118} - (A, D, G), w^{1118};*GMR-Gal4/GMR-Gal4* (B, E, H) and w^{1118};*GMR-Gal4/GMR-Gal4*; *UAS-parkin*$^{2.1}$/*UAS-parkin*$^{2.1}$ (C, F, I).

8.3.5 Expression of *parkin* prevents Gal4-induced apoptosis

The *GMR-gal4* rough eye phenotype is caused by excessive apoptosis in the developing eye disc (Figure 8-3H) (Kramer and Staveley, 2003). As expression of *parkin* can alter the phenotype caused in the adult eye by excessive *Gal4*, the influence of *parkin* upon apoptosis in the developing eye disc was examined. Co-expression of two copies of the *parkin* transgene dramatically reduced the level of apoptosis (Figure 8-3I), though not quite to the control levels (Figure 8-3G).

8.4 Discussion

When *Gal4* is expressed at a low level, little evidence of abnormal phenotypes is apparent. Adverse phenotypes arise when the *Gal4* gene is expressed from a highly active promoter region or multiple copies of the transgene are present or when incubation temperatures are increased (Brand *et al.*, 1994; Kramer and Staveley, 2003). How does Gal4 cause apoptosis? One possibility is that elevated levels of Gal4 may cause transcription of genes involved in apoptosis. This seems unlikely, as there are no UAS sequences (cggagtactgtcctcc) in the *D. melanogaster* genome (Kramer and Staveley, 2003). Alternatively, Gal4 may act as a toxic protein that can activate an apoptotic cascade. As Gal4 mediated apoptosis is inhibited by the caspase inhibitor p35, we suggest that Gal4 may act as a toxic protein that activates the cell suicide machinery.

A potential mechanism by which high levels of Gal4 protein may activate the cell suicide machinery is through the unfolded-protein response (UPR). Accumulation of misfolded

or excessive levels of proteins in the ER leads to 'ER stress', which initiates the UPR, in addition to other responses (Forman *et al.*, 2003; Lindholm *et al.*, 2006; Paschen and Mengesdorf, 2005; Rao *et al.*, 2004). This, in turn, may lead to a reduction in protein synthesis followed, in some instances, by the initiation of ER stress-induced cell death. It is likely that the accumulation of Gal4 may induce a similar response to result in the appearance of the apoptotic phenotypes. Expression of *Gal4* in specific region of the body may provide a useful model for elucidating the molecular mechanisms that play a role in toxic protein-induced apoptosis and could act as a model of toxic protein induced degenerative diseases such as PD.

Parkin can prevent ER stress caused by the accumulation of misfolded proteins via its E3 activity (Imai *et al.*, 2000; Mori, 2000). As *parkin* expression does not seem to suppress the expression of other *GMR-Gal4/UAS* transgene phenotypes, such as *GMR-Gal4/UAS-rpr* (data not shown), we believe that parkin selectively suppresses phenotypes that arise from high levels of *Gal4* expression. It is possible that high levels of *Gal4* expression result in a misfolded or misfolded-like protein that can be a substrate for parkin's ubiquitin ligase function, which tags proteins for destruction. Another possibility is that parkin may prevent Gal4 from initiating a toxic protein-induced apoptotic pathway through an indirect means that has not yet been described. It has been shown that parkin protein levels increase in response to ER stress (Imai *et al.*, 2000) and it is likely that the ectopic expression of *parkin* in conjunction with high levels of Gal4 is enough to abrogate the stress inducing properties of Gal4. This is comparable with the ability of parkin to suppress phenotypes caused by mutant and/or excessive α-synuclein.

Expression of *Gal4* at higher levels should provide a unique model of toxic protein induced cell death. Further analysis of the suppression of Gal4-induced phenotypes by *parkin* expression will provide insight into the role of parkin in opposition to toxic proteins that may lead to Parkinson's disease. The UAS/Gal4 expression system, while extremely useful in the study of ectopic expression of genes in *Drosophila*, has its limitations and should be properly controlled, as expression of *Gal4* is not as benign as first thought.

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Chapter 9: Concluding discussion and future directions

9.1 How do α-synuclein and parkin interact?

Mutations that lead to amino acid changes in, or the triplication of, the *α-synuclein* gene are associated with a form of Parkinson's disease designated ADPD. The first *Drosophila* model of this disease was developed by the directed expression of mutant and wild-type forms of human *α-synuclein*, a gene not native in *Drosophila*, in the *Drosophila* central nervous system (Feany and Bender, 2000). These flies displayed dopaminergic neuronal degeneration coupled with an age-dependent loss of climbing ability. This neuronal degeneration was also demonstrated in the developing eye. The *Drosophila* model of ADPD has been used to examine the effect of various pharmacological agents (Auluck and Bonini, 2002; Pendleton *et al.*, 2002). In studies that comprise this thesis, genetic suppression of this remarkably simple model has been shown by co-expression of *parkin* (see Chapters 2 and 3).

