# EXPRESSION OF BCL-2 HOMOLOGUES IN THE α-SYNUCLEIN-INDUCED PARKINSON DISEASE MODEL IN DROSOPHILA

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# EXPRESSION OF *BCL-2* HOMOLOGUES IN THE

# α-*SYNUCLEIN*-INDUCED PARKINSON DISEASE

# MODEL IN DROSOPHILA

by

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**Abstract**

In Parkinson disease (PD), the age-dependent degeneration of dopaminergic neurons (DA) and loss of locomotor function have been shown to be correlated with prominent mitochondrial abnormalities and dysfunction. A number of genes are associated with inherited forms of PD and most of these genes encode protein products that interact with components of the mitochondria. The pro-survival Bcl-2 proteins are reputed to be the guardians of the mitochondria, an organelle central to the process of cell death in all animals. *Drosophila melanogaster* possess two mitochondria localized Bcl-2-like proteins encoded by *debcl*, which promotes cell death, and *Buffy*, which is pro-cell survival. The Bcl-2 proteins have been shown to have a dual role in the control of cell death and subsequent engulfment of cellular components (autophagy).

In the *α-synuclein*-induced Drosophila model of PD, *Buffy* and *debcl* were overexpressed in the DA neurons and developing eye using the *UAS-GAL4* system of directed gene expression. Longevity and climbing ability of these flies were influenced by these two *Bcl-2* genes: *debcl* enhances the severity of the *α-synuclein*-induced age-dependent loss of climbing ability. On the other hand, *Buffy* suppresses the *α-synuclein*-induced PD-like phenotypes. When overexpressed in the developing neurons of the eye, a similar trend was observed with *Buffy* suppressing the eye defects. Taken together, these results suggest a protective role for *Buffy*, especially under *α-synuclein*-induced protein toxicity.

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## List of Abbreviations

| | |
|---|---|
| AD | autosomal dominant |
| ANOVA | analysis of variance |
| Apaf | apoptosis protease activating factor |
| AR | autosomal recessive |
| ATP | adenosine triphosphate |
| Bcl-2 | B-cell lymphoma 2 |
| BH | Bcl-2 homology |
| Bok | Bcl-2 related ovarian killer |
| bp | base pairs |
| C-terminal | carboxy-terminal |
| CI | confidence interval |
| CMA | chaperone-mediated autophagy |
| CNS | central nervous system |
| DA | dopaminergic |
| *Ddc* | DOPA decarboxylase |
| DNA | deoxyribonucleic acid |
| ER | endoplasmic reticulum |
| FPD | familial forms of Parkinson disease |
| g | gram |
| GAL4 | yeast transcriptional activator for galactose-inducible genes |
| GOF | gain-of-function |

| | |
|---|---|
| GTPase | guanosine triphosphatase |
| Hsp70 | heat shock protein 70 |
| IAP | Inhibitors of apoptosis |
| IFM | indirect flight muscles |
| JNK | Janus kinase |
| kDa | kilo Daltons |
| L | litre |
| LOF | loss-of-function |
| LRRK2 | leucine rich repeat kinase 2 |
| MAPKKK | mitogen activated protein kinase kinase kinase |
| mL | millilitre |
| MOM | mitochondrial outer membrane |
| mRNA | messenger ribonucleic acid |
| mtDNA | mitochondrial DNA |
| n | number of flies |
| N-terminal | nitrogen terminal |
| n/a | not applicable |
| NAC | non-$\beta$ amyloid component |
| NCBI | National Centre for Biotechnology Information |
| PCD | programmed cell death |
| PD | Parkinson disease |
| PI3K | phosphoinositide 3-kinase |
| PINK1 | PTEN-induced kinase 1 |

| | |
|---|---|
| PTEN | Phosphatase and tensin homologue |
| RING | really interesting new gene |
| ROS | reactive oxygen species |
| rpm | revolutions per minute |
| SEM | standard error of the mean |
| SEMs | scanning electron micrographs |
| SOD | superoxide dismutase |
| UAS | upstream activating sequence |
| UPS | ubiquitin proteasome pathway |
| UV | ultra violet |
| WT | wild-type |
| ηg | nanogram |
| α-synuclein | alpha-synuclein |
| μg | microgram |
| μL | microlitre |
| μm | micrometre |
| °C | degree Celsius |

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**Introduction**

*Parkinson Disease*

Parkinson disease (PD) belongs to a subgroup of human diseases referred to in general as neurodegenerative diseases, and is characterized by the progressive loss of specific neuronal populations, resulting in substantial disability and early death (Muqit and Feany, 2002; Lu and Vogel, 2008). PD is the most common movement disorder and the second most common neurodegenerative disease, afflicting about 1 to 2% of the population over 50 years of age. It is associated with selective and profound loss of dopaminergic (DA) neurons resulting in marked clinical features, which include muscle rigidity, resting tremors, postural instability, bradykinesia as well as non-motoric symptoms like autonomic, cognitive and psychiatric problems (Forno, 1996). The neuropathological hallmarks exhibited by PD patients are Lewy Bodies (LB) and Lewy neurites (LN) in surviving neurons. This is due to loss of neuromelanin-containing DA neurons in the S*ubstantia nigra pars compacta* (*SNpc*) with presence of eosinophilic, intracytoplasmic proteinaceous inclusions comprised of α-synuclein and ubiquitin, among other proteins (Forno, 1996; Polymeropoulos *et al*, 1997; Leroy *et al*, 1998). This accumulation of proteins is believed to lead to cellular toxicity and PD pathogenesis.

Most cases of PD are believed to be sporadic with late-onset with no known causes but the discovery of the familial forms of PD (FPD)-associated genes has offered the opportunity to study the mechanisms of both FPD and sporadic PD pathogenesis on model organisms (Lu and Vogel, 2008; Cauchi and Heuvel, 2006). Postmortem studies have implicated defective mitochondrial complex 1 function, and oxidative damage in

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nigrostriatal DA neurons in PD pathology (Lu, 2009). Indeed, most PD research is geared towards the genes with a function at or in the mitochondria.

**PD Gene Loci**

Currently, at least 16 distinct gene loci *PARK1- PARK16* have been described for FPD (Table 1) and some have been characterized at the molecular level (Thomas and Beal 2007, 2011). The identified gene loci are α-synuclein/*PARK1/4* (Polymeropoulos *et al,* 1997), parkin/*PARK2* (Kitada *et al.,* 1998), Ubiquitin C-terminal hydrolase1 (Uchl-1)/*PARK5* (Leroy *et al.,* 1998), Phosphatase and tensin homologue [PTEN] induced kinase 1 (*Pink1*)/*PARK6* (Valente *et al.,* 2004), DJ-1/*PARK7* (Bonifati *et al.,* 2003), and leucine rich repeat kinase 2 (LRRK2)/*PARK8* (Paisan-Ruiz *et al.,* 2004; Zimprich *et al.,* 2004). Other mutated loci that have been implicated in rare FPD are ATP 13A2 (a P-type ATPase)/*PARK9* (Ramirez *et al.,* 2006), Grb10-Interacting GYF Protein-2 (GIGYF2)/*PARK11* (Lautier *et al.,* 2008), HTRA2 (a Serine protease)/PARK 13 (Strauss *et al.,* 2005), Phospholipase A2 (PLA2G6)/*PARK14* (Paisan-Ruiz *et al.,* 2009) and F-box only protein 7 (FBXO7)/*PARK15* (Di Fonzo *et al.,* 2009). Other PARK loci exist whose mutated gene is unknown (Dawson and Dawson, 2003; Thomas and Beal, 2007). Interestingly, most of the PD genes have a direct function at the mitochondria.

**Table 1: Gene loci implicated in familial forms of Parkinson disease.**

| Locus | Gene | Chromosome | Inheritance | Function |
|-------|------|------------|-------------|----------|
| PARK1/ | *α-synuclein* | 4q21 | AD | presynaptic protein, Lewy |
| PARK4 | | 4p14 | | body, lipid and vesicle |
| | | | | dynamics |
| PARK2 | *Parkin* | 6q25.2-q27 | AR | ubiquitin E3 ligase, |
| | | | | mitophagy |
| PARK3 | Unknown | 2p13 | AD | Unknown |
| PARK5 | *Uch L1* | 4p14 | AD | ubiquitin C-terminal |
| | | | | hydrolase |
| PARK6 | *Pink1* | 1p35-36 | AR | mitochondrial kinase |
| PARK7 | *DJ-1* | 1p36.23 | AR | chaperone, antioxidant |
| | | | | oxidative stress |
| PARK8 | *LRRK2* | 12p11.2 | AD | kinase, signalling, |
| | | | | cytoskeletal dynamics, |
| | | | | protein translation |
| PARK9 | *ATP13A2* | 1p36 | AR | lysosomal type 5 P-type |
| | | | | ATPase |
| PARK10 | Unknown | 1p32 | AD | Unknown |
| PARK11 | *GIGYF2* | 2q36-q37 | AD | IGF-1 signalling |
| PARK12 | Unknown | Xq21-q25 | X-linked | Unknown |
| PARK13 | *HtrA2 /Omi* | 2p12 | Unknown | mitochondrial serine |
| | | | | protease |
| PARK 14 | *PLA2G6* | 22q13 | AR | Phospholipase enzyme |
| PARK 15 | *FBXO7* | 22q11 | AR | Ubiquitin E3 ligase |
| PARK 16 | Unknown | 1q32 | Unknown | Unknown |

AD is autosomal dominant and AR is autosomal recessive (Adapted from Thomas, B &
Beal MF 2007; 2011)

*Drosophila as a model organism*

The discovery of FPD-associated gene loci offered an opportunity to study PD in suitable model organisms (Cauchi and Heuvel, 2006; Lu and Vogel, 2008). The combination of our understanding of the molecular basis of PD, and the great advances in Drosophila genetics (Sang and Jackson, 2005; Marsh and Thompson, 2006) have made this organism a very powerful tool for understanding the pathophysiology of PD.

The exploitation of *Drosophila melanogaster* as a model organism is based upon the conservation of fundamental aspects of cell biology in both flies and humans (Cauchi and Heuvel, 2006). Of great importance is the relative ease that PD pathogenesis can be recapitulated in the model organism to reveal both clinical and neuropathological characteristics similar to those observed in humans (Sang and Jackson, 2005). Additional advantages are rapid generation times, cheap culturing requirements, large progeny numbers produced in a single cross and a small highly annotated genome devoid of genetic redundancy.

The presence of PD gene homologues and the high degree of functional conservation contribute to the ability to model PD in *Drosophila melanogaster* (Celotto and Palladino, 2005; Jeibmann and Paulus, 2009). The fly brain has over 300, 000 neurons and is organized into separate specialized areas for learning, olfaction, vision and memory (Wolf and Herbelein, 2003; Cauchi and Heuvel, 2006; Hardaway, 2010). Furthermore, the Drosophila eye is phenotypically easy to detect, tolerant to genetic manipulations and is dispensable for the survival of the fly (Chan and Bonini, 2000; Jeibman and Paulus, 2009). As a powerful genetic system, Drosophila provide the opportunity to carry out

large-scale genetic screens inexpensively and rapidly for mutations that influence related phenomena (Cauchi and Heuvel, 2006). The arsenal of genetic tools include the directed overexpression of transgenes under highly controlled conditions, genetic manipulations by transposon-based methods, and systems that allow directed gene expression.

**Making the Model of PD**

Modelling PD in *Drosophila melanogaster* relies upon two main approaches. The first approach depends upon the overexpression of wild-type or pathogenic forms of transgenes, resulting in a toxic gain-of-function (GOF) mechanism. The two genes *α-synuclein* (PARK1/4) and *Lrrk2* (PARK8) have been modelled in this way. The second approach is based on the genetic inhibition of the endogenous gene to target a loss-of-function (LOF) mechanism. *DJ-1*, *Pink1* and *parkin* models employ this approach (Celotto and Palladino, 2005; Lu, 2009). To achieve either GOF or LOF, transposons-mediated mutagenesis (Rubin and Spradling, 1982) and transgenic RNA interference (RNA*i*) (Fire *et al.* 1998; reviewed in Sharp, 2001) are used. These methods of gene manipulation aided by directed gene expression have made Drosophila one of the most powerful genetic model organisms.

**The UAS/GAL4 System**

The genetic system of choice when modelling PD in Drosophila is the bipartite UAS/GAL4 system in which the transgene is inserted downstream of an upstream activating sequence (UAS) and can be expressed in a tissue and time dependent manner under the control of the yeast transcriptional activator GAL4 (Brand and Perrimon, 1993). In the absence of GAL4, the gene is inactive. Usually Drosophila carrying the transgene

(UAS) is crossed to the flies expressing GAL4 under the control of a cell or tissue specific promoter and in this case the PD protein expression is restricted to only GAL4 expressing tissues. The GAL4 driver lines utilized in PD modelling include the dopaminergic neurons promoter *Ddc* (DOPA decarboxylase), the eye-specific promoter *GMR* (Glass Multimer Reporter), among many others (Gong and Golic, 2003). Defined UAS transgenic strains and Gal4 driver lines have enabled us to redefine how spatio-temporal research is performed in the fruit fly.

**Drosophila models of PD**

*The α-synuclein model*

The *α-synuclein* gene encodes a small, 140 amino acid, soluble, phosphorylated, presynaptic nerve terminal protein, which is the main component of Lewy bodies (LB) in both sporadic PD and FPD and its aggregation is believed to be the main neuropathogenic cause of PD (Feany and Bender, 2000; Michno *et al*, 2005). This production of insoluble protein aggregates has been implicated in neurotoxicity and PD pathogenesis (Feany and Bender, 2000; Auluck *et al.*, 2002; Singleton *et al.*, 2003). The aggregation of α-synuclein has been shown to mediate DA neuron toxicity and, specifically the non-β-amyloid component (NAC) is essential for the aggregation and resulting neurotoxicity in DA neurons. The C-terminal domain was shown to influence α-synuclein toxicity and aggregation (Periquet *et al.*, 2007). α-synuclein is phosphorylated on serine 129 and this selective and extensive phosphorylation of the serine 129 residue promotes fibril formation and contributes to PD pathogenesis (Fujiwara *et al.*, 2002). The mutation of serine 129 to alanine, which prevents its phosphorylation, completely suppressed DA

neuronal loss and its conversion to aspartate, which mimics phosphorylation, increased α-synuclein toxicity. The phosphorylation of serine 129 by the G protein coupled receptor kinase 2 (GPRK2) increased α-synuclein selective neurotoxicity (Chen and Feany, 2005). The role of protein toxicity in disease pathogenesis is clearly exemplified by the aggregation of this protein resulting in PD.

Although *Drosophila* seems to lack a clear *α-synuclein* orthologue, the overexpression of human wild type and two mutant forms of α-synuclein, A30P and A53T (Feany and Bender, 2000), was able to reproduce the key features of PD such as, adult-onset degeneration of DA neurons, filamentous intraneural inclusions containing α-synuclein, and locomotor dysfunction. The directed expression of *α-synuclein* resulted in flies that were viable, accumulated aggregated α-synuclein in perinuclear and neuritic filamentous inclusions similar to Lewy bodies and Lewy neurites, age–dependent loss of dorsomedial DA neurons, neuronal degeneration, age-dependent loss of climbing ability and retinal degeneration (Feany and Bender, 2000; Auluck *et al.,* 2002). These features taken together showed a remarkable model system for understanding the pathophysiology of PD.

