AN ANALYSIS OF APOPTOTIC AND AUTOPHAGIC GENES IN NOVEL DROSOPHILA MODELS OF PARKINSON DISEASE

by

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Abstract

Parkinson disease (PD) is the most common neurodegenerative movement disorder in humans and is strongly associated with the selective loss of dopaminergic (DA) neurons. The clinical features include motor dysfunction, resting tremor and in some cases non-motor features such as autonomic, cognitive and psychiatric disorders. The neuropathologic hallmarks are Lewy bodies and Lewy neurites in surviving neurons which are composed of proteinaceous inclusions comprised of α-synuclein, ubiquitin and other proteins. Pathological mechanisms implicated in PD include aberrant protein aggregation, mitochondrial dysfunction, oxidative stress, and failed cellular processes such as apoptosis, autophagy, proteasomal pathway, and several cellular stressors. To analyse the implication and contribution of some of these cellular processes, we altered the expression of the; classical apoptotic genes namely *Buffy* and *Debcl*; autophagy genes *Atg6* and *Pi3K59F*; the antiapoptotic *transmembrane Bax inhibitor-1 containing motif* (TMBIM) family *Bax inhibitor-1*, *Lifeguard* and *GHITM*; mitochondrial genes such as *HtrA2, MICU1, porin/VDAC, Pink1* and *parkin*; and an increased disease risk gene *pyridoxal kinase* in DA neurons and in the developing eye. We show that the altered expression of key genes can either be detrimental or beneficial to the health of DA neurons as determined by lifespan and locomotor function, in addition to supportive results obtained from biometric analysis of phenotypes from the neuron rich eye. We found that the overexpression of *Buffy*, the sole pro-survival *Bcl-2* homologue in Drosophila could suppress the loss of function-induced phenotypes.

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Dedication

This thesis is dedicated to my late father, for whom this would have been a proud moment, a story to tell his clique about the achievements of his son. I know he has a big smile right now and may God rest his soul in eternal peace.

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List of Symbols and Abbreviations

°C	degree Celsius
α	alpha
ADP	adenosine diphosphate
ADPD	Autosomal dominant Parkinson disease
AIF	apoptosis-inducing factor
ANT	adenine nucleotide translocator
Apaf-1	apoptosis protease-activating factor 1
ARJP	Autosomal recessive juvenile Parkinsonian
ATF6	activating transcription factor 6
Atg	autophagy related protein
ATP	adenosine triphosphate
Bad	Bcl-2-associated agonist of cell death
Bak	Bcl-2 antagonist/killer
Bax	Bcl-2 associated X protein
Bcl-2	B cell lymphoma 2
BH	Bcl-2 homology
Bid	BH3 interacting domain death agonist
Bik	Bcl-2 interacting killer
Bok	Bcl-2 ovarian killer
BOP	BH3 only proteins
CARD	caspases activating recruitment domain
CDD	conserved domain database
CI	confidence interval
CMA	chaperone-mediated autophagy

CoQ	co-enzyme Q
CyO	curly of Oscar
cypD	cyclophilin D
CytC	cytochrome C
DA	dopaminergic
Ddc	dopa decarboxylase
Debcl	death executioner Bcl-2
DIAP	Drosophila inhibitor of apoptosis
DNA	deoxyribonucleic acid
ELM	Eukaryotic linear motif
EOAR	early-onset autosomal recessive
EOPD	early onset Parkinson disease
ER	endoplasmic reticulum
ERAD	ER-associated degradation
ETC	electron transport chain
Gal4	yeast transcription activator
GHITM	growth hormone-inducible transmembrane protein
GMR	Glass multiple reporter
HtrA2	High temperature requirement A2
IAP	inhibitor of apoptosis
IFM	indirect flight muscles
IMM	inner mitochondrial membrane
IRE1α	inositol-requiring enzyme 1α
lacZ	lactose
LB	Lewy bodies
LFG	Lifeguard

LN	Lewy neurites
MAM	mitochondrial-associated ER membranes
MAO	monoamine oxidase
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
mPTP	mitochondrial permeability transition pore
mtDNA	mitochondrial deoxyribonucleic acid
MTS	mitochondrial targeting signal
NAD	nicotinamide adenine dinucleotide
NCBI	National Center for Biotechnology Information
NES	nuclear export signal
NLS	nuclear localization signal
OMM	outer mitochondrial membrane
PARIS	Parkin interacting substrate
PCR	polymerase chain reaction
PD	Parkinson disease
PERK	protein kinase RNA-like kinase
PI3K	phosphatidyl inositol 3 kinase
Pink1	PTEN (phosphatase and tensin homologue)-induced putative kinase 1
RHG	reaper, HID, grim
RNA	ribonucleic acid
RNAi	RNA interference
RNS	reactive nitrogen species
ROS	reactive oxygen species
SEM	standard error of the mean
SNpc	Substantia nigra pars compacta
TFAM	mitochondrial transcription factor A

TH	tyrosine hydroxylase
TMD	transmembrane domain
TMBIM	transmembrane Bax inhibitor-1 containing motif
TNF	tumour necrosis factor
UAS	upstream activating sequence
UPR	unfolded protein response
UPS	ubiquitin proteasome system
UV	ultra violet
VDAC	voltage-dependent anion channel

Chapter 1 - Introduction and Overview

Parkinson Disease

Parkinson disease (PD) is a neurodegenerative disease that is characterized by the progressive loss of specific populations of dopaminergic neurons and can result in substantial disability and early death [1]. PD is the most common movement disorder and afflicts about 1 to 2% of the population over 50 years of age [1, 2]. Patients with PD show profound motor, psychological and emotional dysfunction owing to degeneration and death of dopaminergic (DA) neurons in the *Substantia Nigra pars compacta* (SNpc) [3, 4]. Some of its more common symptoms include muscle rigidity, resting tremors, postural instability, and bradykinesia paired in some cases with non-motor symptoms such as autonomic, cognitive and psychiatric problems [3, 5]. The neuropathological hallmarks exhibited by PD patients are Lewy Bodies (LB) and Lewy neurites (LN) in surviving neurons, which show the presence of eosinophilic, intracytoplasmic proteinaceous inclusions comprised of α-synuclein and ubiquitin as the major components, and other proteins [3, 6, 7]. The identification of familial forms of the disease; of which at least 20 gene loci associated with PD have been identified [8, 9] shows that, together with the sporadic forms, increased oxidative stress, dysfunction in cellular clearance system, mitochondrial dysfunction, and inflammation are common hallmarks in the pathology of both forms of PD.