The expression of *parkin* in the dopaminergic neurons (Chapter 2) and developing eye and wing (Chapters 2-4) does not produce any obvious adverse effects. These experiments indicate that *parkin*, when over-expressed, does not seem to randomly target and tag essential proteins for degradation. This is likely due to the high substrate specificity of parkin (Shimura *et al.*, 2001) and its ability to target itself for degradation (Zhang *et al.*, 2000). This apparently represents an excellent fail-safe mechanism cells have developed to balance the levels of both parkin and its substrates.

The age-dependent loss of dopaminergic neurons caused by expression of *α-synuclein* in *Drosophila* has been shown to be suppressed by the expression of *parkin* (Yang *et al.*,

2003). In addition, our research has found that the co-expression of *parkin* with *α-synuclein* suppresses both retinal degeneration and the premature loss of climbing-induced by the latter (Haywood and Staveley, 2004). Co-immunoprecipitation studies show that parkin will only ubiquitinate O-glycosylated α-synuclein *in vitro* (Chung *et al.*, 2001), a recent tissue culture study shows that parkin ubiquitination of α-synuclein is unaffected by PD associated mutations in α-synuclein (Lim *et al.*, 2005b) and we have demonstrated suppression of the mutant *α-synuclein*-induced phenotype by parkin (Chapter 3). This work suggests that *Drosophila* is able to modify ectopically expressed human α-synuclein. Our experiments indicate that parkin may target α-synuclein for ubiquitination and subsequent degradation *in vivo* in *Drosophila.*

While the wild-type form of α-synuclein appears to undergo O-glycosylation and this allows subsequent ubiquitination by the parkin E3 ubiquitin ligase, it was unclear if the mutant form of α-synuclein could interfere with these processes to result in the disease state. The retinal defects that result from mutant *α-synuclein* expression originally described by Feany and Bender (2000) are prevented by the co-expression of *parkin* (Chapter 3). Moreover, ectopic *parkin* expression in the dopaminergic neurons augments the climbing ability of aged flies that also express mutant *α–synuclein.* These data suggest that the amino acid substitution present in the mutant form of *α-synuclein* does not interfere with the neutralization of its toxic effects by parkin. This suggests that the A30P substitution found in a PD-inducing form of α-synuclein does not impair O-glycosylation of the protein and allows it to continue to be a target of parkin.

In order to select rational potential therapeutic agents, the molecular mechanisms behind disease progression must be characterized. Gene function studies with homologues of disease-causing genes in model organisms have been made practical through the advent of genome projects. The data herein presented shows that over-expression of *Drosophila parkin* has no apparent adverse consequences (Chapters 2 and 4) and that parkin can suppress the PD symptoms in *Drosophila* caused by increased levels of wild type or mutant forms of human α-*synuclein* (Chapters 2 and 3). If the human homologue of *parkin* has the same abilities as its *Drosophila* counterpart then up-regulation of *parkin* could be a viable treatment for α-synuclein induced PD.

9.2 The Gal4 phenomenon: Can it be used to model toxic protein-induced diseases?

The UAS/Gal4 ectopic expression system has made it possible to express genes and test the effects of over-expression of genes of interest in *Drosophila*. This system has been widely used in the *Drosophila* research community and, of concern, a number of papers about the effects of expression of apoptotic genes have been described. These papers on the whole have been well controlled and the results attributed to the expression of the apoptotic gene. What has not been widely studied nor apparently given much consideration is the effect of Gal4 expression alone.

A precise match to the UAS (cggagtactgtcctcc) enhancer sequence, to which the Gal4 transcription factor would bind to and enhance transcription, does not appear to be

present in the *D. melanogaster* genome (Kramer and Staveley, 2003). The *Drosophila* research community has not widely examined the possibility that Gal4 expression has independent effects in *Drosophila*. It has been demonstrated that increased levels of Gal4, either through one versus two *GMR-Gal4* transgenes or increased incubation temperatures of 29°C versus 25°C, leads to morphological defects in the adult eye and increased levels of apoptosis in the larval eye imaginal disc during development (Kramer and Staveley, 2003). We have demonstrated that populations of flies that are homozygous for either insert of the *Ddc-Gal4* transgene exhibit greatly reduced life spans (Chapter 6). Furthermore flies with multiple copies of the *Ddc-Gal4* transgene show increased levels of apoptosis in the larval brain lobes (Chapter 7). Our laboratory has shown that flies with multiple copies of the *Ddc-Gal4* transgene have an inverse relationship between the number of copies of the transgene and life span; also male flies are sterile with three or more copies of the transgene, a phenotype also shown in the *parkin* mutant flies (Sheppard, 2003). The *Ddc-Gal4* driver has been used to examine the expression of transgenes in the dopaminergic neurons including expression of *α-synuclein* to model PD (Feany and Bender, 2000). As *Gal4* transgenes are such an integral part of modern *Drosophila* research it was prudent to investigate this phenomenon further.