In early studies, the directed expression of *Hsp70*, a molecular chaperone up-regulated in stress responses that refolds misfolded proteins, mitigates DA neuronal loss induced by *α-synuclein* in a PD model (Auluck *et al.,* 2002). This chaperone machinery protection results from either α-synuclein refolding or the augmentation of Hsp70 pathway after α-synuclein interference of chaperone activity, possibly through their sequestration. Several studies have been able to suppress *α-synuclein* induced phenotypes

in Drosophila PD models. Notable is the coexpression of the *α-synuclein* transgenes with *parkin*, which reduces retinal degeneration in the developing eye, improves the climbing ability of ageing flies when coexpressed in the DA neurons and slightly increases their survival (Haywood and Staveley, 2006). The overexpression of PTEN induced putative kinase1 (*Pink1*) resulted in the rescue of the α-synuclein-induced phenotypes of premature loss of climbing ability, degeneration of the ommatidial array and developmental defects of the eye (Todd and Staveley, 2008). Investigation of the interaction of α-synuclein with other proteins seems to be an area attracting much attention in formulating therapies and in managing PD.

The coexpression of *Rab1*, a guanosine triphosphatase, with α-synuclein transgenic flies was sufficient to rescue DA neuronal loss (Cooper *et al.*, 2006). α-synuclein was shown to block ER–Golgi vesicular trafficking and the ability of overexpression of *Rab1* to rescue the synucleinopathy might indicate the disruption of basic cellular functions in PD pathogenesis. The inhibition of Sirtuin2, a histone deacetylase (HDAC), by Adenylate kinase 1 or the guanylate kinase, AGK2 rescues α-synuclein-mediated toxicity of dorsomedial DA neurons (Outeiro *et al.*, 2007). The complexity of protein-protein and organelle interactions is an area that needs elucidation, as shown by the ability of α-synuclein to interact with a wide range of proteins and implicate protein toxicity as a major defect in most diseases including PD.

Exposure of *α-synuclein* flies to hyperoxia treatment results in neurotoxicity and DA neuronal degeneration (Botella *et al.*, 2008). This neurodegeneration is a result of DA neurons being specifically sensitive to hyperoxia-induced oxidative stress. The

coexpression of human *Cu/Zn superoxide dismutase* (SOD) with *α-synuclein* A30P reduced the observed neurodegeneration. Lowering the cytoplasmic level of oxidative stress confers protection to DA neurons.

The treatment of *α-synuclein* fly models with certain pharmacological agents such as L-DOPA restored the PD phenotype to normal, and the dopamine agonists pergolide, bromocriptine and SK & F 38393 were also substantially effective. Atropine was found to be effective but to a lesser extent than the other antiparkinson compounds (Pendleton *et al.,* 2002). Indeed, a plethora of proteins and compounds are under investigation but none is as promising as those with a direct role in mitochondrial function.

Genomic investigation of the transcriptional program of *α-synuclein* PD models at pre-symptomatic, early and advanced stages revealed 51 signature transcripts including lipid, energy & membrane mRNAs that were highly distinct and either up-regulated or down-regulated in common cellular pathways (Scherzer *et al.,* 2003). When quantitative proteome analysis of the pre-symptomatic A53T *α-synuclein* Drosophila model of PD was performed, several proteins associated with membrane, ER, actin cytoskeleton, mitochondria and ribosome were found to be either up-regulated or down-regulated (Xun *et al.,* 2008). These variations show the complexity of biological systems and offer novel routes to study the etiology of PD.

*The LRRK2 models*

Leucine rich repeat kinase 2 (*LRRK2*) or dardarin encodes a large 2527 amino acid, multi-domain 280 kDa protein belonging to the ROCO protein super family that contains a leucine rich repeat (LRR) domain, a protein kinase domain of the MAPKKK family, a

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Rho/Ras-like GTPase domain, as well as a WD40 repeat domain (Paisan-Ruíz *et al.*, 2004; Zimprich *et al.*, 2004; West *et al.*, 2005). An additional domain C-terminal to the GTPase domain, termed carboxy-terminal of Ras (COR), has been described too. LRRK2 is a multiple function protein, with these domains being implicated in a host of cellular processes including transformation, focal adhesion, enzyme inhibition, cellular trafficking, stimulation of stress activated kinase (SAPK) among others. The LRRK2 MAPKKK domain has been implicated in the most PD cases, indicating alteration of enzymatic phosphorylation in the pathology of this gene (Taylor *et al.*, 2006; Liu *et al.*, 2008). The increased phosphorylation results from a GOF and leads to the observed neurotoxicity.

Mutations in *LRRK2* gene cause autosomal dominant PD and are present in almost all of the functional domains. This observation, in addition to lack of deletions and truncations along with dominant inheritance, is consistent with a GOF mechanism. The precise physiological activity of this protein is not known, but the presence of multiple functional domains suggests its involvement in a wide variety of cellular functions possibly related to the mitochondria (Paisan-Ruíz *et al.*, 2004; Zimprich *et al.*, 2004; Banerjee *et al.*,2009). The pleomorphic pathology of LRRK2-linked PD has led to the hypothesis that LRRK2 lies in a pathway upstream of other proteins implicated in the pathogenesis of not only PD, but other neurodegenerative diseases (Ross *et al.*, 2006; Taylor *et al.*, 2006) showing α-synucleinopathy and tauopathy and no direct interaction between Lrrk2 and either α-synuclein or tau proteins (Rajput *et al.*, 2006). The multiple active domains in this protein confers additional functions that require a closer look when

investigating PD.

Drosophila has a single orthologue of *LRRK2*, which contains highly conserved motifs, the LRR domain serine/threonine kinase domain, Ras of complex proteins (ROC) domain and the GTPase domain (Lee *et al.*, 2007). In adult flies, the highest expression levels of the *Lrrk* transcript are in the head, indicating a potential role in the fly brain (Lee *et al.*, 2007). *Lrrk* shares transcriptional regulation with human *LRRK2*.

*The Parkin model*

The human parkin gene encodes a 465 amino acid protein containing an N-terminal ubiquitin domain, a central linker region and a C-terminal RING domain composed of two RING finger motifs separated by an in-between RING (IBR) domain (Giasson and Lee, 2001; Betarbet *et al.*, 2005). It functions as an E3 ubiquitin ligase that targets misfolded proteins to the ubiquitin proteasome pathway (UPS) for degradation (Kitada *et al.*, 1998) and loss of this function, due to mutation, leads to autosomal recessive early onset PD (AR-Juvenile Parkinsonism).

Mutant parkin flies are viable, show reduced longevity, a slight developmental delay, male sterility from a defect in spermatogenesis, locomotor defects due to apoptotic muscle degeneration (Greene *et al.*, 2003), reduced body size and cell size, sensitivity to oxidative and environmental stress (Pesah *et al.*, 2004) and loss and degeneration of DA neurons in the protocerebral posterior lateral (PPL) 1 cluster in the adult brain (Whitworth *et al.*, 2005). Overexpression of a human *parkin* mutant (R375W) in Drosophila results in an age-dependent degeneration of specific DA neuronal clusters, concomitant locomotor deficits that accelerate with age and rotenone toxicity susceptible flies. The flies exhibit

prominent pleomorphic mitochondrial abnormalities in their flight muscles (Wang *et al.,* 2007). The examination of the male germ line and the indirect flight muscles (IFM) in parkin null flies revealed mitochondrial defects and/or dysfunction as a common characteristic of pathology in these distinct tissues.

*The Pink1 models*

Human Phosphatase and tensin homologue (PTEN) induced kinase1 (*Pink1*) is a 581 amino acid protein with an N-terminal mitochondrial targeting signal sequence (MTS) and a highly conserved serine/threonine protein kinase domain of the $Ca^{2+}$ calmodulin family (Thomas and Beal, 2007). Mutations in *Pink1* gene have been implicated in AR-early onset PD (Valente *et al.,* 2004; Beilina *et al.,* 2005) with disease pathogenesis probably due to loss of its kinase activity and subsequent protective role.

Drosophila contains a single homologue, encoding a 721 amino acid protein of 80 kDa, with both a mitochondrial targeting motif and serine/threonine kinase domain. Drosophila *Pink1* is found to localize in the mitochondria and *Pink1* transcripts were detectable at all developmental stages with high levels in the adult brain and testes. The human *Pink1* and *Drosophila melanogaster Pink1* show significant homology and functional conservation (Clark *et al.,* 2006; Park *et al.,* 2006). Drosophila *Pink1* PD models were generated by transgenic RNA*i* and transposon-mediated mutagenesis approaches. The resulting *Pink1* flies have a host of phenotypes including viability, abnormally positioned wings, male sterility, short lifespan, apoptotic degeneration, mitochondrial defects, energy depletion, increased sensitivity to multiple stresses including oxidative stress, indirect flight muscle degeneration, ommatidial and DA neuron

degeneration and finally locomotor defects. These phenotypes were remarkably similar to those found in parkin mutant flies (Petit *et al.,* 2005; Clark *et al.,* 2006; Park *et al.,* 2006; Yang *et al.,* 2006; Wang *et al.,* 2006; Dodson and Guo, 2007). It is the similarity between the phenotypes of parkin and *Pink1* flies that led to the suggestion that the two proteins function in the same pathway.

The sterility in *Pink1* mutant flies was due to mitochondrial defects in the spermatids, showing vacuolated nebenkerns and individualization defects. This male sterility was rescued upon expression of Drosophila *Pink1*, human *Pink1* and *parkin*. Pink1 indicatively had a role in spermatogenesis to regulate mitochondrial morphology (Clark *et al.,* 2006). Mitochondrial dysfunction was also implicated in locomotor deficit and IFM degeneration with the IFM having disorganized myofibrils that were highly vacuolated with swollen impaired mitochondria. In addition, these mitochondria had low levels of mtDNA, mitochondrial proteins, ATP and had fragmented cristae. Expression of Drosophila *Pink1*, human *Pink1*, and *parkin* restored the muscle integrity with normal mitochondrial ATP levels and myofibril morphology (Clark *et al.,* 2006; Park *et al.,* 2006; Yang *et al.,* 2006). This indicated that suppression of Drosophila *Pink1* could lead to age-dependent muscle degeneration characterized by extensive mitochondrial dysfunction and DNA fragmentation indicative of apoptotic cell death.

*The DJ-1 models*

*DJ-1* encodes a small, 189 amino acid protein that is ubiquitously expressed and highly conserved with homology to proteases, kinases and small heat shock proteins. It belongs to the ThiJ/ Pfp1 protein superfamily (Thomas and Beal, 2007). It is associated

with various cellular functions that include a redox-sensitive molecular chaperone that is activated in an oxidative cytoplasmic environment, an antioxidant action by scavenging for ROS, ability to stabilize the antioxidant transcriptional master regulator nuclear factor erythroid 2-related factor (*Nrf-2*) by preventing its association with its inhibitor Keap1 and its eventual ubiquitination, ability to increase cellular levels of glutathione by activating the glutamate cysteine ligase, cellular transformation by associating with activated Ras, transcriptional regulation, RNA helicase binding, RNA binding, androgen receptor signalling, spermatogenesis and fertilization, phosphatidyl inositol 3-kinase/Akt signalling through regulation of the tumour suppressor PTEN, and modulation of P53 and thus control of cell death and survival (Kim *et al.*, 2005; Menzies *et al.*, 2005; Yang *et al.*, 2005; Lev *et al.*, 2006; Da Costa, 2007; Dodson and Guo, 2007). It is possible that mutations in DJ-1 affecting its antioxidant function may contribute to PD pathogenesis.

Drosophila possess two homologues of the human *DJ-1* gene designated *DJ-1α* and *DJ-1β*. *DJ-1α* is expressed predominantly in the testes and *DJ-1β* is present in most tissues. *DJ-1β* expression resembles the human *DJ-1* expression pattern (Menzies *et al.*, 2005; Yang *et al.*, 2005). In order to analyze their role, classical genetics and RNA*i* were used to generate mutants that phenotypically were viable, fertile, had a reduced lifespan and showed no pre-adult lethality or defects (Menzies *et al.*, 2005; Meulener *et al.*, 2005; Park *et al.*, 2005; Yang *et al.*, 2005; Lavara-Culebras and Paricio, 2007).

*The Pharmacological models*

Although major insights have been gained from FPD models using genes responsible for PD neuropathology, the etiology of sporadic PD remains unknown. A strong

association between sporadic PD and environmental toxins, especially mitochondrial complex I inhibitors such as rotenone, has been inferred from epidemiological studies. The sub-lethal chronic exposure of wild-type Drosophila flies to rotenone presented with characteristic locomotor dysfunction that was dose-dependent and a dramatic and selective loss of DA neurons in all of the brain clusters (Beal, 2001; Coulom and Birman, 2004; Jeibman and Paulus, 2009). Treatment of flies with paraquat led to impaired climbing capability and a short lifespan.

Treatment of flies with L-DOPA rescued the locomotor deficits but not neuronal death. In addition, the antioxidant melatonin alleviates both behavioral dysfunction and neuronal loss when co-exposed with rotenone (Coulom and Birman, 2004). Treatment of paraquat-impaired flies with cannabinoid receptor agonists (CP 55,940) and a JNK signalling specific inhibitor could rescue the impaired locomotor functions and shortened longevity phenotypes (Jeibman and Paulus, 2009). In addition, several other molecules with antioxidant activity, such as vitamin C, are currently being researched in several Drosophila models of PD.

### The role of the mitochondria in cell death and PD

Apoptosis in mammals is regulated by proteins acting on the mitochondria or released from the mitochondria, which result in caspase (cysteinyl *asp*artate prote*ases*) activation. The role of mitochondria in Drosophila apoptosis is still under investigation (reviewed in Krieser and White, 2010). In all cell death, the activation of caspases is the most important step and seems to be highly conserved in all organisms from *C. elegans* and Drosophila to mammals (Kornbluth and White, 2005). Developmental cell death is

the most understood in Drosophila and requires the activation of caspases (Hay and Guo, 2006). The fly has 7 caspases, 3 long pro-domain "initiator" caspases and 4 "effector" caspases. The initiator caspase Dronc is required for most developmental apoptosis (Xu *et al.,* 2005). Thus far, apoptosis in all organisms seems to require the activation of caspases.

**Bcl-2 proteins and the Apoptotic machinery**

The Bcl-2 family proteins are key regulators for cell death and survival in metazoans and include more than 20 members including Bcl-2, a proto-oncogene. The Bcl-2 family is comprised of up to four conserved Bcl-2 homology (BH) domains designated BH1, BH2, BH3 and BH4, and corresponds to α-helical segments (Gross *et al.,* 1999; Chen and Abrams, 2000; Igaki and Miura, 2004; Quinn and Richardson, 2004; Doumanis *et al.,* 2007). They are made up of two subgroups, the multi-domain 3 or 4 BH domain family and the BH3-only domain family.