Genetics of Parkinson disease

Most cases of PD are sporadic with no known causative factor though environmental and genetic factors are known to contribute to the development of the disease [9]. The

identification of inheritable forms of PD, caused by monogenic mutations have been pivotal to understand the underlying disease process at the molecular level, and in the generation of model systems [10-12]. The complexity of the genetics of PD are highlighted by the numerous gene loci identified, together with a host of disease risk factors. Currently, 15 genes that cause Mendelian forms of PD have been confirmed that display autosomal dominant or autosomal recessive form of inheritance, in addition to at least 25 genetic risk factors that were discovered through genome wide association studies [12]. The PD-linked genes along with the risk factors are implicated in a plethora of cellular functions that are biologically important and include, regulation of miRNAs, vesicular trafficking, mitochondrial homeostasis and biogenesis, cellular clearance systems, ER homeostasis, Golgi homeostasis, among many other cellular processes [9, 11, 12]. The dysfunction of many of these pathways contribute to the disease pathology in both sporadic and familial forms of PD.

Currently, at least 20 distinct gene loci *PARK1* to *PARK20* have been described for familial PD (see Appendix 4) that include; *α-synuclein*/ *PARK1/4* [6], *parkin*/ *PARK2* [13], *Ubiquitin C-terminal hydrolase-1 (Uchl-1)*/ *PARK5* [7], *Phosphatase and tensin homologue [PTEN] induced kinase 1* (*Pink1*)/ *PARK6* [14], DJ-1/*PARK7* [15]*, Leucine rich repeat kinase 2 (LRRK2)*/ *PARK8* [16, 17]. Other mutated gene loci that have been implicated in rare FPD are *Adenosine triphosphate 13A2 (a P-type ATPase)*/ *PARK9* [18], *Grb10-Interacting GYF Protein-2 (GIGYF2)*/ *PARK11* [19], *High temperature requirement A2 (HtrA2)/ PARK 13* [20], *Phospholipase A2 (PLA2G6)*/ *PARK14* [21], *F-box only protein 7 (FBXO7)/ PARK15* [22], *Vacuolar sorting protein 35 (VPS35)/ PARK17* [23, 24]*, eukaryotic translation initiation factor 4 gamma 1 (EIF4G1)/ PARK18*

[25], *dnaJ homologue subfamily C member 6 (DNAJC6)/ PARK19* [26], *synaptojanin 1 (SYNJ1)/ PARK20* [27, 28]. Additional genes have been identified that cause PD; *dnaJ homologue subfamily C member 13 (DNAJC13)* [29], *ATPase H+ transporting lysosomal accessory protein 2 (ATP6AP2)* [30], and *coenzyme Q2 4-hydroxybenzoate polyprenyltransferase (CoQ2)* [31] but require further replication, or lack conclusive linkage, or whose replication studies have not yielded the same results as the original findings [12]. Other FPD causative gene loci exist (*PARK*) whose mutated gene is currently unknown and include *PARK3, PARK10, PARK12* and *PARK16* [8]. The characterization of these genes has led to advances in the understanding of disease pathogenesis and produced robust models in experimental organisms.

Drosophila models of Parkinson disease

A majority of the genes associated with PD show high conservation in a wide variety of organisms and as such can be modelled to study the consequences of their altered expression by assessment of the resultant phenotypes [12, 32, 33]. Drosophila possesses an array of advantages as a model organism that include; a complex nervous system with a DA neuron cluster, large collections of mutant strains, an array of genetic manipulation techniques, easy transgenesis and mutagenesis, and large numbers of approaches for genetic screens. Most of the identified PD genes, at least 15, have a homologue in Drosophila, except for *SNCA* and *FBXO7*: a putative homologue has been reported in our laboratory (Merzetti and Staveley, unpublished work) but nonetheless the expression of the human versions in Drosophila neurons has been achieved [9]. Briefly, a few Drosophila PD models will be described.

The α-synuclein model

The first gene to be identified associated with autosomal dominant PD was *α-synuclein (SNCA/ PARK1/PARK4)* and encodes a small and soluble presynaptic nerve terminal protein with as yet unknown function; that constitute the main component of Lewy bodies (LB) in both sporadic and familial forms of PD and its aggregation is believed to be the main neuropathogenic cause of PD [3, 6, 34]. Types of mutations in the *α-synuclein* gene include point mutations, duplications, triplications, and multiplications and are implicated in PD [8]. These mutations and high levels of α-synuclein seems to aid in the generation of insoluble protein aggregates and high order fibrils that are implicated in neurotoxicity and PD pathogenesis [8, 10, 11]. 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 neurotoxicity in DA neurons. This aggregation is worsened by post-translational modifications such as phosphorylation on serine 129, calpain-mediated cleavage, O-glycosylation, tyrosine nitration, methionine oxidation, and C-terminal truncation [8, 35]. The mechanisms for α-synuclein toxicity are numerous and suggest that; α-synuclein oligomers alters plasma membrane stability or permeability by formation of pores that increase intracellular calcium, α-synuclein interacts with the mitochondria and its components to induce mitochondrial dysfunction that increase ROS and oxidative stress [36, 37], α-synuclein interferes with synaptic transmission to cause accumulation of docked vesicles at the presynaptic membrane and thus reduce the recycling vesicle pool that increases cytosolic levels of dopamine [38], and through its association with cellular pathways, α-synuclein inhibits the ubiquitin-proteasome system, endosomal-lysosomal system, autophagy, ER-Golgi trafficking, Golgi homeostasis, and

promotes apoptosis [39]. The pathways disrupted by α-synuclein may be indicative of how α-synuclein achieves neuronal toxicity and contributes to the aetiology of PD. Although Drosophila seem to lack a clear *α-synuclein* orthologue, the gain of function was achieved by the expression of human wild type and two mutant forms of *α-synuclein*, A30P and A53T [40]. This model, and others that were developed later were able to reproduce the key features of PD such as, adult-onset degeneration and loss of DA neurons, accumulation of proteinaceous inclusions containing α-synuclein that were similar to Lewy bodies, and locomotor dysfunction [40, 41]. The most controversial finding was the degeneration and loss of DA neurons in the Drosophila brain, with inconsistent reports by different groups [33, 42]. This shows a remarkable and robust model system that has been used to understand the pathophysiology of PD. In early studies, the directed expression of *Hsp70*, a molecular chaperone up-regulated in stress responses that refolds misfolded proteins, mitigated DA neuronal loss induced by *α-synuclein* [41]. The propensity for α-synuclein to form aggregates is increased on phosphorylation, α-synuclein is found heavily phosphorylated in the brains of patients with PD, was shown to be conserved in Drosophila [43] and that α-synuclein toxicity may be modulated by phosphorylation. Several studies have shown the suppression of the *α-synuclein*-induced phenotypes, such as the co-expression of the *α-synuclein* transgene with; *parkin,* a ubiquitin E3 ligase [44], *Rab1,* a guanosine triphosphate [45], *Sirtuin2*, a histone deacetylase [46], and *PTEN induced putative kinase1 (Pink1),* a mitochondrial kinase [47]. Investigation of the interaction of α-synuclein with other proteins has been an attractive area of stimulated research and may be important in the formulation of therapies and in management of PD.