The mechanism by which Gal4 induces cell death is unclear. High levels of Gal4 may cause transcription of genes involved in apoptosis in a direct manner. However, this seems unlikely, as no UAS sequences are found in the *Drosophila melanogaster* genome for the Gal4 transcription factor to bind and activate transcription of components of the

apoptotic machinery. Alternatively, excessive Gal4 may act as a "toxic" protein that can activate the cell suicide machinery through the unfolded-protein response (UPR). In brief, the UPR is believed to be initiated by the accumulation of misfolded or excessive levels of proteins in the ER that in turn leads to 'ER stress' (reviewed in Forman *et al.*, 2003, and Rao *et al.*, 2004). Following the initiation of the UPR there is an overall reduction in protein synthesis; if this does not resolve the stressful situation then ER stress-induced cell death is initiated. Accumulation of excessive Gal4 may lead to ER-stress induced cell death. Furthermore, we have demonstrated that the cell death process acts through the caspase pathway as Gal4-induced apoptosis is inhibited by the caspase inhibitor p35 (Chapter 8; Haywood, Kramer, Sheppard and Staveley, submitted). While the specific mechanism through which toxic proteins activate apoptosis is unknown, over-expression of *Gal4* in *Drosophila* tissues may provide a useful model for elucidating the molecular mechanism that play a central role in toxic protein-induced apoptosis. Expression of *Gal4* in specific tissues at specific stages of development may act as a model of a number of toxic protein-induced degenerative diseases including PD.

9.3 Parkin suppresses toxic protein-induced cell death

Parkin can prevent ER-stress caused by the accumulation of misfolded proteins via its E3 activity (Imai *et al.*, 2000; Mori, 2000). Levels of the parkin protein increase in response to ER stress (Imai *et al.*, 2000). If Gal4-induced cell death is the result of an ER-stress-induced apoptotic pathway, it may be likely that the ectopic expression of parkin can act to abrogate the ER-stress inducing properties of Gal4. We have shown that parkin can prevent Gal4-induced apoptosis in the developing eye (Chapter 8, Haywood, Kramer,

Sheppard and Staveley, submitted). Expression of *parkin* does not appear to suppress the expression of other phenotypes driven by *GMR-Gal4*, in particular cell death genes *reaper* and *hid* (data not shown). Note that in these instances the number of *GMR-Gal4* transgenes is only one and hence the level of Gal4 is not at a "toxic" level. This leads us to believe that parkin can selectively suppress the cell death phenotype that arises from excessive *Gal4* expression.

How does *parkin* prevent *Gal4*-induced developmental defects? We speculate that the Gal4 protein may take on a toxic form, such as a misfolded form or a multimeric conglomeration with other proteins, when produced in high levels. As parkin is an ubiquitin ligase, parkin may bind to and mediate the ubiquitination of this form of Gal4 protein to target it for destruction thus preventing Gal4-induced developmental defects. Another possibility is that parkin may prevent Gal4 from initiating a toxic protein-induced apoptotic pathway through an indirect means that has not yet been described. Questions remain as to the mechanism by which *parkin* expression is able to prevent the defects caused by Gal4. Is there a specific interaction between the parkin protein and Gal4? Are there other intermediary proteins involved? We demonstrate that the expression of the anti-apoptotic E3 ubiquitin ligase *DIAP2* does not prevent Gal4-induced disruption of the ommatidial array (Chapter 8), however could other ubiquitin ligases act to prevent Gal4-induced defects? It is possible that high levels of *Gal4* expression result in a misfolded protein target that parkin can recognise and subject to its ubiquitin ligase function and, eventually destruction. This is comparable with the ability of parkin to suppress phenotypes caused by mutant and/or excessive α-synuclein

(Chapters 2 and 3 Haywood and Staveley, 2004; Haywood and Staveley, 2006). Analysis of parkin prevention of Gal4 toxicity may lead to a greater understanding of the role of parkin in repression of toxic protein induced diseases.

9.4 Future research

The results from chapters 2 and 3 demonstrate that there are genetic approaches that can be used to prevent phenotypes induced by *α-synuclein* expression in a *Drosophila* model of PD. Examination of the interaction of parkin with other PD-associated forms of α-synuclein, such as A53T and E46K, will provide a greater understanding of the ability of parkin to suppress PD-like phenotypes in *Drosophila*. Analysis of the function of α-synuclein phosphorylation and nitrosylation through the generation of transgenic flies with non-phosphorylatable or non-nitrosylatable human α-synuclein could also yield unique alternative PD models. Analysis of proteins that interact with these different forms of α-synuclein through immuno-precipitation experiments or through genetic screens for modifiers of mutant α-synuclein phenotypes will increase our understanding of the function of α-synuclein. Of particular importance will be the analysis of interactions between *α-synuclein, parkin* and other genes associated with PD or other neurodegenerative disorders.