The group of multi-domain Bcl-2 proteins has proapoptotic and prosurvival members. The antiapoptotic proteins include Bcl-2, Bcl-X$_L$, Mcl-1, Bcl-w, and A1/Bfl-1. They are characterized by the presence of BH1 to BH4 domains and have been implicated in the maintenance of mitochondrial integrity to prevent cytochrome c release, an important component of the apoptosome. They prevent the escape of other apoptogenic factors found in the inter-membrane space of mitochondria (Gross *et al.,* 1999; Igaki and Miura, 2004; Schwartz and Hockenberry, 2006; Doumanis *et al.,* 2007). The antisurvival members include Bax, Bak and Bok and contain the BH1 to BH3 domains that are required for mitochondrial outer membrane (MOM) permeabilization. The BH3-only subfamily of Bcl-2 proteins is proapoptotic and seems to trigger apoptosis in response to

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developmental cues or cytotoxic damage (Cory and Adams, 2002; Schwartz and Hockenberry, 2006). Most of them act by binding to and neutralizing the antiapoptotic proteins. They include Bid, Bim, Bik, Bad, Bmf, Hrk, Noxa and Puma.

The ratio of proapoptotic to antiapoptotic Bcl-2 proteins may determine the susceptibility of a cell to a death signal. This is achieved through their ability to form homodimers as well as heterodimers, suggesting a neutralizing competition among these subsets of the Bcl-2 protein family. In addition, they are able to transform into integral membrane proteins (Gross *et al.*, 1999). Prior to death signals, these proteins localize to separate sub-cellular compartments. The proapoptotic members are found in the mitochondria, endoplasmic reticulum (ER) or the nuclear membrane (Gross *et al.*, 1999; Cory and Adams, 2002; Schwartz and Hockenberry, 2006; Doumanis *et al.*, 2007). It is believed that the Bcl-2 proteins control apoptosis by controlling caspase activation and by guarding mitochondrial integrity, thereby keeping enclosed a plethora of death activating molecules. The killer molecules include cytochrome c, AIF, endonuclease G, Smac/Diablo and HtrA2 (Gross *et al.*, 1999; Gaumer *et al.*, 2000; Cory and Adams, 2002; Richardson and Kumar, 2002; Bassik *et al.*, 2004; Schwartz and Hockenberry, 2006). Other Bcl-2 functions include inhibition of mitochondrial oxidative stress, regulation of ER $Ca^{2+}$ homeostasis, necrosis, and autophagic death.

Antiapoptotic proteins act to guard the mitochondrion, which is at the centre of the programmed cell death (PCD), from the proapoptotic members who possibly regulate mitochondrial morphological dynamics. These may be by interacting with fusion/fission factors and MOM permeabilization to release apoptogenic factors (Gross *et al.*, 1999;

Karbowski *et al.,* 2006; Brooks and Dong, 2007; Brooks *et al.,* 2007). The Bcl-2 proteins have been implicated in the regulation of cell cycle (Quinn and Richardson, 2004), and apoptosis through controlling apoptotic crosstalk between the mitochondria and the ER (Häcki *et al.,* 2000; Cory and Adams, 2002). It has been shown that apoptotic agents perturbing ER functions induce a novel crosstalk between the ER and mitochondria that can be interrupted by ER-based Bcl-2 proteins.

Cell death is important for embryogenesis, organ development and metamorphosis. Drosophila possesses a cell death regulatory machinery of complexity, similar to that of some mammals. It has most of the apoptotic pathway protein homologues that participate in the intrinsic and extrinsic cell death pathways, induced by p53 and Tumour necrosis factor (TNF) like ligand and receptor respectively. Drosophila has an Apoptotic protease activating factor (Apaf) called *Dark/Dapaf-1/HAC*; seven caspases including *Dredd/Dcp-2, Dronc* which are initiator caspases, *Dcp-1, drICE, DECAY* which are executioner caspases, *Strica*/Dream and *Damm*/Daydream; two *Bcl-2* homologues, *debcl* and *Buffy*; unique killer proteins, namely *reaper (rpr)*, head involution defective (*hid*) and *Grim*, and the caspase inhibitors -Drosophila inhibitor of apoptosis protein- *DIAP1/Thread* and *DIAP2. Sickle* is a killer protein found in Drosophila (Gaumer *et al.,* 2000; Richardson and Kumar, 2002; Igaki and Miura, 2004). The presence of these death/survival protein homologues in Drosophila has made it a very excellent model organism to study cell death and survival.

***Buffy* and *debcl*; Drosophila Bcl-2 homologues**

Drosophila possess two *Bcl-2* homologues, as identified from the Drosophila expressed sequence tag (EST) database, termed *debcl* and *Buffy* (Brachmann *et al.,* 2000; Colussi *et al.,* 2000; Igaki *et al.,* 2000; Zhang *et al.,* 2000). These two proteins share a high degree of similarity to the mammalian proapoptotic protein Bok/Mtd, pore-forming Bcl-2 proteins (Zhang *et al.,* 2000). Both proteins share the BH1, BH2 and the C-terminal transmembrane domains of the Bcl-2 protein family (Chen and Abrams, 2000; Richardson and Kumar, 2002; Igaki and Miura, 2004). Studies of these proteins in Drosophila employed directed gene expression in cell cultures and transgenic constructs, and utilized the GAL4/UAS system of spatiotemporal expression and RNA interference (Brachmann *et al.,* 2000; Colussi *et al.,* 2000; and reviewed in Richardson and Kumar, 2002). Several cellular processes have been studied, especially cell death, to ascertain the role of these proteins in Drosophila and the likely function of their orthologues in mammals.

*Debcl*

The debcl protein has BH1, BH2, BH3 and C-terminal transmembrane domains, and in some quarters, is believed to have a weak BH4 domain (Igaki *et al.,* 2000). The transmembrane domain is the membrane anchor (MA) used in localizing this protein to intracellular membranes). Consistent with its similarity to proapoptotic Bok/Mtd, *in vivo* and *in vitro* studies have shown debcl to be a killer protein. It induces cell death when overexpressed in mammalian and fly cell cultures and in fly tissues (Brachmann *et al.,* 2000; Colussi *et al.,* 2000; Igaki *et al.,* 2000; Zhang *et al.,* 2000; Senoo-Matsuda *et al.,* 2005; reviewed in Cory and Adams, 2002; Richardson and Kumar, 2002; Igaki and

Miura, 2004). The gene is regulated in various developmental stages and both subcellular fractionation and immunofluorescence confirmed it is associated with the outer mitochondrial membrane (MOM) via the MOM-targeting sequence.

The ectopic expression of *debcl* in the developing eye resulted in a rough-eye phenotype due to a loss in photoreceptor morphology as well as a reduced number or a complete loss of photoreceptor neurons (Igaki *et al.,* 2000). Furthermore, overexpression promoted apoptotic cell death, probably through a caspase-independent pathway but dependent on intracytoplasmic membrane localization of this protein. This *debcl*-induced cell death could not be antagonized by p35, a broad spectrum caspase inhibitor, though a different study found that p35 was able to suppress this cell death and was caspase-dependent (Colussi *et al.,* 2000). In addition, this protein genetically interacted with *DIAP1*, *Dark* and mammalian prosurvival Bcl-2 proteins. Debcl was shown to function in the apoptotic response to UV irradiation via the DNA damage response pathway (Brachmann *et al.,* 2000) and participate in stress-induced apoptosis (Sevrioukov *et al.,* 2007). Taken together, these findings show the importance of debcl in cell death.

The pan-neuronal knockdown of *debcl* results in lower locomotor activity and a shorter lifespan in adults, significantly lower ATP levels, increased neurotoxicity as exemplified by neurodegeneration and loss of photoreceptor neurons and retinal structure in Polyglutamine fly models (Senoo-Matsuda *et al.,* 2005). Intriguingly, overexpression of *debcl* suppressed polyQ-induced neurodegeneration including photoreceptor neurodegeneration and early adult mortality. Its down-regulation led to an accumulation of ubiquitinated proteins in Drosophila heads, suggesting that it may protect cells from

cytotoxicity induced by the disruption of proteasome function. In addition, its down-regulation enhanced mitochondrial dysfunction including inhibitor-induced loss of membrane potential, morphological abnormalities and an impairment of the mitochondrial respiratory function.

*Buffy*

Although highly conserved in BH1, BH2, and BH3, Buffy does not have an obvious BH4 domain as it has a prosurvival role as a debcl inhibitor (Quinn *et al.*, 2003). Buffy predominantly localizes to the ER and its N-terminus contains a functional nuclear localization signal (NLS). Without the membrane anchor, Buffy accumulates in the nucleus (Doumanis *et al.*, 2007). In a contrasting study, it was observed to localize to the mitochondria (Quinn *et al.*, 2003). In addition, its expression pattern correlates with that of *debcl* in development of Drosophila and that these two proteins physically interact.

RNA interference (RNA*i*) knockdown of *Buffy* results in embryonic apoptosis, whereas its overexpression inhibits both developmental PCD and ionizing radiation-induced apoptosis (Quinn *et al.*, 2003; reviewed in Quinn and Richardson, 2004). In a genetic epistasis study, Buffy was found to be downstream of the RHG proteins (*Rpr*, *Hid*, *Grim*) and upstream of the apical caspase *Dronc*. Moreover, *Buffy* overexpression could block caspase-dependent cell death. Interestingly, they showed that this protein could induce a $G_1$/early-S phase cell cycle arrest.

*Buffy* knockdown significantly suppresses the proteasome-induced cell death and rotenone- or 3-NP-induced cell death in a PolyQ fly model (Senoo-Matsuda *et al.*, 2005). Work with a Buffy mutant showed that irradiation stress-induced apoptosis mediated by

reaper was blocked by Buffy and resulted in a reduced response to ionizing radiation. They suggested that the Buffy-regulated antiapoptotic pathway was epistatic to the *debcl* proapoptotic pathway (Sevrioukov *et al.,* 2007). The observation that Drosophila Bcl-2 proteins were not required for normal development was disputed by the finding that they exerted a limited control over PCD and were required for pruning cells in the developing CNS (Galindo *et al.,* 2009). A role for debcl in RHG killing has not been shown but it is required for heterologous killing by the murine Bax. Buffy is involved in the protection of mitochondrial integrity and function (Park *et al.,* 2006). When *Buffy* was overexpressed, the Drosophila *Pink1* phenotypes including the levels of the mitochondrial DNA, mitochondrial proteins and ATP were remarkably restored. These are intriguing results as *Pink1* is one of the gene loci implicated in Parkinson disease and its localization is the mitochondria. This suggests a strong involvement and interplay of mitochondrial function with disease pathogenesis.

**Mitochondria Morphology and Dynamics in Cell Death**

Mitochondria show structural changes that include swelling, cristae alterations and fragmentation of the mitochondrial network during cell death (Martinou and Youle, 2006). These changes in flies are caspase-dependent . Swelling is likely due to the permeabilization of the outer mitochondrial membrane (Abdelwahid *et al.,* 2007; Goyal *et al.,* 2007; Means and Hays, 2007). Increased mitochondrial fragmentation is evident during PCD and involves Drp-1, a mitochondrial fission protein. This fragmentation occurs prior to caspase activation in Drosophila cells and an inhibition of the caspases does not block it (Goyal *et al.,* 2007). The alteration of mitochondrial dynamics in

apoptotic cells point to a role of these processes in cell death. Their contribution could be in releasing proapoptotic factors such as Cytochrome C from the mitochondria, mitochondria membrane permeabilization leading to defects in mitochondrial function, and mitochondrial fission sites acting as scaffolding for localization of apoptotic molecules (Karbowski *et al.,* 2002; Martinou and Youle, 2006). Taken together, these changes point to an important role for mitochondrial morphology as controlled by the fission/fusion machinery in cellular homoeostasis and disease.

**Mitochondrial factors and Apoptosis**

The unique killer proteins Reaper, Hid and Grim in Drosophila (RHG) have been shown to localize to the mitochondria (Abdelwahid *et al.,* 2007), which is important for the permeabilization of the mitochondrial membrane and for the effective activation of PCD.

Freed from the mitochondria, cytochrome C and Dronc have been shown to bind to Apaf-1 (Dark), which has both the CARD and WD40 domains required for similar activation in mammals (Kanuka *et al.,* 1999; Rodriguez *et al.,* 1999). Drosophila cytochrome C, Cyt-c-d, appears to be the major form found in germ lines and a loss of function leads to spermatid individualization defects (Arama *et al.,* 2003). Inhibitors of apoptosis (IAP) inhibitors in Drosophila are the RHG proteins, which are controlled by either transcriptional upregulation or by phosphorylation through the MAP kinase pathway (Oberst *et al.,* 2008). HtrA-2/Omi can also bind IAPs and is released from the mitochondria during apoptosis. It can bind DIAP and promote its cleavage and degradation (Challa *et al.,* 2007). Apoptosis inducing factor (AIF) is another

mitochondrial protein that translocates from the mitochondria to the nucleus upon activation of apoptosis and is involved in DNA fragmentation (Joza et al., 2008). These plethora of death activating molecules are released from the mitochondria and seem to play a significant role towards the fate of the cell in life and death decisions.

**Buffy and debcl activity at the Mitochondria**

Buffy protects against mitochondrial damage in *Pink1* LOF mutations by suppressing the resulting phenotypes (Park et al., 2006). When *Buffy* was overexpressed, the *Pink1* phenotypes, excepting flight dysfunction, were remarkably restored, including the levels of mtDNA, mitochondrial proteins and ATP. These results suggested a strong involvement of mitochondrial dysfunction in PD pathogenesis and the Bcl-2 protein family in the protection of mitochondrial integrity and function.

**PD genes at the Mitochondria**

Parkin genetically interacts with components of the mitochondrial fission/fusion machinery in testes and IFM and may be involved in the regulation of mitochondrial integrity (Deng et al., 2008). Furthermore, the knockdown of mitochondrial assembly regulator factor (*Marf*, a mitofusin orthologue in Drosophila) or Optic atrophy 1 (*Opa1*) or the overexpression of dynamin related protein 1 (*drp1*) rescued the phenotypes of muscle degeneration, cell death and mitochondrial abnormalities in parkin mutants. Parkin is selectively recruited to dysfunctional mitochondria with low membrane potential (Narendra et al., 2008) and promotes autophagy of damaged mitochondria, implicating a failure to eliminate dysfunctional mitochondria in the pathogenesis of PD.

DJ-1β mutants have been shown to be sensitive to oxidative stress and when exposed

to paraquat displayed a severe loss of locomotor ability due to mitochondrial dysfunction (Park *et al.,* 2005). This implicates DJ-1 in a defensive role against oxidative stress in the mitochondria especially since DJ-1 localizes to the mitochondria.

Parkin and Pink1 may function in a common pathway that protects cells against mitochondria-dependent cell death induced by toxic insults. The suppression of *Pink1* loss of function phenotypes by parkin expression was not due to a general protective role but a specific one against mitochondrial dysfunction (Clark *et al.,* 2006; Park *et al.,* 2006; Yang *et al.,* 2006; reviewed in Dodson and Guo, 2007; Poole *et al.,* 2008; Banerjee *et al.,*2009). In general parkin acts downstream of Pink1 to maintain mitochondrial integrity and function. Indeed, Pink1 was recently shown to control the localization of parkin to the mitochondria by direct phosphorylation on its linker region and this phosphorylation enables parkin to translocate to the mitochondria (Kim *et al.,* 2008). The Pink1/ parkin pathway has been shown to play fundamental roles in regulating mitochondrial biogenesis or mitochondrial dynamics, such as fission/ fusion events.