The impact of antioxidants on *α-synuclein* flies has been investigated, the co-expression of human Cu/Zn superoxide dismutase (SOD) with *α-synuclein* A30P reduced the observed neurodegeneration [48]. Moreover, flies fed with antioxidants slightly altered the *α-synuclein*-induced phenotypes such as reported on a blueberry extract [49]. This showed that lowered cytoplasmic levels 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, whereas the dopamine agonists pergolide, bromocriptine and SK & F 38393 were substantially effective [50]. Atropine was found to be effective but to a lesser extent than the other anti-Parkinson compounds. Genomic investigation of the transcriptional program of *α-synuclein* models at pre-symptomatic, early and advanced stages revealed 51 signature transcripts that include lipid, energy & membrane mRNAs that were highly distinct and either up-regulated or down-regulated in common cellular pathways [51] and the dysregulated proteins are associated with membranes, ER, actin cytoskeleton, mitochondria and ribosomes [52]. These variations show the complexity of biological systems that are modified by α-synuclein in a model of Parkinson's disease.

The Parkin model

The *parkin/PARK2* gene encodes a cytosolic ubiquitin E3 ligase that selectively targets misfolded proteins to the ubiquitin proteasome system (UPS) for degradation [53, 54]. Parkin consists of an N-terminal ubiquitin-like domain (Ubl), and four zinc-coordinating RING-like domains: RING0, RING1, an in-between RING (IBR), and RING2 [55]. Over 170 mutations in this gene have been identified which are responsible for familial early-

onset PD (EOPD) [7, 13, 56] and has been implicated in dysfunction in a number of cellular processes such as UPS, autophagy and endosomal-lysosomal pathway. Parkin is involved in the ubiquitination of substrates in cooperation with ubiquitin E2 enzymes, it first needs to be activated from its inactive or auto-inhibited form [55, 56]. Pink1 and parkin are implicated in the same pathway that clears dysfunctional mitochondria via a specialized form of autophagy called mitophagy [55-57]. Parkin is activated through phosphorylation on ser65 by Pink1 and by phospho-ubiquitin. Upon activation, parkin modifies numerous cytosolic and outer mitochondrial membrane (OMM) proteins [56], key to mitophagy is its ability to add phospho-ubiquitin chains to defective mitochondria and target it for clearance [56, 58], and ER-associated degradation-like extraction of proteins from the OMM (OMMAD) [56, 59]. There is consensus that parkin and Pink1 participates in the same pathway to rid cells of defective mitochondria and that Pink1 senses the damage and recruit parkin. Other functions of parkin are discussed elsewhere in this chapter.

The generation of *parkin* transgenic flies has contributed to the wealth of information available on the biological functions of parkin to date [33, 60]. 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 [61], reduced body size and cell size, sensitivity to oxidative and environmental stress [62], and degeneration and loss of DA neurons in the adult brain [63]. In addition, the overexpression of a human *parkin* mutant variant (R375W) in Drosophila resulted in an age-dependent degeneration of specific DA neuronal clusters, concomitant locomotor deficits that accelerated with age due to mitochondrial abnormalities [64]. The insights

from studying Drosophila *parkin* mutants are immense and have highlighted the importance of this model organism in modelling human neurodegenerative diseases.

The Pink1 model

The second autosomal recessive EOPD gene to be identified was *PARK6/Pink1* that encodes the Phosphatase and tensin homologue (PTEN)-induced kinase1 (Pink1) [14, 65, 66], the protein sequence of which contains an N-terminal mitochondrial targeting signal sequence (MTS) and a highly conserved serine/threonine protein kinase domain of the Ca^{2+} calmodulin family. Mutations in *Pink1* gene resulted in nigrostriatal neuronal loss with Lewy bodies as observed in post-mortem brains of patients with PD due to loss of its kinase activity [67]. Pink1 is a nuclear encoded gene that is translated in the cytosol and via the MTS is imported into the mitochondria where it is believed to act as a mitochondria damage sensor [68]. Studies have demonstrated different localization, with some to the inner mitochondrial membrane, intermembrane space, or to the outer mitochondrial membrane [67]. Pink1 is important in mitochondrial function as it is involved in the removal of dysfunctional mitochondria.

Drosophila *Pink1* is found to localize in the mitochondria and is detected at all developmental stages with high levels in the adult brain and testes, and show significant homology and functional conservation with the human version [69, 70]. The *Pink1* mutant flies have a host of phenotypes that included 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, ommatidial and DA neuron degeneration and finally locomotor defects.

These phenotypes were remarkably similar to those found in *parkin* mutant flies [69-73]; 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 has a role in spermatogenesis to regulate mitochondrial morphology [69]. 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 mitochondrial DNA (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 [69, 70, 73]. This indicated that suppression of Drosophila *Pink1* could lead to age-dependent muscle degeneration characterized by extensive dysfunction and DNA fragmentation that is indicative of apoptotic cell death.

Further studies in Drosophila and cell systems demonstrated that parkin is recruited by Pink1 to depolarized mitochondria to mediate selective autophagic removal of the damaged mitochondria [60]. The finding that Pink1 directly phosphorylates parkin [74] or phosphorylates ubiquitin which in turn activates parkin, led to the unravelling of the Pink1/Parkin pathway, in which Pink1 detected the damaged mitochondria and signals parkin to ubiquitinate the defective mitochondria and thus target them for autophagic removal [56, 75]. An alternative mechanism for parkin recruitment is the phosphorylation of mitofusin 2 (Mfn2) by Pink1 which then acts as a parkin receptor [76]. Upon

recruitment to the outer mitochondria membrane, parkin ubiquitinates numerous targets that are degraded through the proteasome and attaches chains of ubiquitin to the damaged mitochondria in readiness for mitophagy. The Pink1/Parkin pathway has been implicated in the regulation of mitochondrial quality control by interaction with the fission/fusion machinery [77-79], regulates localized translation of select nuclear-encoded respiratory chain complex mRNAs [80], regulates selective mitochondrial respiratory chain turnover [81], and retards mitochondrial motility by regulation of Miro, a component of the motor/adaptor complex, that quarantines damaged mitochondria [82]. Pink1 can modify other PD-associated phenotypes, such as its rescue of α-synuclein [47], or HtrA2 [83], and possible association with DJ-1 [53]. The fly model was first to demonstrate that Pink1 and parkin are key regulators of mitochondrial quality control through genetic epistatic studies.