The *parkin* gene may have a more central role in PD than we currently realise. Certainly parkin can act to ubiquitinate α-synuclein *in vitro* (Shimura *et al.*, 2001) and abrogates α-synuclein-induced phenotypes *in vivo* (Chapters 3 and 4, Haywood and Staveley, 2004;

Yang *et al.*, 2003). Recently, parkin was shown to interact with DJ-1 in varying ways that depended on the aberration in the DJ-1 gene (Moore *et al.*, 2005). Parkin has also been shown to have a novel, proteasomal-independent, catalytic activity that mediates a non-classical, K63-linked ubiquitin multi-chain assembly on synphilin-1 that is distinct from the classical, degradation-associated, K48-linked ubiquitination (Lim *et al.*, 2005a; Lim *et al.*, 2005b). Interestingly, two other PD-linked gene products, α-synuclein and UchL1, have recently also been associated with K63-linked ubiquitination (Lim *et al.*, 2005b). Understanding how parkin interacts with other genes associated with inherited forms of PD I believe may be integral to understanding PD. We still do not understand why dopaminergic neurons are selectively vulnerable in PD patients.

The ability to generate a potential model of toxic protein-induced cell death, through *Gal4* expression in specific parts of the *Drosophila* anatomy, has two resulting points of interest. First of all, it indicates that excessive expression of *Gal4* has consequences. Any genetic analysis/research utilising this system must ensure that it is properly controlled, particularly when using cell death genes under the control of the UAS/Gal4 bipartite system. As the expression of *Gal4* is fundamental in the expression of many different genes in *Drosophila*, it is vital that we are aware of the consequences of *Gal4* expression alone. We have shown that expression of *Gal4* is not as benign as was first thought and a solid understanding of the nature of the phenotypes that arise from *Gal4* expression is required (Chapters 6 and 7). Second, that *Gal4* expression could be used to generate a potential model of toxic protein-induced disease is another useful tool in the tool belt of a scientist. While further work needs to be done to examine this potential

model it could be used in genetic screens to find modifiers of the Gal4 phenotype. Expression of genes that modify this phenotype may have a role in toxic protein-induced diseases and could provide further insight into the basic mechanisms that cause such diseases.

As we found that expression of *parkin* can suppress the Gal4 phenotype further examination of this interaction may provide a wealth of information (Chapter 8). Do these two proteins physically interact? Does parkin ubiquitinate Gal4 *in vivo*? By analysing the properties of Gal4, when co-expressed with *parkin*, we should be able to determine if it can be ubiquitinated. Should parkin ubiquitinate Gal4 directly then further analysis, through mutagenesis studies, will yield clues to the substrate specificity function of parkin. In conclusion selective targeting of toxic proteins for degradation appears to be a useful approach to address neurodegenerative conditions such as PD. The use of parkin and its apparent involvement in many aspects of inherited PD could lead to the development of therapeutic strategies.

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Appendix I - Overexpression of *phosphatidylinositol 3-OH kinase (PI3K)* in dopaminergic neurons dramatically reduces life span and climbing ability in *Drosophila melanogaster*

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Abstract

Parkinson's disease (PD) is a prevalent neurodegenerative disease marked by the selective loss of dopaminergic neurons that is accompanied by resting tremors and other symptoms. The study of organismal models of PD, including the well-studied α-synucleinopathic model, in *Drosophila melanogaster* has lead to a greater understanding of the biological basis of the disease. In an attempt to establish additional *Drosophila* models of PD via the manipulation of cell survival signaling, the UAS/Gal4 system was used to overexpress two forms of *phosphatidylinositol 3-OH kinase* (*PI3K*) in the dopaminergic neurons of flies. The directed expression of *PI3K* in this manner dramatically reduces life span and climbing ability while an inhibitory form, a dominant negative version of *PI3K*, reduces life span in a far less dramatic way. These novel models should provide the basis for a series of investigations into the role of cell survival signaling in Parkinson's disease.

Introduction

Parkinson's disease (PD) is a common, age-related neurodegenerative disease characterized by muscle rigidity, resting tremors, and postural instability (Lansbury and Brice, 2002; Spacey and Wood, 1999). Post-mortem analysis of patients reveals that PD appears to be due to the selective loss of dopaminergic neurons in the *substantia nigra* region of the brain. The underlying cause of this distinctive loss of neurons may be classified as either sporadic or familial in origin. Although the underlying mechanism is not well understood, defects in several genes as well as a number of environmental toxins have been linked to the cause of this neuronal loss. As it is difficult to research the

pathogenesis of PD in living patients, a number of animal models (Dawson, 2000; Hashimoto *et al.*, 2003), including a well-established *Drosophila* model (Feany and Bender, 2000), have been developed to investigate aspects of PD.