The heterozygous LOF mutations in *drp1*, which encodes a key mitochondrial fission-promoting component (Frank *et al.,* 2001), are largely lethal in a Pink1 and parkin mutant background (Poole *et al.,* 2008). The overexpression of *drp1* or knockdown of *Opa1* and *Mfn2*, two mitochondrial fusion-promoting factors, suppressed the flight muscle degeneration, cell death and mitochondrial morphology defects induced by Pink1 or parkin mutations. In addition, an eye phenotype resulting from increased activity of the Pink1/ parkin pathway is suppressed by perturbations reducing mitochondrial fission or enhanced by perturbations reducing mitochondrial fusion. Mitochondrial morphology is

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maintained by a dynamic balance between the opposing actions of mitochondrial fusion-controlled by *Marf* and *Opa1* and mitochondrial fission- controlled by *drp1*. Genetic interactions exist between Pink1 and the testes specific mitofusin fly homologue Fuzzy onion (*Fzo*) and between Pink1 and *drp1* in the male germ line and IFM (Deng *et al.*, 2008). The role of mitochondrial dynamics in PD pathology show the important role this cellular activity plays in the balance between cell death and survival.

A study carried out in our lab showed that overexpression of Drosophila *Pink1* results in the rescue of *α-synuclein* induced phenotypes of premature loss of climbing ability, degeneration of the ommatidial array and developing eye defects (Todd and Staveley, 2008). The recent identification of Htra2/Omi as a Pink1 substrate (Plun-Favreau *et al.*, 2007) and possibly its involvement in the Pink1/ parkin pathway together with another mitochondrial localized protein, Rhomboid-7 (Whitworth *et al.*, 2008) suggests the importance of this organelle in the pathophysiology of PD. A host of other proteins are being investigated for their possible role in the maintenance of mitochondria integrity and PD etiology, mostly proteins involved in autophagy and mitophagy.

RATIONALE

The discovery of mitochondrial defects in most cases of PD has prompted research into the role of proteins with a function in the mitochondria. Where Bcl-2 proteins likely play a role as guardians of the mitochondria, we conducted this study to analyze whether the PD-like phenotypes seen in the *α-synuclein* model would actually be influenced by overexpression of the two known *Bcl-2* homologues, *Buffy* and *debcl* in the dopaminergic neurons and in the developing compound eye of *Drosophila melanogaster*.

## Materials and Methods

### Drosophila media and culture

Stocks and crosses were maintained on a standard medium containing 65 g/L cornmeal, 50 ml/L molasses, 10 g/L yeast, 5.5g/L agar and ~900 ml/L water. Fresh food was prepared by Dr. Brian E. Staveley approximately twice a month and treated with 2.5 ml/L propionic acid and 5 ml/L of 10% in ethanol methylparaben to prevent growth of mold. Seven millilitre aliquots of media were poured into vials, allowed to solidify, and refrigerated at 4°C to 6°C. Stocks were maintained on solid media for two to three weeks before transfer onto new media to reculture. Stocks were kept at room temperature (22 ± 2°C) while crosses and experiments are carried out at 25°C and 29°C.

### Drosophila stocks

UAS-Buffy (Quinn et al., 2003) was generously provided by Dr. Leonie Quinn (University of Melbourne), $y^I w^{67c23}$; $P \{EPgy2\} debcl^{EY09743}$, and $y^I w^{67c23}$; $P \{EPgy2\} Buffy^{EY11259}$ (Bellen et al., 2004) were from Bloomington Drosophila Stock Center (Table 2). UAS-α-symuclein (Feany and Bender, 2000) was generously provided by Dr. M. Feany of Harvard Medical School. The standard line $w^{1118}$ was received from Dr. Howard Lipshitz (University of Toronto), the standard lines UAS-GFP and UAS-lacZ$^{4-1-2}$ were obtained from the Bloomington Drosophila Stock Center. Dr. J. Hirsch (University of Virginia) generously provided Ddc-Gal4 flies (Li et al., 2000), and GMR-GAL4$^{12}$ flies (Freeman, 1996) were obtained from the Bloomington Drosophila Stock Center at Indiana

University.

*Derivative lines*

The *UAS-α-synuclein/CyO; Ddc-GAL4/TM3* was generated and tested by Dr. Brian Staveley using standard homologous recombination methods and was used to overexpress *α-synuclein* in the dopaminergic neurons using the dopa decarboxylase (*Ddc*) driver. The *GMR-GAL4 UAS-α-synuclein/CyO* line was generated by Dr. Brian Staveley and tested by myself and was used to overexpress *α-synuclein* in the developing eye using the Glass Multiple Reporter (*GMR*) driver. PCR reactions and gel electrophoresis were used for analysis of recombination events.

**Table 2: Genotypes of fly stocks and crosses used in the study.**

| GENOTYPE | ABBREVIATION | EXPRESSION | BALANCER | REFERENCE |
|---|---|---|---|---|
| CONTROL LINE | | | | |
| *w; UAS-lacZ[4-1-2]* | *lacZ* | | | Brand *et al.* 1994 |
| DRIVER LINES | | | | |
| *w; GMR-GAL4[12]* | *GMR-GAL4* | Eye | | Freeman, 1996 |
| *w; Ddc-GAL4* | *Ddc-GAL4* | Dopaminergic neurons | | Li *et al.*, 2000 |
| EXPERIMENTAL LINES | | | | |
| *UAS-Buffy.Q* | *UAS-Buffy.Q* | | | Quinn *et al.*, 2003 |
| *y[1]w[67c23]; P {EPgy2} Buffy[EY11259]* | *Buffy[EY11259]* | | | Bellen *et al.* 2004 |
| *y[1]w[67c23]; P {EPgy2} debcl[EY05741]* | *debcl[EY05741]* | | | Bellen *et al.* 2004 |
| *w; UAS-α-synuclein* | *α-synuclein* | | | Feany and Bender, 2000 |
| DERIVATIVE LINES | | | | |
| *w; UAS-α-synuclein/CyO; Ddc-GAL4/TM3* | *α-synuclein;Ddc-GAL4* | Dopaminergic neurons | CyO; curly wings (Curly) | |
| *w; GMR-GAL4 UAS-α-synuclein/CyO* | *α-synuclein; GMR-GAL4* | Eye | TM3; Tubby body and short bristles (Stubble) | |

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*DNA extraction*

Between 10 and 15 adult flies were collected and frozen at -80°C overnight, and DNA was extracted (Gloor *et al.*, 1993). The flies were placed in 500 µl of a simple DNA extraction or "squishing" buffer with proteinase K and completely homogenized. The tubes were incubated at 37°C for 30 minutes, 250 µl of phenol and 250 µl of chloroform were added and mixed by inversion and centrifuged at 4°C at 10,000 rpm for 10 minutes. The top aqueous layer was collected and 1 ml of 95% ice cold ethanol and 10 µl of 3M sodium acetate were added and precipitated at -20°C for 1 hour (or overnight). The tubes were centrifuged at 4°C at 10,000 rpm for 15 minutes and the supernatant discarded then pellets were washed with 250 µl of 70% ethanol and further centrifugation at 4°C at 10,000 rpm for 3 minutes was done. Pellets were retained and the tubes spun down for 30 seconds, and the pellets were left to dry in the air, resuspended in 500 µl of $ddH_2O$ and stored at -20°C.

For rapid DNA extraction, a secondary method was applied which involved squishing one fly in 50 µl of $ddH_2O$ under ice. The tube was centrifuged at 4°C at 10,000 rpm for 15 minutes and the supernatant was collected by pipetting out the top layer and 1 to 5 µl was used directly in the PCR reaction.

*Determination of DNA quality and quantity*

The DNA extracted was qualified and quantified using the NanoDrop spectrophotometer (Thermo Scientific) that shows the concentration (ng/µl) and purity of the DNA by using the 260/280 ratio. The NanoDrop was first blanked using 2 µl of

ddH$_2$O and then 2 µl of DNA sample was loaded, readings for the concentration (ng/µl) and quality (260/280 ratio) were taken in triplicates and averaged and then recorded (Table 3).

*Analysis of the derivative lines*

The *GMR-GAL4/ UAS-α-synuclein/CyO* line was tested by using a PCR reaction to determine the amplification of DNA products from primers designed from the *Homo sapiens* synuclein, alpha (non A4 component of amyloid precursor) (SNCA), transcript variant 1 mRNA, NCBI reference sequence: NM_000345.3 using the NCBI primer design tool (Table 3).

The PCR reaction master mix was prepared by adding 5 µl of DNA to 5 µl of 10X PCR buffer (Qiagen), 2 µl of dNTPs, 2 µl of the forward primer, 2 µl of the reverse primer (Invitrogen), 2 µl of 25 mM MgCl$_2$ ions (Qiagen), 30 µl of ddH$_2$O and 2.1 µl of HotStarTaq Plus DNA polymerase to form 50 µl volumes. The PCR thermal cycler profile used is shown in Table 4.

The appropriate PCR product was determined by loading a mixture of 2 µl of 6X gel loading dye (NE BioLabs) and 10 µl of PCR product on a 1.5% agarose gel with 4 µl of 10 µg/ml ethidium bromide per 100 ml agarose. 2-5 µl of 1 mg/ml 2-log DNA ladder (NE BioLabs) was added and the gel electrophoresed at 120 V for 45 minutes. The gel was visualized in a UV transilluminator ChemiImager 4400 Ready (Alpha Inotech Corporation).

**Table 3. The primers designed to detect the presence of _α-synuclein_**

|  | Forward Primer | Reverse Primer |
|---|---|---|
| Sequence (5' to 3') | TGTGCCCAGTCATGACA TTT | CCACAAAATCCACAGC ACAC |
| Length | 20 nucleotides | 20 nucleotides |
| Melting temperature (Tm) | 60.0 | 60.0 |
| Percent GC content | 45.0 | 50.0 |
| Initial concentration (nM) | 165.4 | 133.7 |
| Volume added to make 200 μM (μL) [Volume A] | 827.0 | 668.0 |
| Volume A used to make 10 μM working stock (μL) | 10.0 | 10.0 |
| Volume ddH$_2$O added to volume A (μL) | 190.0 | 190.0 |
| Total volume working stock (μL) | 200.0 | 200.0 |

**Table 4. The PCR thermal profile used to detect _α-synuclein_**

| Thermal Cycler Conditions | Temperature (°C) | Duration (minutes) |
|---|---|---|
| Hot start | 95.0 | 5.0 |
| 3 Step cycling -Denaturation | 93.0 | 0.5 |
|        -Annealing | 60.0 | 0.5    35 CYCLES |
|        -Elongation | 72.0 | 4.0 |
| Final extension | 72.0 | 10.0 |
| Hold | 4.0 | |

*Ageing Analysis*

Several single vial matings of three to five females plus three to five males were made of each genotype. A cohort of adult heterozygous male flies were collected upon eclosion. Approximately two hundred to four hundred flies were aged per genotype, at a density of $\leq 20$ flies per vial. Adults were kept on fresh media replenished every other day. Flies were observed and scored every two days for presence of deceased adults. Flies were considered dead when they did not display any movement upon agitation (Staveley *et al.*, 1990). Longevity data was analyzed using the GraphPad Prism 5.0 software. Survival curves were compared using the log-rank test, a statistical test that compares the actual and expected number of failures (death) between survival curves at each individual failure event. Significance was determined at 95%, at a P-value less than or equal to 0.05 with Bonferroni correction of the family wise P value.

*Climbing Assay*

Several single vial matings of three to five females plus three to five males were made of each genotype. A cohort of adult heterozygous male flies were collected upon eclosion and scored for their ability to climb (Todd and Staveley, 2004). Adults were kept on fresh media replenished every other day. Every 7 days, 50 males from every genotype were assayed for their ability to climb 10 centimetres in 10 seconds in a clean climbing apparatus. Data was collected over a period of 90 days or until all the flies being assayed were all dead. Flies were maintained on standard cornmeal-yeast-molasses-agar medium

at 25°C. Climbing analysis was performed using the GraphPad Prism 5.0 statistical software. Climbing curves were fitted using non-linear regression and compared using a 95% confidence interval with a 0.05 P-value.

### *Scanning Electron Microscopy of the Drosophila eye*

Several single vial matings of three to five females plus three to five males were made of each genotype at 29°C and a cohort of adult heterozygous male flies collected upon eclosion and aged for three days on standard cornmeal-yeast-molasses-agar before being frozen at -80°C. Whole flies were mounted on SEM studs, desiccated overnight and coated in gold before photography at 170X magnification with a Hitachi S-570 scanning electron microscope was done. For each cross at least 20 eye images were analyzed using the NIH ImageJ software (Abramoff et al. 2004) and biometric analysis was performed.

**Results**

*Overexpression of Bcl-2 genes in the dopaminergic neurons*

The key manifestation of PD is the age-dependent degeneration of the DA neurons. The selective death and degeneration of these neurons led us to investigate the effects of *Buffy* and *debcl* in these neurons. These genes were overexpressed in the DA neurons to assess whether they had any effect on the DA neurons. A standard line, that overexpressed *lacZ* was used as a control for examining the effects on either ageing or climbing.

**Lifespan of standard and experimental lines**

The ageing analysis was carried out in parallel to the climbing assays in order to account for changes in climbing ability as a result of premature senescence. The experimental and control lines were crossed to the driver line (*Ddc-GAL4*) to overexpress the *Bcl-2* homologues and *lacZ* in the DA neurons. The results indicated there was a significant difference in the longevity of the flies when the *Buffy* transgenes were overexpressed in the DA neurons (Figure 1). The median lifespan was 62 days for the control flies and 64 days for the *Buffy* expressing (Table 5). The log-rank test showed the curves were significantly different (P<0.0001) from the control curve. The results show that overexpressing both *Buffy^{EY11259}* and *UAS-BuffyQ* increases the lifespan of these flies, while overexpressing *debcl* has no significant effect on longevity of these flies.
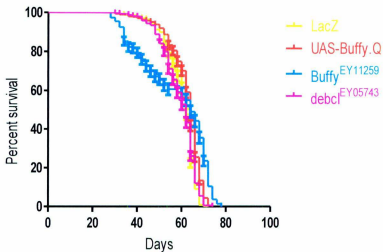
**Figure 1: Lifespan when *Buffy* and *debcl* are overexpressed in the dopaminergic neurons.** Directed overexpression of *Buffy^EY11259* and *UAS-Buffy.Q* in the dopaminergic neurons increases longevity. Longevity of flies overexpressing *UAS-Buffy.Q* (n=313) and *Buffy^EY11259* (n=267) in the neurons is significantly increased compared to *UAS-lacZ* control (n=321). Overexpressing *debcl^EY05743* had no significant effect on longevity when compared to control flies. Longevity is shown as percent survival (P < 0.01, determined by log-rank). The genotypes are *Ddc-GAL4/UAS-lacZ, UAS-Buffy.Q; Ddc-GAL4, Buffy^EY11259; Ddc-GAL4* and *debcl^EY05743; Ddc-GAL4.*

**Table 5. The Log-Rank (Mantel-Cox) curve comparison of directed expression of _Bcl-2_ genes in the dopaminergic neurons.** Survival curves were analyzed using log-rank test.

| Genotype | # of Deaths | Median Survival (Days) | Chi Square | P value |
|---|---|---|---|---|
| _UAS-lacZ_ | 321 | 62 | n/a | n/a |
| _UAS-Buffy.Q_ | 313 | 64 | 36.53 | <0.0001 |
| _Buffy^{F31I239}_ | 267 | 64 | 51.13 | <0.0001 |
| _debcl^{E105743}_ | 341 | 62 | 0.09 | ns |