The HtrA2 model

Loss of function mutations in the *PARK13/HtrA2* gene that encodes *H*igh *t*emperature *r*equirement *A2* (HtrA2 also known as Omi) that interfere with its protease activity are strongly associated with PD [20]. HtrA2 is a nuclear encoded mitochondrial localized serine protease with an N-terminal mitochondrial targeting signal and exhibits proapoptotic and cell protective properties; it is very closely related to the Drosophila Reaper, Hid and Grim proapoptotic proteins by the presence of the inhibitor of apoptosis (IAP) inhibitory Reaper-like motif [84]. A141S and G399S are two mutant alleles that were found in PD patients and were determined to be causative to PD [20], but have since been challenged after being found to segregate in non-PD population [85, 86]. In a

subsequent study, a new mutation R404W was characterized in the PDZ domain that abrogated the protease function of HtrA2 [87], and thus confirmed its role in PD susceptibility. Briefly, HtrA2 is sequestered within the mitochondrial intermembrane space and is released into the cytosol during apoptosis where it binds IAPs and thus blocks them from inhibiting caspases [88]. The binding of Omi/HtrA2 to IAPs activates the protease function. The common facet of PD is mitochondrial dysfunction and especially the loss of mitochondrial Complex I function in the midbrain of PD patients highlights the importance of the mitochondria in the pathology of PD [37, 89, 90]. This protein may play a role in cellular protection due to stress.

In *Drosophila melanogaster*, HtrA2 has been shown to have apoptotic functions through cleavage of IAP1 in the vicinity of the mitochondria, lending credence to the mitochondrial cell death pathway [60, 91, 92]. Modelling of PD in Drosophila is robust and has been insightful in understanding the role of several PD-linked genes in disease pathology [33, 93, 94]. The link between *HtrA2* and other PD-linked genes has been demonstrated in both mammals and the fly, including the Pink1 kinase [83, 95] which was found to phosphorylate HtrA2 in response to p38 SAPK activated pathway, and the E3 ubiquitin ligase, parkin [83, 96]. The link between HtrA2 and the Pink1/Parkin pathway has been challenged by an *in vivo* study that relied on a loss of function [97]. Whether HtrA2 functions downstream of the Pink1/Parkin pathway or if it affects mitochondrial homeostasis, the loss of its protease activity is known to lead to neuronal degeneration. Phenotypic analysis of *HtrA2* mutants revealed male infertility, stress-mediated apoptosis [96], and mild mitochondrial defects [97]. The release of HtrA2 from the mitochondria in Drosophila is dependent upon cellular stress such as UV irradiation

and cleaves Drosophila inhibitor of apoptosis (DIAP1) to activate apoptosis [91, 92, 98]. HtrA2 was found to interact with the Pink1 but this association was independent of parkin, it was determined that the HtrA2-induced phenotypes were of a mild nature than those of Pink1 [83], though a different study did not find this association [97]. The studies so far reveal the strong association between HtrA2 loss of function and susceptibility to PD.

Mitochondrial dysfunction in Parkinson disease

Mitochondrial dysfunction is one of the mechanisms implicated in the pathogenesis of PD where post-mortem studies have revealed evidence for oxidative stress [99, 100] as a result of defective mitochondrial Complex I function [101, 102], and oxidative damage in nigrostriatal DA neurons in the pathology of PD. The identification of mutations in genes strongly associated with familial forms of PD that have a mitochondrial function has highlighted the role of defective mitochondrial function in the pathogenesis of PD [103, 104]. Mitochondrial dysfunction appears to be a common hallmark in both sporadic and familial forms of PD, that include impaired electron transport chain, damaged mitochondrial DNA, defective calcium buffering and abnormal mitochondrial morphology and dynamics [36, 37, 75]. The effects of dysfunctional mitochondria are widespread in DA neurons that consequently result in cellular oxidative stress, apoptosis, autophagy and inflammation.

Mitochondrial electron transport chain complexes in PD

Earliest evidence on the involvement of mitochondria in the pathology of PD was from the discovery that MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) causes PD-like

symptoms in humans [105]; MPTP is metabolized to its toxic form MPP+ (1-methyl-4-phenylpyridinium ion) by mitochondrial monoamine oxidase B (MAO B) and is concentrated in the mitochondria through an energy-dependent process. It then specifically inhibits the oxidation of nicotinamide adenine dinucleotide (NAD)-linked substrates by blocking the electron transfer through Complex I [68]. Complex I (NADH-ubiquinone oxidoreductase) is a catalyst for electron transfer from NADH into ETC subunits (Figure 1.1) and therefore, acts as one of the entry point for electrons from the mitochondrial matrix into the ETC, which makes it an important site for ROS production [90]. MPP+ also inhibits the activity of α-ketoglutarate dehydrogenase complex (KGDHC), a key tricarboxylic acid (TCA) enzyme and thus impedes ATP synthesis to possibly induce an "energy crisis" [68].

Compounds such as rotenone, paraquat, and maneb, among others interfere with Complex I activity and result in elevated ROS levels that increases oxidative stress, and have been linked to parkinsonism. The presence of deletions in mitochondrial DNA (mtDNA) of patients with PD are known to affect other respiratory chain enzymes such as cytochrome c oxidase [106]. It appears the close proximity of mtDNA to ROS generated by the impaired respiratory chain, and a lack of efficient DNA repair mechanism, contributes to the vulnerability of mtDNA to mutations [90]. A generalized defect in the assembly of complexes I to V has been observed, and has been implicated in the elevated levels of ROS and the subsequent oxidative stress. A direct association between a subunit of