A promising series of investigations into the biological basis of PD have been initiated through the generation of a PD model by the conditional expression of human *α-synuclein* in transgenic *Drosophila* (Feany and Bender, 2000). The expression of *α-synuclein*, in both a pan-neural and dopaminergic neuron-specific manner, produces an age-dependent loss of dopaminergic neurons. The neuronal loss is accompanied with the premature loss of climbing ability and the formation of cytoplasmic inclusions in the dopaminergic neurons. In addition, expression of *α-synuclein* in the developing eye results in an age-dependent degeneration of the retina. In further experiments, the dopamine precursor levodopa, dopamine receptor agonists, and the anticholinergic agent atropine act to counter the age-dependent loss of climbing ability (Pendleton *et al.*, 2002). Expression of the molecular chaperone gene *hsp70* with *α-synuclein* prevents dopaminergic neuronal degeneration (Auluck *et al.*, 2002). The expression of *parkin* can suppress the loss of dopaminergic neurons (Yang *et al.*, 2003), the premature loss of climbing ability and the age-dependent degeneration of the retina (Haywood and Staveley, in preparation) induced by α-synuclein in *Drosophila*. In addition, another model has recently been established with the description of mutants in the *parkin* gene (Greene *et al.*, 2003). The *Drosophila* models of PD are proving to be very effective tools in the investigation of the biological basis of this disease.

Dopaminergic neurons may die as a result of apoptosis in PD (for review Lev *et al.*, 2003). This process may be caused by the accumulation of endogenous toxic proteins or environmental toxins. Exploration of the role of cell survival signaling in the selective loss of dopaminergic neurons in *Drosophila* may provide further insight into the basis of PD. The insulin receptor (InR)/ PI3 kinase/ akt anti-apoptotic signaling pathway is highly conserved between mammals and *Drosophila* (Datta *et al.*, 1999; Fernandez *et al.*, 1995; Leevers *et al.*, 1996; Oldham *et al.*, 2000; Staveley *et al.*, 1998). To initiate this signal, insulin or insulin-like growth factors bind to receptor tyrosine kinases at the cell membrane and activate the protein phosphatidylinositol 3-OH kinase (PI3K) via phosphorylation (Vanhaesebroeck *et al.*, 2001). In turn, PI3K phosphorylates inositol lipids on the inner membrane of the cell, which leads to the co-localization of akt and phosphoinosotide-dependent kinase 1 (PDK-1) and, as a result, the activation of akt. An anti-apoptotic or cell survival signal results from activated akt. Consequently, manipulation of the InR/PI3K/akt pathway in the dopaminergic neurons of *Drosophila melanogaster* may produce selective apoptotic death of those cells and produce flies with symptoms similar to other models of PD. As PI3K is an essential component of this pathway, it is a good candidate for manipulating cell survival signaling.

The UAS/Gal4 ectopic expression system (Brand and Perrimon, 1993) was used to overexpress wild type and mutant forms of PI3K in the dopaminergic neurons. Climbing and longevity assays were performed and the results demonstrate that overexpression of PI3K dramatically reduces climbing ability and viability of the flies from the time of

eclosion. Overexpression of an inhibitory PI3K also reduces the length of life span when compared to controls but does not prematurely reduce the climbing ability of the flies.

Materials and Methods

Fly stocks and culture: The *Ddc-Gal4*$^{4.3D}$ and *Ddc-Gal4*$^{4.36}$ transgenic lines (Li *et al.*, 2000) were obtained from Dr. Jay Hirsh at the Department of Biology, University of Virginia. The *UAS-PI3K-dp110* and *UAS-PI3K-dp110*^{D954A} flies were obtained from Dr. Sally Leevers at the Ludwig Institute for Cancer Research and the Department of Biochemistry and Molecular Biology, University College, London. The w^{1118} strain was provided by Dr. Howard D. Lipshitz of the Hospital for Sick Children and the University of Toronto. All flies were cultured on standard cornmeal/yeast/agar medium at 25°C.

Transgene Expression: The UAS/Gal4 ectopic expression system (Brand and Perrimon, 1993) was used to express wild type and mutant forms *of phosphatidylinositol 3-OH kinase* (*PI3K*; Leevers *et al.*, 1996) in the dopaminergic neurons using *Ddc-Gal4* transgenes (Li *et al.*, 2000). The progeny of crosses of the *Ddc-Gal4* lines to transgenic *UAS-PI3K-dp110* flies will express the catalytic subunit of PI3K (dp110) in the dopaminergic neurons. The same *Ddc-Gal4* driver lines were crossed to *UAS-PI3K-dp110*^{D954A} to induce the expression of an inhibitory form of this subunit of PI3K. The controls were produced by crossing w^{1118} to the *Ddc-Gal4* transgenics.