**Climbing analysis of the standard and experimental lines**

The locomotor ability assay was carried out to investigate whether the overexpression of *Bcl-2* genes in the dopaminergic neurons would have an impact on these sensitive neurons. The result indicated that there was no statistically significant difference when the control (*UAS-lacZ*) was compared to either *UAS-Buffy.Q* or *debcl*[E3057-43], but was significant when compared to *Buffy*[E3/1259] (Figure 2). The *Buffy*[E3/1259] flies climbed for longer than the other *Bcl-2* overexpressing flies and lost their climbing ability later (Table 6) with a P<0.0322.
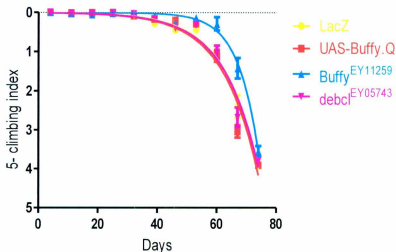
**Figure 2: Climbing analysis when *Bcl-2* homologues are overexpressed in the dopaminergic neurons.** Directed overexpression of *Buffy*[EY11259] resulted in a significant increase in climbing ability as determined by non-linear fitting of the climbing curves and comparing at 95% confidence interval. *UAS-Buffy.Q* and *debcl*[EY05743] had no significant impact on the locomotor ability when overexpressed in dopaminergic neurons. The genotypes are *Ddc-GAL4/UAS-lacZ, UAS-Buffy.Q; Ddc-GAL4, Buffy*[EY11259]*; Ddc-GAL4* and *debcl*[EY05743] *; Ddc-GAL4.*

**Table 6. A comparison of climbing index curves for the directed overexpression of the *Bcl-2* in the dopaminergic neurons.** The 95% confidence interval was compared between the *UAS-lacZ* flies and the *Bcl-2* flies.

| Genotype | # of flies | Mean of difference | 95% CI | R square | P value | Significance |
|----------|-----------|--------------------|--------|----------|---------|--------------|
| *UAS-lacZ* | 50 | n/a | n/a | n/a | n/a | n/a |
| *UAS-Buffy.Q* | 50 | -0.02 | -0.176 to 0.128 | 0.01 | 0.73 | ns |
| *Buffy*<sup>EY11259</sup> | 50 | 0.25 | 0.026 to 0.47 | 0.38 | 0.03 | significant |
| *debcl*<sup>EY05743</sup> | 50 | 0.01 | -0.058 to 0.085 | 0.02 | 0.69 | ns |

***Overexpression of Bcl-2 genes in the α-synuclein model of PD***

The coexpression of the experimental lines with α-synuclein (*UAS-α-synuclein/CyO; Ddc-GAL4/TM3*) was undertaken to determine the effects of overexpressing the *Bcl-2* homologues *Buffy* and *debcl$^{E05741}$* in the dopaminergic neurons when coexpressed with α-synuclein. Both ageing and climbing ability were analyzed and compared to results obtained in α-synuclein expressing control flies.

**Ageing analysis of the standard and experimental lines in the α-synuclein model**

The coexpression of the *Bcl-2* homologues with α-synuclein had significantly different survival curves (Figure 3). The coexpression of *UAS-Buffy.Q* with α-synuclein in the dopaminergic neurons showed an increase in the lifespan of these flies, but no difference was seen when coexpressed with *Buffy$^{EY11239}$* (Table 7). Strikingly, the coexpression of debcl$^{EY05745}$ with α-synuclein led to a significant increase in the lifespan of these flies.
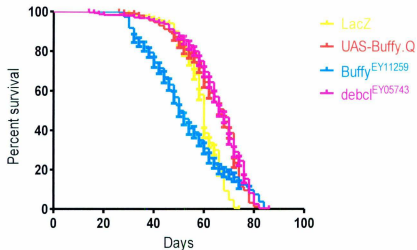
**Figure 3: Longevity of flies coexpressing *α-synuclein* and *Bcl-2* family members in the dopaminergic neurons.** Directed overexpression of *UAS-Buffy.Q* and *debcl*<sup>EY05743</sup> in the DA neurons increases longevity whereas flies overexpressing *Buffy*<sup>EY11259</sup> had no significant improvement in their lifespan. Longevity of flies overexpressing *UAS-Buffy.Q* (n=315) and *debcl*<sup>EY05743</sup> (n=306) in the neurons is significantly increased compared to *lacZ* control (n=321). Longevity is shown as percent survival (P < 0.01, determined by the log-rank test). Genotypes are *UAS-α-synuclein; Ddc-GAL4/UAS-lacZ, UAS-α-synuclein/ UAS-Buffy.Q; Ddc-GAL4, UAS-α-synuclein/Buffy*<sup>EY11259</sup>*; Ddc-GAL4* and *UAS-α-synuclein/ debcl*<sup>EY05743</sup>*; Ddc-GAL4.*

**Table 7. Log-rank (Mantel-Cox) comparison of survival curves for the directed overexpression of _Buffy_ and _debcl_ in the _α-synuclein_-dependent Drosophila model of Parkinson disease.** Survival curves were analyzed using log-rank test.

| Genotype | # of Deaths | Median Survival | Chi Square | P value |
|---|---|---|---|---|
| _UAS-lacZ_ | 323 | 60 | n/a | n/a |
| _UAS-Buffy:Q_ | 315 | 68 | 103.3 | <0.0001 |
| _Buffy_[J7/1259] | 266 | 51 | 0.84 | 0.3596 ns |
| _debcl_[E10574] | 306 | 67 | 120 | <0.0001 |

**Climbing analysis of the standard and experimental lines in the α-synuclein model**

Loss of climbing ability is one of the phenotypes displayed by the α-synuclein model (Feany and Bender, 2000), and thus the assay is important in detecting the role being played by the overexpressed gene. We investigated the effect of coexpressing the *Bcl-2* genes with *α-synuclein* on the locomotor ability of these flies. The overexpression of *debcl*[E1053A] resulted in a marked reduction in the climbing ability of these flies, whereas the overexpression of the two *Buffy* lines rescued the loss of climbing ability displayed by the *α-synuclein* model of PD.

*Debcl overexpression decreases climbing ability*

The overexpression of *debcl* in dopaminergic neurons resulted in a remarkable reduction in climbing ability (Figure 4). The *debcl* overexpression flies showed a decreased climbing ability over time, with these flies losing their ability earlier than the control flies which were also overexpressing *α-synuclein*. The decreased climbing ability was significantly different compared to the control with a P-value of 0.0231** with a mean difference of -0.8551 and confidence intervals of between -1.328 to -0.383.
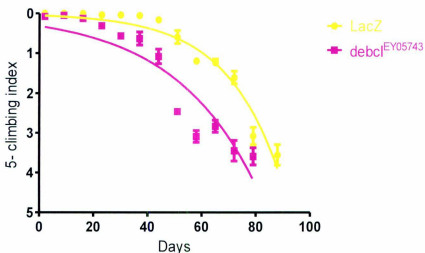
**Figure 4: The coexpression of *debcl* with *α-synuclein* result in an age-dependent loss in climbing ability.** The directed overexpression of *debcl* in the DA neurons decreased the climbing ability over time compared to the control (*lacZ*). Analysis was by non-linear fitting of the climbing curves and comparing the 95% confidence intervals. The genotypes are *UAS-α-synuclein; Ddc-GAL4/UAS-lacZ* and *UAS-α-synuclein/ debcl^{EY05743} ; Ddc-GAL4.*

*Buffy rescues climbing ability in α-synuclein model*

It was observed that overexpressing *Buffy* in the DA neurons had a significant effect on the climbing ability of these flies compared to the control (Figure 5). The coexpression of *Buffy* with *α-synuclein* in dopaminergic neurons resulted in a significant increase in the climbing ability of the affected flies.

Overexpression of *Buffy* in the dopaminergic neurons alongside *α-synuclein* suggests that *Buffy* counteracts the *α-synuclein* protein toxicity by significantly improving the climbing ability of these flies. The *Buffy* flies were able to climb over an extended period of time compared to the control flies, which lost their climbing ability at an early-onset. This was statistically significant at a P value less than 0.05. The 95% CI for *UAS-Buffy.Q* was between 0.06829 to 0.1066, while that of *Buffy^{EY11298}* was between 0.06608 to 0.1103 and thus the climbing curves were significantly different from the control flies with 95% CI at 0.04722 to 0.05965.
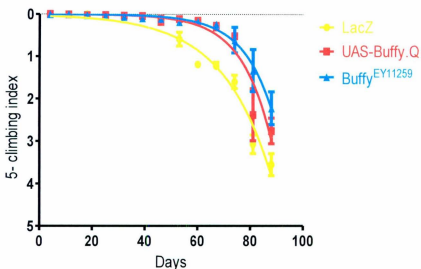
**Figure 5: The coexpression of *Buffy* in the *α-synuclein* model of PD rescue the age-dependent loss in climbing ability.** The directed overexpression of both *UAS-Buffy.Q* and *Buffy^EY11259* in the dopaminergic neurons remarkably increased the climbing ability over time compared to the control (*lacZ*). Analysis was by non-linear fitting of the climbing curves and significance was determined by comparing the 95% confidence interval. The genotypes are *UAS-α-synuclein; Ddc-GAL4/UAS-lacZ, UAS-α-synuclein/UAS-Buffy.Q; Ddc-GAL4,* and *UAS-α-synuclein/Buffy^EY11259; Ddc-GAL4.*

*Recombinant line for GMR-GAL4*

The recombinant line *GMR-GAL4 UAS-α-synuclein/CyO* was developed from *GMR-GAL4[2]* and *UAS-α-synuclein* lines by standard homologous recombination methods. The recombinant line was tested for the presence of *UAS-α-synuclein* using PCR with primers designed as described. The recombinant chromosome was determined by electrophoresing the PCR product on agarose gel and checking the product size on the gel against the predicted product size from the primer design. The predicted PCR product was 165 bp long and the gel band corresponded to this value (Figure 6). The negative control was *UAS-lacZ* (Figure 6, Lanes 2 to 7) and there was no corresponding 165 bp PCR product, the only product was approximately between 30 to 50 bp long and likely represented primer dimers.

Arrow indicates possible primer dimers with an approximate size of 30 to 50 bp
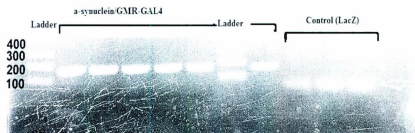
**Figure 6: Agarose gel electrophoresis image for the detection of *α-synuclein* recombinant chromosomes. Top panel,** 2-log DNA ladder (lanes 1 and 7), *UAS-α-synuclein/GMR-GAL4* (lanes 2 to 6 and 8) and *UAS-lacZ* (lanes 9 to 11). **Bottom panel,** 2-log DNA ladder (lanes 1 and 11), *UAS-lacZ* (lanes 2 to 7), *UAS-α-synuclein/Ddc-GAL4* (lanes 8 to 10 and 12 to 14), *UAS-α-synuclein* (lanes 15 to 20). The predicted band was 165 bp long, the smaller band approximately 30 to 50 bp long likely represent primer dimers as indicated by arrows on the gel images.

*Eye analysis*

Eye development in Drosophila is very precise and the development of each ommatidium and the organization of the ommatidial array is tightly controlled (Thomas and Wassarman, 1999). The eye is a photoreceptor and thus a neuron. Under this precept, we investigated by biometric analysis whether overexpressing the *Bcl-2* genes would have any influence on the development of these specialized neurons. We first overexpressed the *Bcl-2* genes in the eyes using the eye specific driver *GMR-GAL4* and, secondly we overexpressed the *Bcl-2* genes in the Drosophila α-synuclein model using the derivative line *GMR-GAL4/ UAS-α-synuclein/CyO*, which was already overexpressing *α-synuclein* in the background.

**Investigation of *Bcl-2* overexpression in the eye**

The *Bcl-2* homologues and the standard line *UAS-lacZ* were all expressed in the eye to determine whether they caused a rough eye phenotype or other defects during eye development. Analysis of scanning electron micrographs (Figure 7) of eyes overexpressing these genes revealed that there was no significant difference in the area of the ommatidium (Figure 8-A) or the number of bristles (Figure 8-B) when these genes were overexpressed in the eye using the *GMR-GAL4* driver (Table 8).
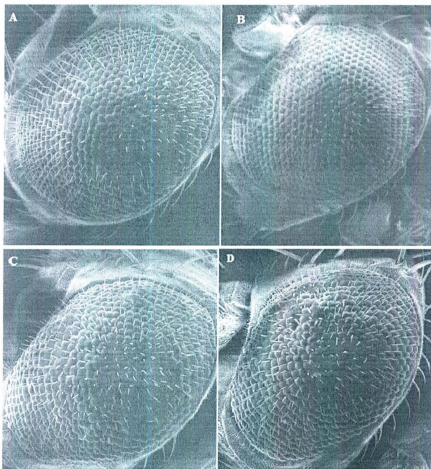
**Figure 7: Scanning electron micrographs when *Buffy* and *debcl* are overexpressed in the eye.** (A) *GMR-GAL4; UAS-lacZ*, (B) *GMR-GAL4/ UAS-Buffy Q*,(C) *GMR-GAL4/Buffy^{JY11239}* and (D) *GMR-GAL4/debcl^{EP895743}*. Biometric analysis shows no significant differences in the ommatidium area (P<0.05) or the number of bristles (P<0.05).

A.
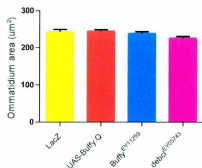


B.
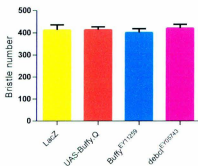


**Figure 8: Directed expression of *Bcl-2* genes in the eye with the eye-specific driver *GMR-GAL4*.** There was no significant difference in the area of ommatidium and the number of bristles when *Bcl-2* genes were overexpressed in the eye as determined by a one-way ANOVA and Dunnett's multiple comparison test (P<0.05 and 95% CI), error bars are SEM. Genotypes are *GMR-GAL4; UAS-lacZ, GMR-GAL4/ UAS-Buffy.Q, GMR-GAL4/Buffy^{EY11298}* and *GMR-GAL4/debcl^{E7087A1}*.

**Table 8: A summary of biometric analysis when *Buffy* and *debcl* are overexpressed in the eye.** The ommatidium area and bristle number were compared using one-way ANOVA and Dunnett's multiple comparison test.