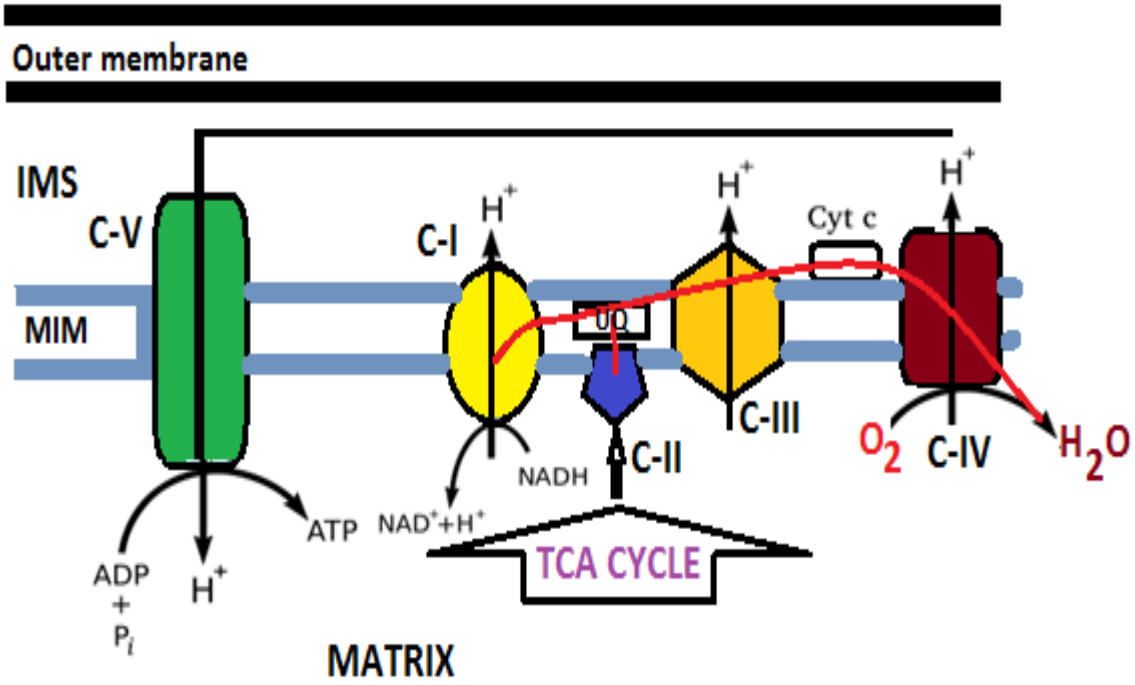

Figure 1.1 Mitochondrial respiratory chain

A simplified representation of the mitochondrial electron transport chain (ETC) in which Complex I to IV and Complex V also known as ATP synthase are shown. The ETC is coupled to the oxidative phosphorylation (OxPhos) by a proton gradient across the inner mitochondrial membrane. In Complex I (NADH: ubiquinone oxidoreductase), two electrons are removed from NADH and transferred to Ubiquinone (UQ) which is reduced to ubiquinol (UQH_2). Complex I then translocate four protons from the matrix to the intermembrane space (IMS). Complex II (Succinate dehydrogenase) transfers more electrons to the UQH_2 pool from succinate (a TCA intermediate) via FAD. No protons are generated into the IMS in this pathway. Cytochrome *c* reductase or Complex III sequentially transfers two electrons from UQH_2 to two molecules of cytochrome *c*. Four electrons are then removed from four molecules of cytochrome *c* by Complex IV (cytochrome *c* oxidase) and transferred to molecular oxygen to form water. Protons are released to the IMS. This forms a proton gradient or electrochemical gradient that is utilised by ATP synthase or Complex V to make ATP via a proton flux back into the matrix. These redox reactions can be leaky and as thus prematurely release electrons to oxygen forming superoxide radicals. The main sites for reactive oxygen formation are Complex I and III.

Complex I (composed of at least 49 different subunits), GRIM-19 and a PD susceptibility gene, HtrA2 has been shown [68, 105]; GRIM-19 enhances the pro-apoptotic activity of HtrA2.

Mitochondrial permeability transition pore (mPTP) and PD

The mitochondrion is composed of an outer membrane (OMM), that is permeable to small molecules, and an inner membrane (IMM), that is selectively permeable to many molecules [107, 108] and only allow specific substrates into the matrix by use of transporters. Mitochondrial membrane transition results from an increase in IMM permeability to molecules that have a molecular mass of less than 1500 Da due to the reversible opening of a large protein complex pore [107, 109, 110]. This opening is facilitated by depletion of ATP and ADP, calcium overload, fatty acid levels, phosphate levels, and oxidative stress [108]. This causes the non-specific entry of water and solutes into the mitochondria which cause the mitochondrial matrix to swell, disruption of oxidative phosphorylation, loss of mitochondrial membrane potential and reduction in respiratory chain function [108-110]. In addition to the dissipation of the mitochondrial transmembrane electrochemical proton gradient, termination of ATP synthesis, loss of respiratory substrates and nucleotides from the matrix, excess generation of ROS, release of calcium and apoptogenic proteins, the sustained opening of the mPTP results in cell death.

The molecular constituents of the mPTP complex remain controversial but studies show the major proteins thought to comprise the mPTP are: voltage-dependent anion channel (VDAC) located in the OMM, adenine nucleotide translocator (ANT) present in the IMM,

and cyclophilin D (cypD) found in the matrix [108, 109]. Other proteins believed to be components of the mPTP are F_0-F_1 ATP synthase (Complex V), translocator protein (TSPO), mitochondrial phosphate carrier (PiC), and paraplegin, among others. This area remains controversial and only future studies will confirm the components of the mPTP. What is not in dispute is the role the mPTP plays in disease pathogenesis, by being a key component of mechanisms such as apoptosis, autophagy, necrosis and "necroptosis". The strongest connection of mPTP to PD was the observation that calcium-induced mitochondrial dysfunction in PD-related neuronal death by MPP+ and dopamine which stimulated the release of calcium from the mitochondria [68], caused mitochondria to swell, release cytochrome c, and membrane depolarization.

Role of PD-linked genes in mitochondrial dysfunction

Monogenic causes of PD are known to contribute to less than 10% of the total cases, and the gene products seem to have a direct or indirect role in mitochondrial function and consequently in disease pathogenesis [68]. The non-amyloid beta component of α-synuclein makes it prone to aggregate into fibrils which are the main component of Lewy bodies found in both sporadic and familial forms of PD [6, 7]. The effects of α-synuclein on mitochondrial dysfunction are both direct and indirect, for example, the application of mitochondrial toxins is known to result in the formation of α-synuclein aggregates and inclusions [111], while the inhibition of the proteasomal pathway causes the accumulation of α-synuclein leading to mitochondrial dysfunction [112]. The mitochondrial import and accumulation of α-synuclein impairs Complex I in the SNpc and striatum of PD patients [113]. *In vivo* and *in vitro* studies have highlighted a strong pathogenic role of α-

synuclein in mitochondrial dysfunction, mutant α-synuclein causes mitochondrial degeneration, mtDNA damage, and an impaired ETC [114]. Within the mitochondria, α-synuclein is known to interact with components of the ETC in a deleterious manner, such as Complex I, Complex IV cytochrome oxidase [115], and adenylate translocator [116]. The presence of a cryptic mitochondrial targeting signal in the N-terminus of α-synuclein facilitates its translocation into the mitochondria [113]. In addition, α-synuclein selectively conforms to the mitochondria [117] due to the physiological environment present in neurons. This leads to mitochondrial fragmentation and decreased respiration [118]. In a similar way, α-synuclein appears to regulate mitochondrial calcium levels as it enhances ER-mitochondrial interactions [119]. The association of α-synuclein with the mitochondria, especially Complex I, has been shown to lead to increased apoptosis through caspase-dependent and caspase-independent pathways, with most neuronal death occurring by autophagy [120, 121]. The association of α-synuclein with the adenylate translocator (ANT), a component of the permeability transition pore complex (PTPC), leads to membrane depolarization and mitochondrial morphological changes that result in translocation of AIF to the nucleus and increased neuronal apoptosis [116]. The blocking of the chaperone-mediated autophagy by α-synuclein results in cellular stress that activates apoptosis [122]. In brief, α-synuclein seems to alter mitochondrial homeostasis, cellular bioenergetics and disrupt several cellular systems that are critical to cell survival. The loss of the E3 ubiquitin ligase activity of parkin, responsible for an early onset juvenile form of autosomal recessive parkinsonism, is known to result in accumulation of toxic protein aggregates by a failure of the clearance system [13, 123] and gross mitochondrial defects [61, 62]. Parkin-dependent ubiquitination is known to control