Aging assay: Adult male flies were collected within 24 hours of eclosion and scored for viability every two to three days to determine the adult life span characteristics as

previously described (Staveley *et al.*, 1990). Flies were maintained under non-crowded conditions of approximately 5 to 15 individuals upon standard cornmeal/yeast/agar medium at 25^0C.

Climbing assay: The climbing ability of male flies of the same age were assayed every four days to determine their locomotor abilities throughout their life span as previously described (Feany and Bender, 2000). To be precise, the proportion of a cohort of ten (or fewer) flies to climb a distance of 8 centimetres within a period of 18 seconds was determined. In total, twenty trials were carried out at each time point. From this data, the average number of flies that successfully completed the climb at each time point was calculated.

Data Analysis: Data from the aging and climbing assays were compiled and graphed using Microsoft Excel.

Results and Discussion

Transgenic flies expressing one of the two forms of *PI3K* in the dopaminergic neurons were tested for viability with an aging assay (Figure AI-1). Overexpression of *PI3K-dp110* with both of the *Ddc-Gal4* transgenes greatly decreased the life span of the flies. The median age of survival (50%) for flies expressing *PI3K-dp110* was between 18 and 20 days when expressed by $Ddc\text{-}Gal4^{4.3D}$ and between 12 and 14 days when expressed by $Ddc\text{-}Gal4^{4.36}$. Expression of the dominant negative form of *PI3K* ($PI3K\text{-}dp110^{D954A}$) produced a small decrease in survival. The median age of survival (50%) was between