## A. Ommatidium area

| Genotype | Mean area (um²) | Mean difference | Significance (P<0.05) | 95% confidence interval |
|---|---|---|---|---|
| *UAS-lacZ* | 243.23 | n/a | n/a | n/a |
| *UAS-Buffy;Q* | 245.26 | -2.02 | no | -20.77 to 16.73 |
| *Buffy^{EY11259}* | 239.01 | 4.23 | no | -14.52 to 22.98 |
| *debcl^{CB05743}* | 226.57 | 16.66 | no | -2.088 to 35.41 |

## B. Bristle number

| Genotype | Mean number | Mean difference | Significance (P<0.05) | 95% confidence interval |
|---|---|---|---|---|
| *UAS-lacZ* | 413.01 | n/a | n/a | n/a |
| *UAS-Buffy;Q* | 414.14 | -1.14 | no | -75.86 to 73.57 |
| *Buffy^{EY11259}* | 401.29 | 11.71 | no | -63.00 to 86.43 |
| *debcl^{CB05743}* | 421.29 | -8.29 | no | -83.00 to 66.43 |

**Expression of *Bcl-2* homologues in the α-synuclein model of PD**

The *Bcl-2* homologues were coexpressed in the background of *α-synuclein* to determine their effect on the PD model in the Drosophila eye. The derivative line *GMR-GAL4 UAS-α-synuclein/CyO* was used to overexpress a single copy of *α-synuclein* in the background. Our standard line *lacZ* and the experimental lines *Buffy*, and *debcl* were crossed to this derivative line and whole area of eye, bristle number and ratio of eye area disruption were analyzed (Figure 9). Ratio of eye area disruption was calculated by dividing the total area of the eye with the total disrupted area of the eye.

The directed expression of *debcl* in the eye resulted in smaller eyes than the control flies when the total area of the eye was measured but no significance was seen with the *Buffy* flies (Figure 10). The mean area of the *debcl* flies eye was 85108 um$^2$, while the control had a mean area of 96791 um$^2$ (Table 9-A). When the interommatidial bristles were counted, there was no significant difference in the number in either of the *Bcl-2* genes (P<0.05)(Table 9-B).

The ratio of the disrupted eye area show that there was significantly less disruption in the eyes of flies overexpressing *Buffy*, while no significance was seen when *debcl* was overexpressed (P<0.05). The ratio of *UAS-Buffy.Q* was 0.25 corresponding to a 25% of the eye area being disrupted, while *Buffy$^{EY11298}$* was 0.27 (Table 9-C). The control flies had an area of disruption corresponding to 37% of the whole eye area, while there was no statistical significance in the ratio of disruption for the *debcl$^{E59743}$* flies with a ratio of 0.41.
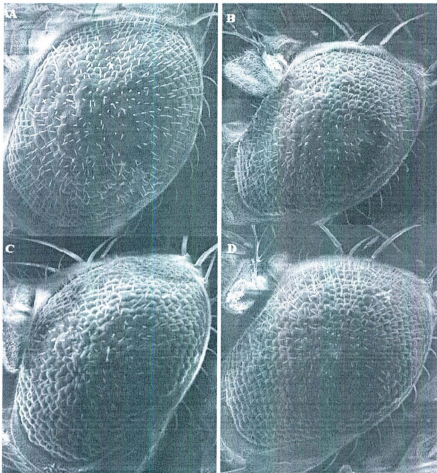
**Figure 9: Scanning electron micrographs when *Buffy* and *debcl* are overexpressed in the eye.** (A) *GMR-GAL4 UAS-α-synuclein;UAS-lacZ*, (B) *GMR-GAL4 UAS-α-synuclein/UAS-Buffy;Q*, (C) *GMR-GAL4 UAS-α-synuclein/Buffy[EY14259]* and (D) *GMR-GAL4 UAS-α-synuclein/debcl[E7057A3]*. Biometric analysis showed significant differences in the ommatidium area for *debcl* flies (P<0.05) but no significance was observed in the number of interommatidial bristles (P<0.05).

A.



B.



C.
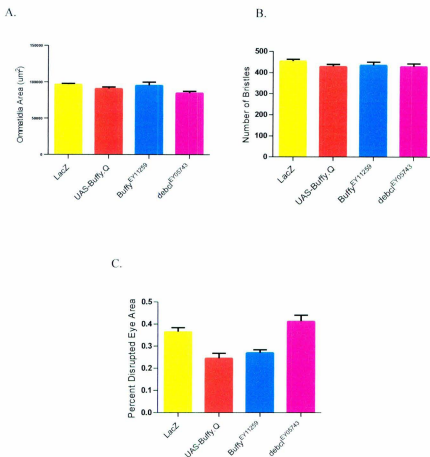


**Figure 10: Coexpression of *α-synuclein* with *Bcl-2* genes.** There was significant difference in the area of ommatidium of *debcl* flies (A), and no significance in the number of bristles (B) when *Bcl-2* genes were overexpressed in the eye but the ratio of disrupted eye surface (C) was significantly different in flies overexpressing *Buffy* but no significance was determined in the flies overexpressing *debcl*, as determined by one-way ANOVA and Dunnett's multiple comparison test (P<0.05 and 95% CI), error bars are SEM. Genotypes are *GMR-GAL4 UAS-α-synuclein;UAS-lacZ, GMR-GAL4 UAS-α-synuclein/UAS-Buffy Q, GMR-GAL4 UAS-α-synuclein/Buffy<sup>EY11259</sup> and GMR-GAL4 UAS-α-synuclein/debcl<sup>E00/743</sup>.*

**Table 9: A summary of biometric analysis when the _Bcl-2_ genes are overexpressed in the eye.** The ommatidia area, bristle number and ratio of disrupted eye area were compared using one-way ANOVA and Dunnett's multiple comparison test (P<0.05).

**A. Ommatidia (Whole eye) area**

| Genotype | Mean area (um$^2$) | Mean difference | Significance (P<0.05) | 95% confidence interval of difference |
|---|---|---|---|---|
| _UAS-lacZ_ | 96791 | n/a | n/a | n/a |
| _UAS-Buffy.Q_ | 91148.5 | 5643 | No | -5954 to 17239 |
| _Buffy$^{EY11259}$_ | 95529.5 | 1261 | No | -10335 to 12858 |
| _debcl$^{E305743}$_ | 85108 | 11683 | Yes | 86.41 to 23280 |

**B. Bristle number**

| Genotype | Mean number | Mean difference | Significance (P<0.05) | 95% confidence interval of difference |
|---|---|---|---|---|
| _UAS-lacZ_ | 454.4 | n/a | n/a | n/a |
| _UAS-Buffy.Q_ | 429.64 | 24.76 | No | -17.93 to 67.45 |
| _Buffy$^{EY11259}$_ | 436.1 | 18.3 | No | -25.40 to 62.00 |
| _debcl$^{E305743}$_ | 429.9 | 24.5 | No | -19.20 to 68.20 |

**C. Ratio of Disrupted eye area**

| Genotype | Mean ratio | Mean difference | Significance (P<0.05) | 95% confidence interval of difference |
|---|---|---|---|---|
| _UAS-lacZ_ | 0.37 | n/a | n/a | n/a |
| _UAS-Buffy.Q_ | 0.25 | 0.12 | Yes | 0.06 to 0.18 |
| _Buffy$^{EY11259}$_ | 0.27 | 0.09 | Yes | 0.05 to 0.14 |
| _debcl$^{E305743}$_ | 0.41 | -0.04 | No | -0.12 to 0.028 |

## Discussion

The Bcl-2 proteins are thought to be the guardians of the mitochondria, involved in the life and death decisions at the cellular level by initiating mitochondrial remodelling, mitochondrial outer membrane permeabilization and the release of apoptotic factors from the mitochondria (Wang and Youle, 2009; Tanner *et al.*, 2010). This delicate balance is maintained by the activity of the pro-survival and anti-survival members of the Bcl-2 family. The various members are considered to be either pro-survival, which in *Drosophila melanogaster* is the single member *Buffy*, or anti-survival, such as *debcl* (Brachmann *et al.*, 2000; Colussi *et al.*, 2000; Igaki *et al.*, 2000; Zhang *et al.*, 2000).

In previous studies, the overexpression of *Buffy* has been shown to suppress the *Pink1* mutant phenotypes (Park *et al.*, 2006) and suggest a role for this protein in 1) interacting with the Pink1 protein and other mitochondrial proteins or 2) in a pathway that regulates mitochondrial function and integrity. Studies show that both *Buffy* and *debcl* have little involvement in cell death during development (Sevrioukov *et al.*, 2007; Galindo *et al.*, 2009), though they have a role in regulating cell death that occurs in response to external stimuli (Sevrioukov *et al.*, 2007; Galindo *et al.*, 2009) and recently a role in the mitochondrial pathway for the activation of cell death during Drosophila oogensis (Tanner *et al.*, 2011), all which point to an important role for these proteins in aspects of cell death. Indeed, early studies demonstrated that *debcl* acts in a "pro-death" mechanism while *Buffy* has been shown to play both roles of anti- and pro-survival (Quinn *et al.*, 2003; Wu *et al.*, 2010) depending on the stimuli.

A direct role for the Bcl-2 proteins in mitochondrial dynamics has been shown in the activation of cell death in *Drosophila melanogaster* during mid-oogenesis (Tanner *et al.*, 2011) and in a Parkinson disease model, mutant for Pink1, a mitochondrial associated kinase (Park *et al.*, 2006). The possible role of the mitochondria in PD pathogenesis makes the α-synuclein-induced model of PD (Feany and Bender, 2000) a very attractive model for investigating the role of Bcl-2 proteins in interacting with PD proteins, and indirectly their role in regulating mitochondria function.

The recapitulation of PD-like symptoms in *Drosophila melanogaster* and especially the age-dependent loss of climbing ability led to the investigation of possible gene products that could counteract this phenotype (Feany and Bender, 2000; Auluck *et al.*, 2002; Haywood and Staveley, 2004). Mitochondrial dysfunction has been implicated in PD pathogenesis and thus we investigated the effect of overexpressing two known Drosophila *Bcl-2* homologues *Buffy* and *debcl* to understand their effect on PD-like phenotypes and indirectly whether they have any mitochondrial protective role. The overexpression of *Buffy* in a *Pink1* PD model restored normal levels of mtDNA, mitochondrial proteins and ATP suggesting that Buffy has a protective and pro-survival role (Park *et al.*, 2006). The use of the climbing assay to determine the role of the various gene products in rescuing the α-synuclein-induced phenotypes has been widely applied (Feany and Bender, 2000; Haywood and Staveley, 2004; Haywood and Staveley, 2006; Todd and Staveley, 2008). This assay allows for scoring of flies based on their loss of climbing ability and is a key indicator of the effect the overexpressed gene has on the phenotype.

Persons with PD have gait abnormalities, cognitive and psychiatric problems which can result in substantial disability and early death (Forno, 1996). The α-synuclein model of PD in Drosophila showed no difference in lifespan between the control and wild type, A53T and A30P *α-synuclein* flies (Feany and Bender, 2000). In our study, *Buffy* was overexpressed in the DA neurons under the control of the *Ddc-GAL4* driver, there was a significant difference in their longevity, with *Buffy* expressing flies living slightly longer than the control flies. It is yet unclear from our studies how *Buffy* is able to prolong the life of these flies when overexpressed in the DA neurons, but since it has been shown the resulting death of these sensitive neurons is partly due to defects in mitochondrial complex I function (Lu, 2009), the pro-survival *Buffy* likely plays a protective role in these neurons to increase longevity by protecting the mitochondria. The overexpression of the pro-death Bcl-2 member *debcl* in the DA neurons, did not have any significant effect on the lifespan of these flies. It is possible that the overexpression of *debcl* is not sufficient to counter the protective balance of *Buffy*, or more of this protein in conditions of normal cell function has no significant effect and is likely to exert its proapoptotic function under cellular stress-induced conditions.

Locomotor dysfunction is one of the behavioural manifestations of PD. The α-synuclein model developed by Feany and Bender (2000) showed an age dependent loss in climbing ability, with the mutant flies being unable to climb above the first section of the climbing apparatus in the last days of their lives. When we overexpressed the *Bcl-2* genes in the DA neurons under the control of the *Ddc-GAL4* driver, the *Buffy^{EY11259}* flies were the only ones to have a climbing index significantly different from the control flies. The

*Buffy^{E31I259}* flies climbed for longer and lost the climbing ability later than the control flies. This is possibly due to the *Buffy* flies living longer than the control flies and is likely due to the protective role that Buffy confers to the mitochondria. The anti-survival *debcl* flies showed no significant difference in their climbing ability when compared to the control flies. It seems that *debcl* does not lead to a reduction of locomotor function when overexpressed in the DA neurons. Taken together, these results would indicate an early protective role for *Buffy* in the DA neurons even in the absence of induced cellular stress, but however, *debcl* showed no degeneration of the DA neurons under normal cellular function.

In the *Drosophila melanogaster* model of PD, the *UAS-α-synuclein/CyO; Ddc-GAL4/TM3* line, which overexpresses *α-synuclein* in the DA neurons, when crossed to *UAS-Buffy;Q*, showed a significant difference in the ageing curves. *UAS-Buffy;Q* flies had a survival median of 68 days which was slightly longer lifespan compared to the control flies. In *Buffy^{E31I259}* flies, the survival median was 51 days, which was not significantly different from the control flies whose median survival was 60 days. A reduced lifespan has been reported in *debcl* knockdown studies (Senoo-Matsuda *et al.*, 2005), but not in *Buffy* studies. In our study, the overexpression of *debcl* in the DA neurons actually significantly increased the lifespan of these flies, indicating that *debcl* likely plays a protective role by increasing the rate of apoptosis in affected cells and thus possibly maintaining healthy neurons. This protective role was shown for *debcl* in PolyQ induced neurodegeneration (Senoo-Matsuda *et al.*, 2005) and further highlights the complex role played by the Bcl-2 proteins in making life and death decisions.

When transgenic flies overexpressing *α-synuclein* in the DA neurons were crossed to *Buffy^{EY11298}* and *UAS-Buffy.Q*, the resulting progeny showed a remarkable recovery in their climbing ability compared to the control flies, which were expressing *α-synuclein* in the DA neurons in the background under the control of the *Ddc-GAL4* driver. These results suggest that overexpressing *Buffy* in the DA neurons counteracts the α-synuclein induced phenotype of locomotor dysfunction over lifespan. We had hypothesized that when *debcl* is overexpressed in the DA neurons in a α-synuclein PD model, the resulting flies would show an exacerbation in their locomotor dysfunction. Flies overexpressing *debcl* (*debcl^{E30574}*) displayed a reduced climbing ability compared to the control flies and were remarkably similar to flies overexpressing a double dosage of *α-synuclein* (see Appendix I). This indicates that *debcl* acts to worsen the α-synuclein induced loss of climbing ability. Indeed, this suggests that it is the presence of excess *Buffy* products that triggers the observed improved climbing ability in these flies. It would therefore suggest that tipping the balance towards the pro-survival Buffy remarkably rescues the α-synuclein PD model from climbing dysfunction. Taken together, these results indicate an important role for *Buffy* in counteracting α-synuclein PD pathogenesis in as yet unknown mechanisms.

The Drosophila eye is composed of between 700 to 800 ommatidia made up of photoreceptor cells, cone cells, pigment cells and bristle cells (Baker, 2001). The overexpression of *α-synuclein* in the developing eye results in a rough eye phenotype that can be analyzed for counteraction by the overexpressed gene product (Haywood and Staveley, 2004; Todd and Staveley, 2008). The use of *GMR-GAL4* to drive the

overexpression during late eye development has been shown to affect the eye morphology. This makes the fly eye an ideal model for the study of cell death and survival. Retinal degeneration and ommatidial array defects (Feany and Bender, 2000) are phenotypes observed when α-synuclein is overexpressed in the *Drosophila melanogaster* eyes using the eye-specific driver *GMR-GAL4*.

Directed overexpression of *Bcl-2* homologues in the eye showed no significant differences with the control when compared for ommatidium area or in the number of bristles. This indicated that elevated levels of the Bcl-2 proteins, Buffy and debcl do not alter the normal development of the eye and seems to have a limited role in neurogenesis under normal cellular conditions.