several pathways that mediate transcriptional regulation, protein trafficking, and neuroprotection. *Parkin* mutations have been shown to impair mitochondrial Complex I and IV activities in human leukocytes of patients with PD [124], and in *parkin* knock out mice [125]. Parkin mediates mitochondrial biogenesis through transcription and translation by direct import into the mitochondria and interaction with TFAM [126]. Parkin-mediated biogenesis and promotion of cell survival occurs via its proteasomal degradation of PARIS [55]. Importantly parkin is pivotal in mitochondrial dynamics and mitophagy [127], as it is selectively recruited to impaired mitochondria by Pink1, and facilitates their removal via mitophagy [59]. In the Pink1/Parkin pathway, Pink1 senses mitochondrial damage and recruit's parkin, which ubiquitinates the damaged mitochondria and targets it for cellular clearance through mitophagy. Another model for parkin recruitment to damaged mitochondria involve the voltage-dependent anion channels (VDAC) [128], which serve as mitochondrial docking sites. The Pink1/Parkin pathway promotes mitochondrial fission/fusion and controls mitochondrial dynamics [69, 77, 78]. Parkin plays an important role in mitochondria homeostasis, by being directly involved in the biogenesis and clearance of defective mitochondria.

Mutations in *Pink1/PARK6* cause EOAR PD and result in severe mitochondrial dysfunction [14, 69]. Pink1 has a mitochondrial targeting signal, it has been shown to localize to the IMM, intermembrane space [67, 129] and the OMM [67]. Studies have shown a wide range of mitochondrial defects in Pink1 mutants, that include abnormal mitochondrial morphology, reduced membrane potential, elevated generation of ROS, deficits in respiratory chain complexes I to IV activity, and ATP synthesis defects [68]. As previously discussed, Pink1 is a sensor of damaged mitochondria, and as such, is

pivotal in mitochondrial quality control [56]. Pink1 accumulates on dysfunctional mitochondria, and its kinase activity is essential for parkin recruitment [130]. In healthy mitochondria, Pink1 is rapidly degraded but on defective mitochondria, Pink1 accumulates and becomes stabilized on the OMM where it recruits parkin and activates its E3 ubiquitin ligase activity [59].

Apoptosis is one of the mechanisms suggested for the progression of PD and investigation of neuronal apoptosis in PD has found elevated levels of anti-apoptotic proteins, Bcl-2 and Par-4 in DA neurons [131]. Other gene products believed to have a vital function in the maintenance of mitochondrial integrity and turnover, such as, *Pink-1*, *parkin*, and *HtrA2* have been implicated in the aetiology of PD [8]. Pink1 can also activate parkin indirectly through phosphorylation of ubiquitin, which in turn activates parkin. Pink1 has other substrates that promote mitophagy, such as: Miro1 [82] whose phosphorylation and degradation arrests mitochondrial motility; Mitofusin2 which upon phosphorylation by Pink1 binds parkin and leads to its degradation; others include HtrA2 and tumour necrosis factor receptor-associated protein 1 (TRAP-1).

DJ-1/PARK7, a susceptibility gene that cause rare cases of EOAR parkinsonism, has antioxidant and transcription modulation activity and is thought to localize to the mitochondria [15, 132]. DJ-1 translocates to the mitochondria upon oxidative stress and promotes neuroprotection. Mutant DJ-1 DA neurons show a deficiency in Complex I activity and consequently generates more ROS, reduction in mitochondrial membrane potential, abnormal morphology and leads to accumulated dysfunctional mitochondria [133].

Mutations in *Lrrk2/PARK8* cause autosomal dominant PD [16, 17], Lrrk2 is a multi-functional protein, it is thought to localize to the mitochondria [134]. PD patients show a decrease in mitochondrial membrane potential, low ATP levels and elongated mitochondria. Lrrk2 interacts with DLP1 to induce mitochondrial fission, it further stimulate mitophagy through its interaction with ULK1, a key component in the initiation of autophagy [135]. There seems to be an intricate relationship between mitochondrial dysfunction and PD-associated gene products, to maintain mitochondrial fidelity, homeostasis and dynamics.

Molecular mechanisms of Parkinson disease

Neuronal apoptosis

The term apoptosis was coined by Kerr and his colleagues in 1972, a Greek word used to describe leaves falling from trees or petals from flowers, in reference to the particular morphology of physiological cell death [136]. By definition apoptosis refers to the morphologic features of cell death that manifest through cell shrinkage, nuclear condensation, membrane blebbing, fragmentation into membrane bound apoptotic bodies, and membrane changes that targets the dying cells for phagocytosis [137, 138]. These are the distinct characteristics that differentiate apoptosis from other types of cell death. The three successive stages of apoptosis are: 1) commitment to death triggered by extrinsic or intrinsic signals; 2) cell killing by activation of caspases and other proteases; and 3) engulfment of dead neurons by phagocytic cells such as macrophages and glial cells [138, 139]. Apoptosis is an important cellular mechanism that is tightly controlled.

Extrinsic death pathway

The sensors mediating neuronal apoptotic signals from the extracellular to the intracellular death machinery are known as death receptors [140]. Neuronal apoptosis induced via the surface death receptors is mediated by the tumor necrosis factor receptor (TNFR) superfamily that is composed of Fas/Apo-1, TNFR-1 and p75NTR (Figure 1.2) [141]. The receptors contain an extracellular ligand binding cysteine motif and an intracellular death domain. Upon ligand binding for example FasL, TNF-related apoptosis-inducing ligand (TRAIL), TNF-α, or NGF to their respective surface receptors (Figure 1.2), the assembly of the death-inducing signaling complex (DISC) is initiated and induces the clustering of the death domains of the receptor [140]. Fas-associated death domain (FADD), an adapter protein, binds through its death domain to the clustered death domains of the receptor. FADD has a death effector domain (DED, which is a type of caspase activation and recruitment domain or CARD) which it uses to recruit the procaspase-8 (FLICE/MACH) and auto-cleaves the DED that releases the active form of caspase-8. Activated caspase-8 cleaves effector caspases, such as caspase-3 and mediates neuronal killing [140, 142]. One of the substrates for caspase-8 is the pro-apoptotic Bcl-2 member Bid; when cleaved (tBid), it translocates to the mitochondria where it mediates the disruption of the OMM [143]. This is one of the key evidences for crosstalk between the two mechanisms of neuronal apoptosis. This extracellular pathway appears less important in neurons than the one mediated by p75NTR/p75-NGFR/TNFR2 (Figure 1.2), which cooperates with Trk receptors [144]. Several modifiers of this pathway in neurons have been identified, key among them is the anti-apoptotic Lifeguard (LFG/TMBIM2) also known as neural membrane protein 35 (nmp35), that is predominantly expressed in