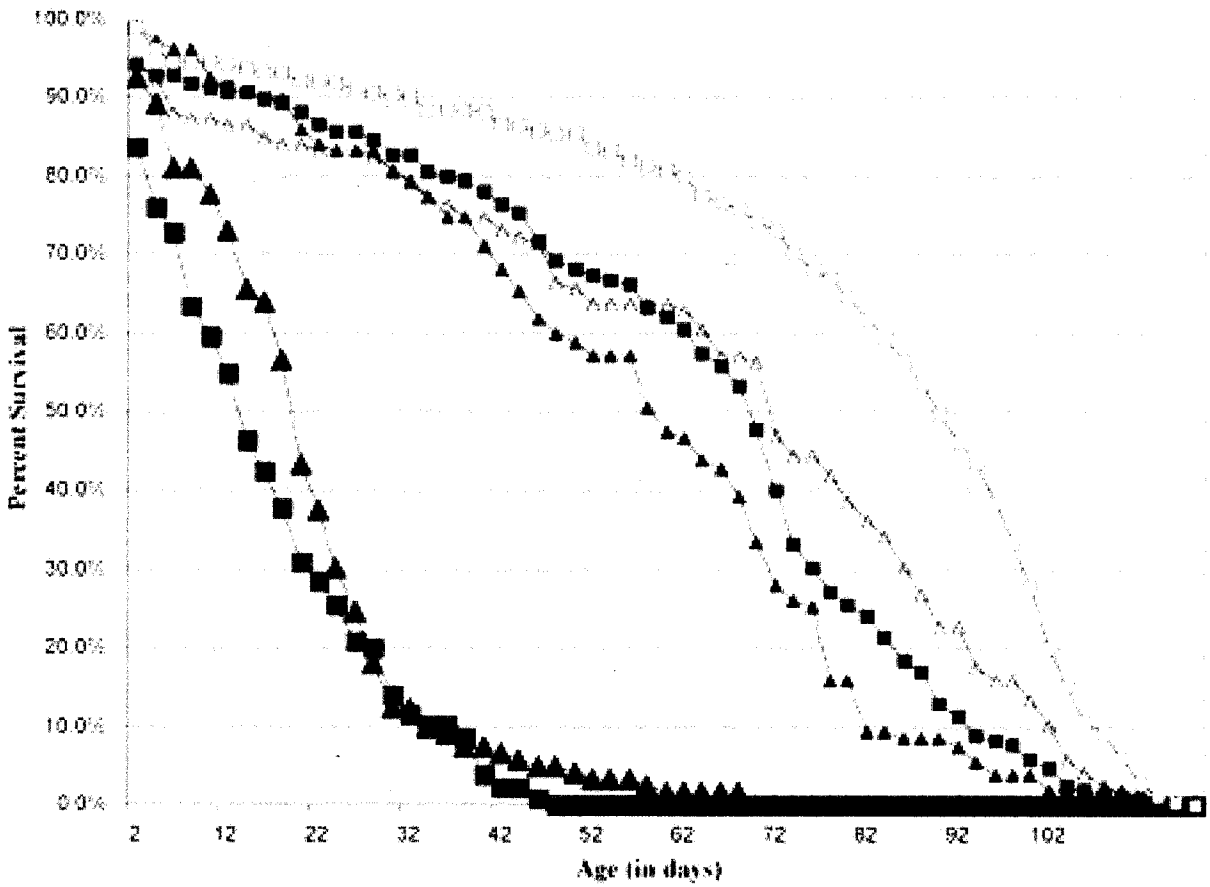

Figure AI- 1: Survival of flies expressing wild type (*PI3K*) and dominant negative *PI3K* (*PI3K^{DN}*) in the dopaminergic neurons.
Adult males that express the wild type version of *PI3K* in the dopaminergic neurons *Ddc-Gal4$^{4.36}$/UAS-PI3K-dp110* (large solid triangles) and *Ddc-Gal4$^{4.3D}$/UAS-PI3K-dp110* (large solid squares) have a greatly reduced life span when compared to controls. Expression of the dominant negative *PI3K* transgene under the same circumstances, *Ddc-Gal4$^{4.36}$/UAS-PI3K-dp110^{D954A}* (small solid triangles) and *Ddc-Gal4$^{4.3D}$/UAS-PI3K-dp110^{D954A}* (small solid squares), leads to a slightly reduced life span, when compared to the *Gal4*-expressing controls, *Ddc-Gal4$^{4.36}$/+*: (small open triangles) and *Ddc-Gal4$^{4.3D}$/+*: (small open squares). The number of individuals aged was as follows: *Ddc-Gal4$^{4.36}$/UAS-PI3K-dp110*, n = 122; *Ddc-Gal4$^{4.3D}$/UAS-PI3K-dp110*, n = 129; *Ddc-Gal4$^{4.36}$/UAS-PI3K-dp110^{D954A}*, n = 107; *Ddc-Gal4$^{4.3D}$/UAS-PI3K-dp110^{D954A}*, n = 195; *Ddc-Gal4$^{4.36}$/+*, n = 119; *Ddc-Gal4$^{4.3D}$/+*, n = 280.

58 and 60 days under the control of the *Ddc-Gal4*$^{4.36}$ driver and between 68 and 70 days with the *Ddc-Gal4*$^{4.3D}$ transgene. The *Gal4* heterozygotes, *Ddc-Gal4*$^{4.3D}$ and *Ddc-Gal4*$^{4.36}$, were tested and the results show a median age of survival between 70 and 72 days for the former and between 82 and 84 for the latter. The expression of *PI3K-dp110*^{D954A} resulted in a decrease in median survival of approximately 14 days when compared to the *Ddc-Gal4* heterozygote controls while the expression of *PI3K-dp110* resulted in a major decrease in life span by between 50 and 70 days.

To monitor the effects upon locomotion, the climbing ability of these transgenic flies were tested (Figure AI-2). Flies that express the wild type version *PI3K-dp110* under the control of *Ddc-Gal4* climb poorly while those expressing *PI3K-dp110*^{D954A} appear to climb as well as the controls throughout the duration of the experiment.

In addition to the defects in climbing ability and the greatly reduced life span, flies overexpressing *PI3K-dp110* exhibit a blistered wing phenotype shortly after emerging from the pupae cases (data not shown). Within a day or so, most adult *Ddc-Gal4/UAS-PI3K-dp110* flies have shriveled wings. This defect may be indirectly caused by neuronal loss.