The *Bcl-2* homologues were overexpressed along with α-synuclein in the developing compound eye using the *GMR-GAL4 UAS-α-synuclein/(CyO)* line. The coexpression of *debcl* with α-synuclein resulted in eyes smaller than the control flies and these flies had a normal number of interommatidial bristles when compared to the control flies. In flies overexpressing *Buffy*, the eyes were not significantly different from the control ones when whole eye area and interommatidial bristle number were compared. However, the flies coexpressing *Buffy* with α-synuclein developed eyes with less disruption in the development of the eye when compared to the control eyes. This points to a role for this protein in the regulation of eye development during stress-induced by α-synuclein toxicity. It is likely that during the protection of the mitochondria from protein toxicity insults by α-synuclein overexpression, *Buffy* ensures an almost normal neuronal differentiation resulting in eyes with less disruption. Taken together, these results show a

likely role for *Buffy* in neurogenesis and cell survival.

Studies have shown that Buffy is localized to the mitochondria (Quinn *et al.,* 2003) and endoplasmic reticulum (ER) (Doumanis *et al.,* 2007), two very important organelles in the development of PD, and a role for both mitochondrial and ER stress in the pathogenesis of PD has been demonstrated (Bouman *et al.,* 2011). However, debcl has been shown to localize to the mitochondria (Doumanis *et al.,* 2007) and this is likely due to a function at the membranes of these organelles or within these organelles themselves.

The observed PD symptoms have been attributed to damaged DA neurons. In general, neurons are sensitive to mitochondrial function changes since they utilize higher levels of energy to function (Su *et al.,* 2010). Synaptic transmission, axonal/dendritic transport, ion channel activity, and ion pump activity are energy-taxing processes (Kann and Kovacs, 2007). As such any mitochondrial stress or damage alters neuronal function and survival (Chan, 2006). Central to mitochondrial integrity and function are the mitochondrial fusion and mitochondrial fission processes that control mitochondrial dynamics.

Significant deficits in subunits and activity of mitochondrial respiratory chain complex 1, and mtDNA deletions in the DA neurons are a common feature of PD pathology. Identified familial PD genes are localized to and involved in mitochondrial function including *Pink1, parkin,* and *DJ-1* (Henchcliffe and Beal, 2008). In Drosophila the overexpression of mutant *Pink1* alters mitochondrial dynamics including the mtDNA and ATP levels and the overexpression of *Buffy,* which encodes a Bcl-2 protein protects against these phenotypes (Park *et al.,* 2006). Pink1 is involved in mitochondrial turnover

by autophagy (Dagda *et al.,* 2009), where the altered mitochondrial morphology and reduced ATP production targets the mitochondria to autophagy (macroautophagy) or mitophagy. In this role, Pink1, parkin and other proteins have been linked. The dysregulation of this process causes the accumulation of abnormal proteins and/or damaged organelles due to a failure by the cells to degrade proteins under normal conditions and under conditions of stress.

The vesicular nature of Lewy bodies led to the suggestion that the autophagic-lysosomal pathway contributes to the formation or dissolution of Lewy bodies (Forno, 1996). In mice treated with MPTP, a mitochondrial toxin that induces mitochondrial dysfunction and oxidative stress, other than α-synuclein, LC3 (Macrotubule associated protein 1A/1B light chain 3 or Atg8), autophagic vacuoles and macroautophagic components are found to accumulate in Lewy bodies (Xilouri and Stefanis, 2010) . It is still unclear whether lysosomal dysfunction is secondary to accumulation of autophagic vacuoles, but evidence suggests an interplay in failure of the protein degradation system and macroautophagy.

The accumulation of α-synuclein in Lewy bodies and Lewy neurites has been attributed to failure of the UPS degradation system (Imai *et al.,* 2000; Auluck *et al.,* 2002) but recent studies show α-synuclein could be degraded by the lysosomal pathway and especially macroautophagy and the chaperone-mediated autophagy (CMA) (Xilouri and Stefanis, 2010). The inhibition of macroautophagy leads to the accumulation of WT α-synuclein showing it has an important role in normal α-synuclein turnover (Webb *et al.,* 2003). The post-translational modifications of α-synuclein interferes with its degradation

by CMA and the degradation of other products by CMA (Martinez-Vicente *et al.,* 2008). The toxic effects of α-synuclein have been determined to be CMA dysfunction, lysosomal dysfunction, or inhibition of an early point in autophagosome formation by Rab1a interaction (Winslow *et al.,* 2010; Xilouri and Stefanis, 2010). Induction of macroautophagy by treatment with rapamycin or overexpressing Atg7 or Beclin-1 rescued the α-synuclein induced phenotypes.

The autophagosome or autophagic vacuole engulfs cytosolic constituents such as organelles, proteins and lipids. Atg, Beclin-1 and several other proteins are involved in the formation of the phagosome (Xilouri and Stefanis, 2010). Bcl-2 family of proteins bind Beclin-1/Atg6, a BH3 only protein, in a multimeric complex that is involved in vesicle nucleation stage of the autophagosome formation (Maiuri *et al.,* 2007; Sinha and Levine, 2008; Xilouri and Stefanis, 2010). The discovery of the BH3 domain, a binding site for interaction between the antiapoptotic Bcl-2 proteins and required for inhibition, in Beclin-1 showed that not only do Bcl-2 proteins regulate apoptosis, but function as antiautophagic proteins (Sinha and Levine, 2008). Bcl-2 proteins seem to play a crucial role in maintaining the autophagic homoeostasis since phosphorylation of Beclin-1 that weakens this interaction promote autophagy (Zalckvar *et al.,* 2009). Thus Bcl-2 proteins have a dual role in regulating apoptosis and autophagy.

In a recent study in *C. elegans,* overexpression of *α-synuclein* was shown to inhibit mitochondrial fusion by binding to the outer mitochondria membrane, resulting in an age-dependent mitochondrial fragmentation (Kamp *et al.,* 2010). This fragmentation was rescued upon coexpression of *Pink1, parkin* and *DJ-1* but not the mutant versions of these

proteins. Similarly, mutant α-synuclein (A53T) was shown to induce neuronal cell death by upregulating autophagy (macroautophagy) leading to mitochondrial removal (Choubey *et al.,* 2010). Using the same α-synuclein mutant, overexpression of *α-synuclein* in DA neurons was shown to interfere with mitochondria complex I, leading to an age-dependent decrease in substrate specific respiration along with an increase in mitophagy (Chinta *et al.,* 2010). It seems the accumulation of α-synuclein interferes with the normal functioning of the mitochondria by either binding to the outer mitochondrial membrane, the inner mitochondrial membrane or associates with mitochondria membrane proteins, such as adenylate translocator (Zhu *et al.,* 2011), a component of the mitochondria permeability transition pore, leading to deformed mitochondria and depolarization of the mitochondrial membrane potential.

The overexpression of *Pink1* enhances starvation-induced macroautophagy by interacting with Beclin-1 (Michiorri *et al.,* 2010), and has a role in mitophagy (Narendra *et al.,* 2008; 2010). It seems *Buffy* plays a protective role in the mitochondria since its overexpression counteracts the loss of *Pink1*, and in our study, it counteracts the induced PD phenotype of age-dependent loss of climbing ability. Mounting evidence points to disorders in macroautophagy homoeostasis, and especially mitophagy, in the pathogenesis of PD. Bcl-2 proteins have been shown to play a dual role in apoptosis and autophagy and some theories suggest they have an overly active role for mitochondrial removal, leading to neuronal death and altering synaptic function, which manifest as motor and non-motoric dysfunction. Since Bcl-2 proteins have been shown to block Beclin-1 dependent autophagy by inhibiting the formation of the Beclin-1/Vps34 PI3K complex (Pattingre *et*

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*al.*, 2005), we are inclined to theorize that *Buffy* plays a similar role in *Drosophila melanogaster*. Excessive levels of autophagy leads to cell death, and as such *Buffy* may act to balance the levels of autophagy via the Beclin-1 pathway to remove damaged mitochondria but inhibit excessive removal of dysfunctional mitochondria, counteracting the PD-like symptoms in *Drosophila*. The accumulation of α-synuclein promotes excessive levels of macroautophagy leading to removal of mitochondria, and it is possible that once most neurons are depleted of mitochondria, synaptic function is interrupted. *Buffy* seems to restore this balance.

### Conclusion

Bcl-2 proteins interact with the fusion/fission machinery to affect mitochondrial dynamics to regulate apoptosis, whereas they interact with familial PD genes such as *Pink1* to maintain mitochondrial integrity and with *Beclin-1* to maintain a healthy autophagic system in healthy cells to regulate mitophagy. Further work is required to clearly elucidate how *Buffy* and *debcl* associate with the familial PD genes and how they fit into the regulation of mitochondrial integrity and the tightly controlled autophagy.

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## Appendix 1

While conducting our study we analyzed the effect of *α-synuclein* gene dosage and our results are presented here written in the journal format.

**Effects of increased *α-synuclein* expression on the Drosophila α-synuclein model of Parkinson Disease.**

**ABSTRACT.** α-synuclein toxicity is proposed to be one of the causes for the symptoms that manifest in PD. Since the discovery of familial forms of PD, the study of *α-synuclein* has shown that the mutated gene product is involved in PD and is a result of dysfunctional protein aggregation and accumulation. By expressing a single copy and two copies of *α-synuclein* in the fly PD model, we show that locomotor ability and eye development as determined by eye area, interommatidial bristles number and ratio of eye disruption is more compromised in the flies expressing two copies of *α-synuclein* than in flies expressing a single copy. We suggest that increased severity in phenotype is due to increased α-synuclein protein toxicity.

## INTRODUCTION

Parkinson disease (PD) is the most common movement disorder and the second most common neurodegenerative disease, afflicting about 1 to 2% of the population over 50 years of age. It is associated with selective and profound loss of dopaminergic (DA) neurons, resulting in marked clinical features, which include muscle rigidity, resting tremors, postural instability and bradykinesia, as well as non-motoric symptoms, like autonomic, cognitive and psychiatric problems (Forno, 1996). There is presence of Lewy bodies which are intracytoplasmic inclusions containing α-synuclein, and ubiquitin among other protein aggregates. It is the accumulation of these proteins that is believed to lead to cellular toxicity and PD pathogenesis.

Drosophila is an important organism to model human degenerative disorders because the fly brain has over 300, 000 neurons and is organized into separate specialized areas for learning, olfaction, vision and memory (Wolf and Herbelein, 2003; Cauchi and Heuvel, 2006; Hardaway, 2010). Furthermore, the Drosophila eye is phenotypically easy to detect, tolerant to genetic manipulations and is dispensable for the survival of the fly (Chan and Bonini, 2000; Celotto and Palladino, 2005; Jeibman and Paulus, 2009). The genetic system of choice when modelling PD in Drosophila is the bipartite UAS/GAL4 system (Brand and Perrimon, 1993). We used this bipartite system to overexpress *α-synuclein* in the DA neurons and eyes of *Drosophila melanogaster*.

The directed expression of *α-synuclein* results in flies that are viable, accumulate aggregated α-synuclein in perinuclear and neuritic filamentous inclusions similar to Lewy bodies and Lewy neurites, age–dependent loss of dorsomedial DA neurons, neuronal degeneration, age-dependent loss of climbing ability, retinal degeneration (Feany and Bender, 2000; Auluck *et al.*, 2002), and ommatidial degeneration (Todd and Staveley,

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2008). This taken together, showed a remarkable model system for understanding the pathophysiology of PD.

To investigate whether the PD-like phenotypes become worse when more α-synuclein is accumulated, we overexpressed *α-synuclein* in the dopaminergic (DA) neurons and eyes of *Drosophila melanogaster*.

## MATERIALS AND METHODS

*Drosophila media and culture.*

Stocks and crosses were maintained on a standard medium containing 65 g/L cornmeal, 50 ml/L molasses, 10 g/L yeast, 5.5g/L agar and ~900 ml/L water. Fresh food was prepared by Dr. Brian E. Staveley approximately twice a month and treated with 2.5 ml/L propionic acid and 5 ml/L of 10% in ethanol methylparaben to prevent growth of mold. Seven millilitre aliquots of media was poured into vials, allowed to solidify, and refrigerated at 4°C to 6°C. Stocks were maintained on solid media for two to three weeks before transfer onto new media to reculture. Stocks were kept at room temperature (22 ± 2°C) while crosses and experiments are carried out at 25°C and 29°C.

*Drosophila stocks and lines*

UAS-α-synuclein (Feany and Bender, 2000) was generously provided by Dr. M. Feany of Harvard Medical School, *UAS-lacZ* was obtained from the Bloomington Drosophila Stock Center, Dr. J. Hirsch (University of Virginia) generously provided *Ddc-Gal4* flies (Li *et al.*, 2000), and *GMR-GAL4[12]* flies (Freeman, 1996) were obtained from the Bloomington Drosophila Stock Center at Indiana University.

The *UAS-α-synuclein/CyO; Ddc-GAL4/TM3* was generated and tested by Dr. Brian Staveley using standard homologous recombination methods and was used to overexpress *α-synuclein* in the dopaminergic neurons using the dopa decarboxylase (*Ddc*) driver. The *GMR-GAL4/ UAS-α-synuclein/CyO* line was generated by Dr. Brian Staveley and tested by myself by standard PCR procedures and was used to overexpress *α-synuclein* in the developing eye using the Glass Multiple Reporter (*GMR*) driver.

*Ageing assay*

Several single vial matings of three to five females plus three to five males were made of each genotype. A cohort of adult heterozygous male flies were collected upon eclosion. Approximately between two hundred and four hundred flies were aged per genotype, at a density of ≤ 20 flies per vial. Adults were kept on fresh media replenished every other day. Flies were observed and scored every two days for presence of deceased adults. Flies were considered dead when they did not display any movement upon agitation (Staveley *et al.*, 1990). Longevity data was analyzed using the GraphPad Prism 5.0 software. Survival curves were compared using the log-rank test, a statistical test that compares the actual and expected number of failures (death) between survival curves at each individual failure event. Significance was determined at 95%, at a P-value less than or equal to 0.05.

*Climbing analysis*

Several single vial matings of three to five females plus three to five males were made of each genotype. A cohort of adult heterozygous male flies were collected upon eclosion and scored for their ability to climb (Todd and Staveley, 2004). Every 7 days, 50 males from every genotype were assayed for their ability to climb 10 centimetres in 10 seconds in a sterile climbing apparatus. Data was collected over a period of 90 days or until all the flies being assayed were all dead. Flies were maintained on standard cornmeal-yeast-molasses-agar medium at 25°C. Climbing analysis was performed using the GraphPad Prism 5.0 statistical software. Climbing curves were fitted using non-linear regression and compared using 95% confidence interval.

*Scanning electron microscopy of the Drosophila eye*

Several single vial matings of three to five females plus three to five males were made of each genotype at 29°C and a cohort of adult heterozygous male flies collected upon eclosion and aged for three days on standard cornmeal-yeast-molasses-agar before being frozen at -80°C. Whole flies were mounted on SEM studs, desiccated overnight and coated in gold before photography at 170X magnification with a Hitachi S-570 scanning electron microscope was done. For each cross at least 20 eye images were analyzed using the NIH ImageJ software (Abramoff et al. 2004) and biometric analysis performed. The ratio of the area of disruption was calculated from the total area of the eye divided by the total disrupted area. Disrupted area was considered as an area occupying two to three fused ommatidia.