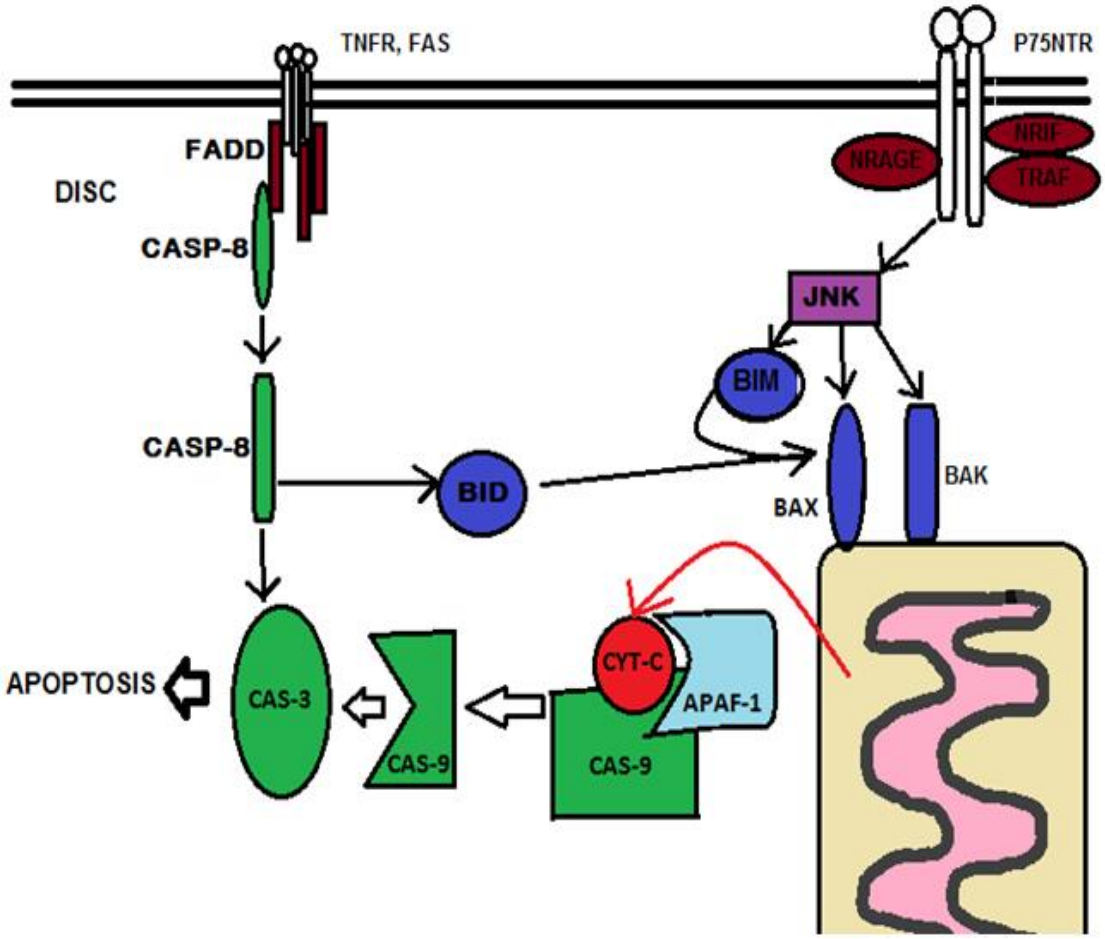

Figure 1.2 The extrinsic cell death pathway

A schematic representation of the extrinsic death pathway that show the key molecules. The assembly of the death inducing signalling complex (DISC) starts with the binding of a ligand to the TNF or FAS receptors. FADD has death domains which it uses to bind to the receptor's death domains and death effector domains (DED, a type of a caspase activation and recruitment domain) which it uses to recruit pro-caspase-8. Once pro-caspase-8 binds to FADD, it is auto-cleaved at the DED and the active form released that activates caspase-3. Caspase-8 can also activate the mitochondrial death pathway via activation of the Bcl-2 protein Bid.

the nervous system and has been shown to block cell death induced by the FasL but not by TNFα [145]. This demonstrates the specificity of extracellular death receptors and their ligands to highlight the divergence of death pathways. Extracellular death signals promote neuronal death by the inhibition of survival pathways and the promotion of the activation of pro-apoptotic molecules that ultimately elicit an intrinsic death signal that activates caspases or other proteases involved in cell death.

Intrinsic death pathway

The intrinsic pathway that is mostly controlled by the Bcl-2 proteins depends on a complex interplay between the anti-apoptotic and the pro-apoptotic members of this family of proteins [146]. The intrinsic pathway, also known as the Bcl-2-regulated death pathway (Figure 1.3), monitors the intracellular environment and relays this information mostly through the Bcl-2 proteins to the mitochondria [147]. When death-inducing signals, such as damaged DNA, oxidative stress, or other cytotoxic stress outweigh the survival signals, then the mitochondrial membrane is breached through a concerted effort of pro-apoptotic Bcl-2 proteins and cytochrome c and additional death inducing molecules are released [143, 148]. The death effectors in this pathway can either proceed by a caspase-dependent or caspase-independent pathway.

The Bcl-2 family of proteins have a central role in the regulation of the intrinsic apoptotic pathway. Whereas in mammals they exist as a family of proteins [149], there are only two members in Drosophila: anti-apoptotic Buffy [150] and pro-apoptotic debcl [151-154]. This protein family has both anti-apoptotic and pro-apoptotic members that contain one or more Bcl-2 homology (BH) domains [144, 155, 156]. The major anti-apoptotic members