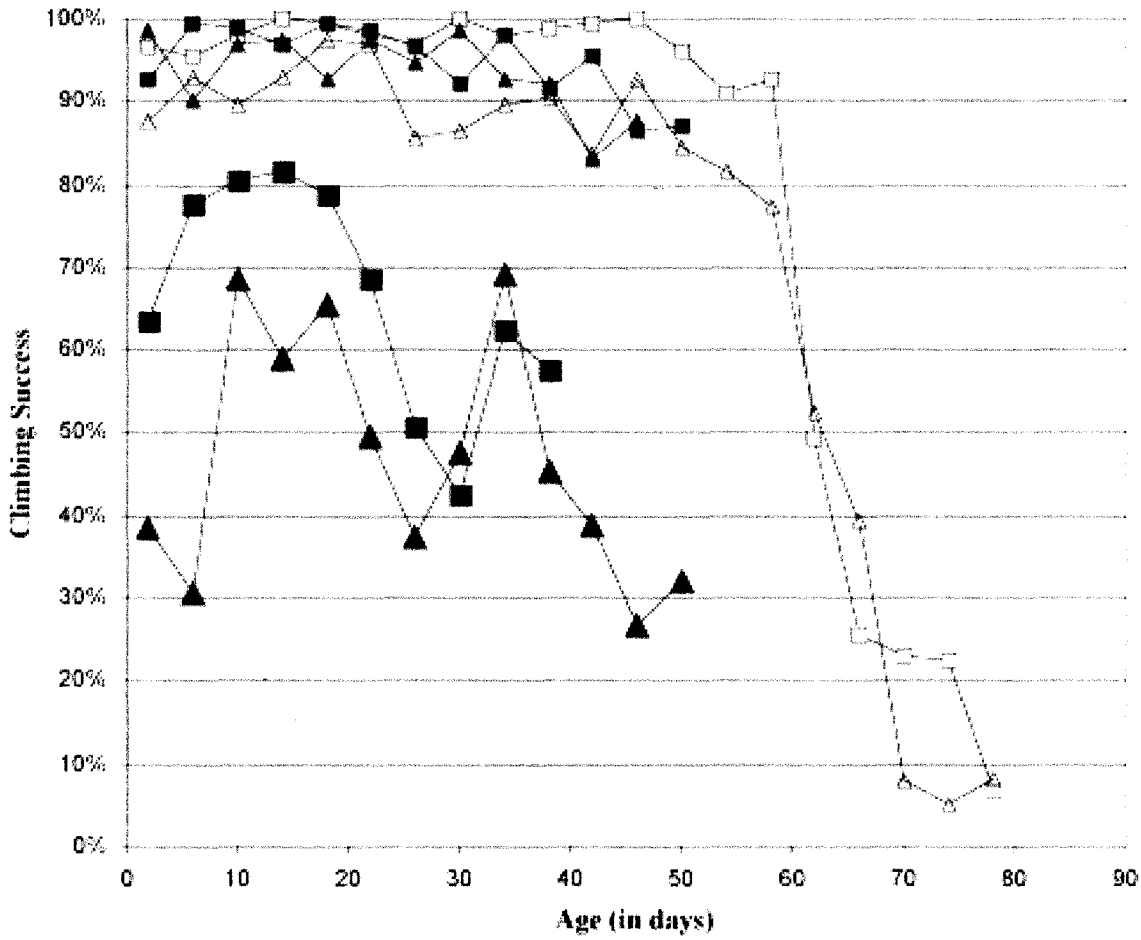

Figure AI- 2: The measurement of climbing ability of flies expressing wild type (*PI3K*) and dominant negative *PI3K* (*PI3K*^{DN}) in the dopaminergic neurons.
Adult males that express the wild type version of *PI3K* in the dopaminergic neurons Ddc-Gal4$^{4.36}$/UAS-PI3K-dp110 (large solid triangles) and Ddc-Gal4$^{4.3D}$/UAS-PI3K-dp110 (large solid squares) have a poor ability to climb when compared to controls. Expression of the dominant negative PI3K transgene under the same circumstances, Ddc-Gal4$^{4.36}$/UAS-PI3K-dp110^{D954A} (small solid triangles) and Ddc-Gal4$^{4.3D}$/UAS-PI3K-dp110^{D954A} (small solid squares), maintain the ability to climb in a manner similar to the controls, Ddc-Gal4$^{4.36}$/+ (small open triangles) and Ddc-Gal4$^{4.3D}$/+ (small open squares). The climbing experiments were discontinued when death reduced the number significantly.

Overexpression of *PI3K* in the dopaminergic neuron during development may lead to selective apoptotic death of these neurons. Contrary to the common role of *PI3K* in supporting cell survival, overexpression of *PI3K* has been shown to cause apoptosis. In cultured rat embryo fibroblasts, prolonged activation of *PI3K* in the absence of other stimuli (serum) results in apoptosis (Klippel *et al.*, 1998). Prolonged overexpression of *PI3K* increases in the proportion of cells in G2/M and induces apoptosis in *Drosophila* (Vanhaesebroeck *et al.*, 2001). This may be due to deregulation of the cell cycle or the induction of an apoptotic feedback program by the hyperactivation of many signaling pathways. The selective loss of the dopaminergic neurons via a cell death mechanism could be responsible for the observed poor climbing ability and reduced life span of adult *Ddc-Gal4/UAS-PI3K-dp110* flies.

Although active PI3K acts to prevent apoptosis of cells, larvae survive for twenty days without PI3K (Weinkove *et al.*, 1999). In contrast, the inhibitory form of *PI3K* has been shown to cause cell death when expressed in embryos (Scanga *et al.*, 2000). In our experiments, the expression level of *PI3K-dp110*^{D954A} may have been sufficient to induce neuronal loss only in late life. The small decrease in life span may have resulted from this late loss in neurons in the *Ddc-Gal4/UAS-PI3K- dp110*^{D954A} flies.

In conclusion, this experiment analyzed the viability and climbing ability of flies expressing two forms of *PI3K* in an attempt to model characteristics of Parkinson's disease. Unexpectedly, the ectopic expression of *PI3K* showed dramatically reduced life span coupled with poor climbing ability. Unlike Parkinson disease patients, the

locomotor dysfunction begins early, rather than arising in a gradual manner, which may be due to the larval expression of *PI3K*, and subsequent loss of dopaminergic neurons at that stage. The dominant negative version of *PI3K* reduced life span by a modest amount but did not seem to influence the ability of these flies to climb. In summary, our experiments show that the overexpression of *PI3K* in dopaminergic neurons can produce defects that may recapitulate some aspects of Parkinson's disease *in Drosophila melanogaster*.

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