## RESULTS

*Overexpression of α-synuclein in the dopaminergic neurons*

An age-dependent loss and degeneration of DA neurons is implicated in the pathophysiology of PD (Forno, 1996), and especially the protein toxicity resulting from the accumulation and aggregation of α-synuclein in this neurons to form Lewy bodies. We overexpressed *α-synuclein* in the DA neurons and assayed the flies for longevity and locomotor ability.

**α-synuclein does not alter lifespan when overexpressed in the DA neurons.**

Directed expression of *α-synuclein* in the DA neurons using the *Ddc-GAL4* driver did not result in a significant change in the lifespan of this flies, as determined by comparing the survival curves by Log-rank (Mantel-Cox) test (Figure 1-A). The median survival for *α-synuclein* flies was 58 days compared to the *lacZ* flies, which was 62 days. The P value was 0.2019 (P<0.05) with a 95% CI of between 0.2120 to 1.926.

**α-synuclein flies have locomotor ability similar to control flies**

The climbing analysis when *α-synuclein* is expressed in the DA neurons revealed no significant difference when compared to the control flies overexpressing *lacZ* (Figure 1-

B). The 95% CI for the *lacZ* flies was between 0.0788 to 0.0978 and that of *α-synuclein* flies was between 0.0727 to 0.0938 with a P value of 0.9212 (ns). This is possibly due to the driver we were using, *Ddc-GAL4* [III], which has previously been shown to have subtle phenotypes.

*Directed overexpression of α-synuclein in the DA neurons in the α-synuclein model of PD.*

An age-dependent loss of climbing ability is one of the recapitulated PD-like symptoms in flies (Feany and Bender, 2000), and is an important assay for detecting any changes in the phenotype. We hypothesized that elevated levels of *α-synuclein* would exacerbate the seen *α-synuclein*-induced phenotype of age-dependent loss of climbing ability. We analyzed the lifespan and the locomotor ability of this flies to determine the effect of the elevated levels of α-synuclein.

**Overexpression of *α-synuclein* does not alter lifespan.**

The coexpression of *α-synuclein* with *α-synuclein* (*UAS-α-synuclein/CyO; Ddc-GAL4/TM3*) was done to determine the effects of elevated levels of *α-synuclein* in a system that is expressing a single copy of *α-synuclein*. The *α-synuclein* flies showed no significant difference when compared to the control flies overexpressing *lacZ* (Figure 2-A). The median survival was 60 days for the *lacZ* flies, which were overexpressing a single copy of *α-synuclein* in the DA neurons, and 58 days for the *α-synuclein* flies, which were overexpressing two copies of *α-synuclein* in the dopaminergic neurons, the chi square for the Log-rank (Mantel-Cox) test was 0.0093 with a P value of 0.9233 (Not significant), and a 95% CI of between 0.2120 to 1.926. This indicates that survival is not dependent on the dosage of α-synuclein.

***α-synuclein* overexpression decreases climbing ability**

Flies expressing *α-synuclein* in an α-synuclein background in the DA neurons, when assayed show a decreased locomotor function compared to control flies (*lacZ*), which were expressing a single copy of *α-synuclein* (Figure 2-B). This suggests that α-synuclein induced phenotype of decreased climbing ability is dose related and the observed decrease in climbing ability of this flies is due to an elevated α-synuclein dosage. The statistical analysis show a P-value of 0.0069** with mean of the difference being -0.6940 and a confidence interval of between -1.15 to -0.2376.

*Eye Analysis*

Eye development in Drosophila is very precise, the development of each ommatidium and the organization of the ommatidial array being tightly controlled (Thomas and Wassarman, 1999). We first overexpressed *α-synuclein* in the eyes using the eye specific driver *GMR-GAL4* and secondly, we overexpressed *α-synuclein* in the eye of Drosophila α-synuclein model using the derivative line *GMR-GAL4 UAS-α-synuclein /CyO,* which was overexpressing *α-synuclein* in the background.

*Expression of α-synuclein in the eye with GMR-GAL4.*

   Analysis of SEMs of eyes overexpressing a single copy of *α-synuclein* compared to the control flies overexpressing *lacZ* revealed differences in eye development (Figure 3-A), but notable was a slight decrease in the overall area of the eye of *α-synuclein* flies.

**α-synuclein expression in the eye alters area of the eye.**

   We found that the whole area of the eye ($107802 \pm 1311$; $116459 \pm 2153$) (Figure 3-B I) and the area of a single ommatidium ($216.6 \pm 4.826$; $243.2 \pm 6.332$) (Figure 3-B II) were slightly reduced for *α-synuclein* flies when compared to the control flies $P<0.05$ (Figure 3) respectively. The number of interommatidial bristles did not show any significant difference (Figure 3-B III), with the control flies having a mean number of 413 $\pm 22.92$ and the *α-synuclein* flies having a mean of $347.57 \pm 28.99$ bristles.

*Expression of α-synuclein in the eye of α-synuclein-dependent model of PD*

   Investigation of the directed expression of *α-synuclein* was conducted with *GMR-GAL4 UAS- α-synuclein/CyO,* which was expressing one copy of *α-synuclein* in the eye. We compared the SEMs of the control flies, expressing one copy of *α-synuclein* and flies that were expressing two copies of *α-synuclein.*

**Overexpressing α-synuclein in the PD model alters eye development.**

   We found that elevated levels of *α-synuclein* slightly altered overall eye development. The whole eye area ($85346.4 \pm 2250$) (Figure 4-B I), bristle number ($341.7 \pm 9.276$) (Figure 4-A II) and the ratio of disrupted area ($0.4673 \pm 0.0322$) (Figure 4-B III) for *α-synuclein* were significantly different from that of the control flies with whole eye area ($96791 \pm 1288$), bristle number ($454.4 \pm 8.871$) and ratio of disrupted area ($0.3152 \pm 0.0187$) (Figure 3). This suggests that elevated expression of *α-synuclein* alters the normal development of the eye.

**DISCUSSION**

   The accumulation of *α-synuclein* is implicated with the development of PD and the intracytoplasmic inclusions commonly referred to as Lewy bodies have been shown to contain aggregates of *α-synuclein,* ubiquitin and other proteins (Forno, 1996; Polymeropoulos *et al*, 1997; Leroy *et al*, 1998). This accumulation of proteins is believed to lead to cellular toxicity and PD pathogenesis. The *α-synuclein*-induced PD model developed in Drosophila displayed an age-dependent loss in climbing ability among other PD-like symptoms (Feany and Bender, 2000). We investigated whether the overexpression of *α-synuclein* would exacerbate the loss of climbing ability associated with α-synuclein PD model flies.

   We first investigated what the effects of overexpressing *α-synuclein* in the DA neurons would be on the lifespan and locomotor ability. The resulting flies did not show any significant difference in longevity or in their climbing ability, the later was a surprise

since previous studies have shown an age-dependent loss in climbing ability (Feany and Bender, 2000; Haywood and Staveley, 2004; Haywood and Staveley, 2006). We attributed this anomaly to the use of the DA neurons-specific driver, *Ddc-GAL4*, which was on the 3rd chromosome, and has been shown to have very subtle phenotypes. Secondly, we crossed *UAS-α-synuclein/CyO; Ddc-GAL4/TM3* line with *α-synuclein* that resulted in the progeny of these flies having a double dosage of α-synuclein, the resulting flies showed a severe age-dependent loss in their climbing ability, but had a similar lifespan to that of *UAS-α-synuclein; Ddc-GAL4/UAS-lacZ* flies, which were overexpressing a single copy of *α-synuclein* and *lacZ*. This points to the toxicity of α-synuclein in PD pathogenesis, and by extension that α-synuclein dosage is important as a measure of PD severity, and the ability of α-synuclein to confer PD-like phenotypes when overexpressed.

The directed expression of *α-synuclein* in the eye of flies with *GMR-GAL4* revealed significant differences in the morphology of the eye when compared to the *lacZ* expressing flies. The area of the whole eye and ommatidium was slightly decreased in *α-synuclein* flies but the interommatidial bristle number was not changed. This would suggest that expressing *α-synuclein* in the eye of flies affect neurogenesis but it seems to be limited to cell size. This might be attributed to the loss or death of the neurons due to *α-synuclein*-induced toxicity. Expression of *α-synuclein* in flies that were overexpressing a single copy of *α-synuclein* with the *GMR-GAL4 UAS- α-synuclein/CyO* line slightly affected the development of the eye and in particular, 1) the overall area of the eye was reduced, 2) the interommatidial bristles were reduced in number, and 3) the ratio of disrupted area of the eye was also slightly higher when compared to the control flies which were overexpressing *lacZ* and a single copy of *α-synuclein*. It is possible that the elevated levels of α-synuclein result in greater biological protein toxicity that cause the system for clearing malformed proteins to be stressed and lead to more neuronal cell death.

Recent work has suggested that α-synuclein toxicity results in chaperone-mediated autophagy and lysosomal dysfunction by interfering with its ability to degrade α-synuclein and other products (Auluck *et al.*, 2002; Martinez-Vicente *et al.*, 2008; Winslow *et al.*, 2010; Xilouri and Stefanis, 2010) and seem to lead to the up-regulation of autophagy. Indeed, neuronal death has been attributed to mitochondrial damage resulting from stress induced by α-synuclein and causing an age-dependent decrease in substrate specific respiration along with an increase in mitophagy (Chinta *et al.*, 2010). It therefore, seems that accumulation of α-synuclein promotes mitochondrial depletion and interferes with synaptic function.

The results would suggest that higher levels of α-synuclein expression lead to a more severe form of PD-like symptoms and more studies are required to exactly pinpoint the role α-synuclein plays in the pathophysiology of PD.


## ACKNOWLEDGEMENTS

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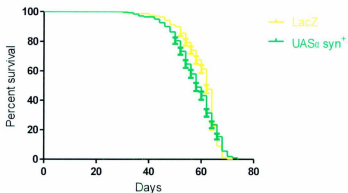
**Figure Legends**

**Figure 1: Lifespan and locomotor ability when *α-synuclein* is overexpressed in the dopaminergic neurons. A.** Directed overexpression of *α-synuclein* in the dopaminergic neurons has no effect on lifespan when flies overexpressing *α-synuclein* (n=324) and *lacZ* (n=321) were compared. Longevity is shown as percent survival (P < 0.05, determined by log-rank). **B.** The climbing curves were analyzed by Non-linear fitting and compared at 95% CI, there was no significance between the two curves. Error bars are SEM, the genotypes are *Ddc-GAL4/UAS-lacZ* and *Ddc-GAL4;UAS-α-synuclein.*

**Figure 2: The coexpression of *α-synuclein* in the *α-synuclein* model of PD exacerbate the age-dependent loss in climbing ability. A.** The directed overexpression of both *α-synuclein* and *lacZ* in the dopaminergic neurons had no effect on lifespan when single copy of *α-synuclein* (*lacZ* n=323*)* and two copies of *α-synuclein* (*α-synuclein* n=337*)* were overexpressed. The survival curves were compared by Log-rank (P<0.05) B. *α-synuclein* overexpression slightly decreased the climbing ability over time compared to the control flies (*lacZ*). Analysis was by Non-linear fitting of the curves and significance was determined by comparing the 95% CI. The genotypes are *UAS-α-synuclein; Ddc-GAL4/UAS-lacZ, UAS-α-synuclein/ UAS-α-synuclein; Ddc-GAL4.*

**Figure 3: The directed expression of *α-synuclein* in the eye. A.** Scanning electron micrographs of the eye when *α-synuclein* and *lacZ* are overexpressed. **B.** The whole area of the eye (I) and the area of a single ommatidium (II) were significantly different (**) from the control flies P<0.05. The number of bristles (III) did not show any significant difference, with the control flies having a mean number of 413 and the *α-synuclein* flies having a mean of 347.57 bristles. The genotypes were *GMR-GAL4; UAS-lacZ* and *α-synuclein/GMR-GAL4.*

**Figure 4: The overexpression of α-synuclein in the Drosophila model of PD. Panel A;** Scanning electron micrographs of both the control flies *lacZ* (A), overexpressing a single copy of *α-synuclein*, and α-synuclein flies (B), overexpressing two copies of *α-synuclein*. **Panel B;** Biometric analysis of the eyes showing significance (*) for the whole area of the eye (I), the bristle number (II) and the ratio of disrupted eye area (III) when compared to the control flies (P<0.05). The genotypes were *GMR-GAL4 UAS-α-synuclein;UAS-lacZ,* and *GMR-GAL4 UAS-α-synuclein/UAS-α-synuclein.*
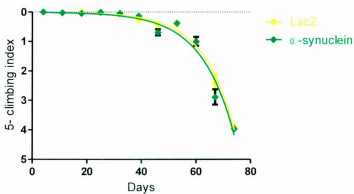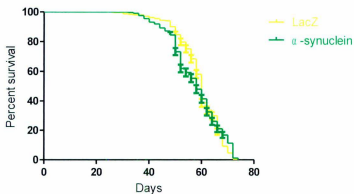
A.



B.



Figure 1: Lifespan and locomotor ability when *α-synuclein* is overexpressed in the dopaminergic neurons.
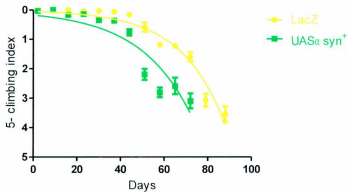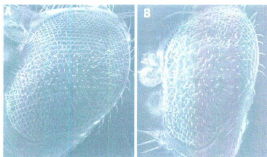
**A.**



**B.**



**Figure 2: The coexpression of *a-synuclein* in the *a-synuclein* model of PD exacerbate the age-dependent loss in climbing ability.**
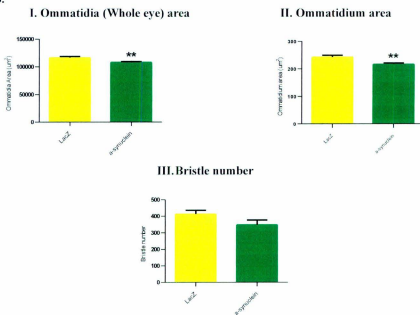
A.



B.

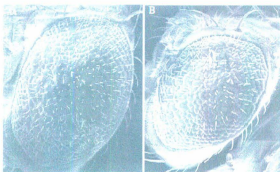**I. Ommatidia (Whole eye) area**



**II. Ommatidium area**



**III.Bristle number**



Figure 3: The directed expression of *α-synuclein* in the eye.

**A.**



**B.**

**I.   Ommatidia (Whole eye) area**



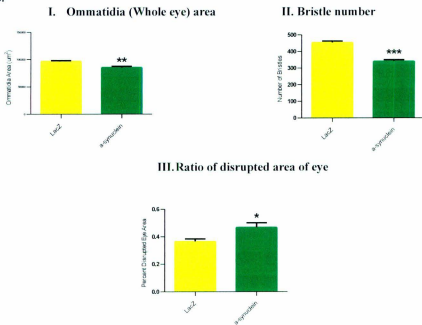**II. Bristle number**



**III. Ratio of disrupted area of eye**



**Figure 4: The overexpression of α-synuclein in the Drosophila model of PD.**