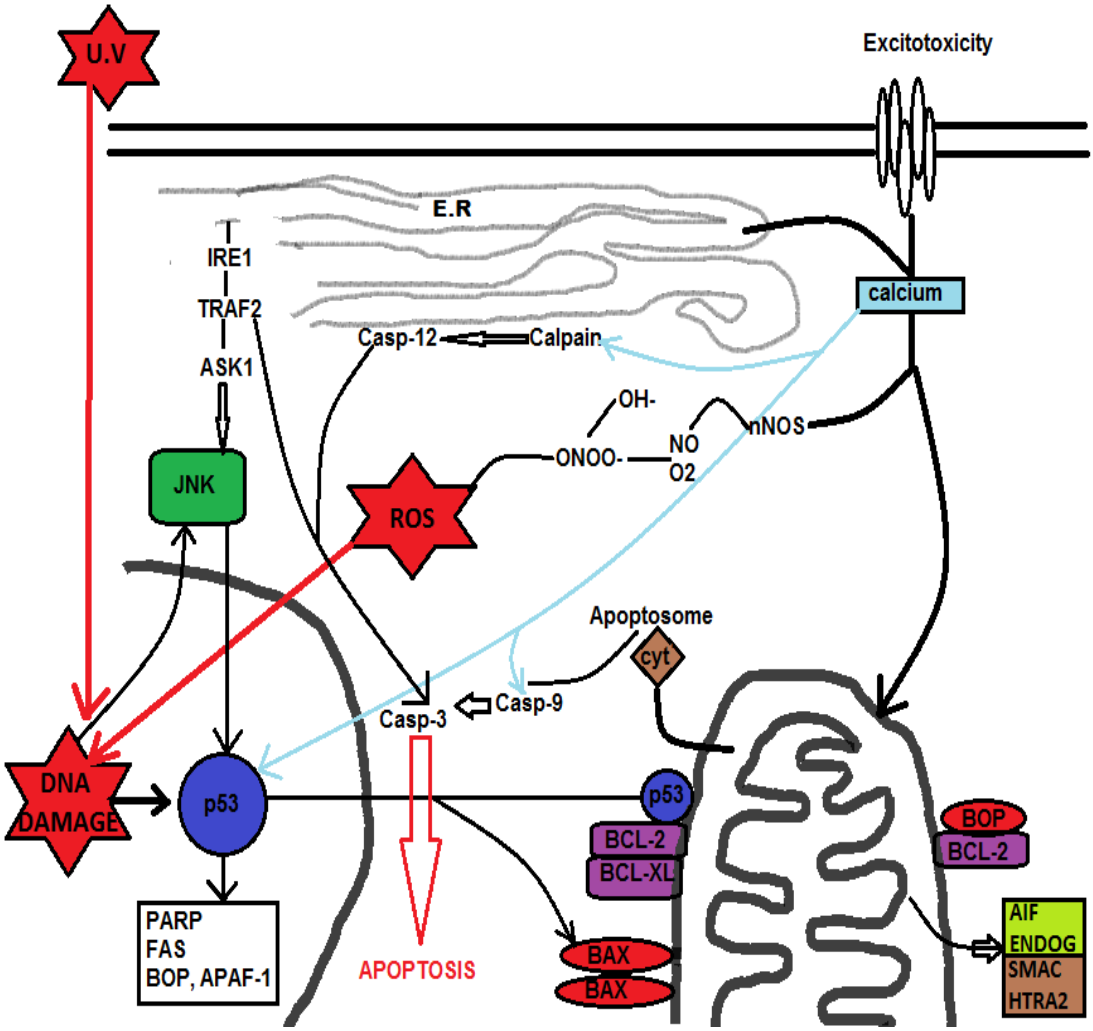

Figure 1.3 The intrinsic cell death pathway

The pathway is initiated upon induction of death signals from damaged DNA, oxidative stress, or cytotoxic stress that surpasses the survival signals. This then leads to a breaching of the outer mitochondrial membrane that results in the release of apoptogenic molecules such as cytochrome *c* that induces the formation of the apoptosome together with apoptosis protease activating factor (Apaf-1). The apoptosome then recruits pro-caspase-9 which is activated and in turn cleave pro-caspase-3 to activate it. A non-caspase cell death pathway can be initiated by calcium signalling through calpains which may ultimately activate caspases. Apoptosis inducing factor (AIF) is also caspase-independent and initiates death by causing chromatin condensation and DNA fragmentation. Endonuclease G (EndoG) once released from the mitochondria translocate to the nucleus and nicks DNA and RNA.

are Bcl-2, Bcl-xL, Bcl-W, A1 and Mcl-1, which are normally characterized by the presence of four BH domains [148, 149, 157]. These anti-apoptotic members localize to the outer mitochondrial membrane (OMM), as does the single fly homologue Buffy. In addition, the mammalian and fly proteins have been shown to localize to the endoplasmic reticulum and to the perinuclear membrane. The pro-apoptotic members of the Bcl-2 family are categorized into two subgroups, the first is the multi-domain proteins made up of Bax, Bak, Bok and Bcl-xS consisting of between three and four BH domains, and the second subgroup that has more structural diversity and is often referred to as BH3 only proteins (BOP) made up of proteins such as Bad, Bid, Bim, DP5/Hrk, Puma, Noxa among others [155]. In mammals the Bcl-2 proteins have a dual role, being both anti- and pro-apoptotic and not only bind and inactivate Apaf-1, but also stabilize the mitochondria membranes preventing the release of a plethora of death molecules [148, 158]. In flies, Buffy, the anti-apoptotic Bcl-2 homologue has been shown to suppress death from various stimuli [157], but it is the RHG proteins; reaper, HID (head involution defective) and grim that regulate cell death by interacting with IAPs [159]. The anti-apoptotic members of Bcl-2 action is to inhibit the pro-apoptotic proteins through heterodimerization, and the BH3 domain has been shown to be central to this role, especially in BOP pro-apoptotic members, that form heterodimers with anti-apoptotic members of the Bcl-2 family [138, 144]. These proteins appear to be cellular rheostats that balance death or survival signals, and alter the permeability or conductance of mitochondrial membranes which result in the release of death molecules among which cytochrome c is key to the formation of the apoptosome [156]. The Bcl-2 proteins have been shown to suppress death in neurons after the withdrawal of NGF, and block

developmental cell death and neuronal injury, showing expression of these proteins can override the extrinsic death signal and trophic factor withdrawal.

On induction of apoptosis from intrinsic signals, Apoptosis protease activating factor (Apaf-1) forms a multimeric complex with mitochondrial- released cytochrome c, and recruits and activates procaspase-9 [160, 161]. The interaction between Apaf-1 and procaspase-9 is mediated through the caspases-activating recruitment domains (CARD) present in both proteins. The activated caspase-9 then cleaves and activates caspase-3. The formation of the apoptosome requires ATP [162] and cytochrome c, the controlled release of cytochrome c from the mitochondria therefore offers a key step in the regulation of neuronal apoptosis. Several molecules have been shown that physically interact with cytochrome c in the mitochondria and blocks its release, even during Bax-induced apoptosis. One such molecule is a member of the Transmembrane Bax inhibitor-1 containing motif 5 (TMBIM5) [145] or growth hormone-inducible transmembrane protein (GHITM), also known as *mi*tochondrial morphology and *c*ristae *s*tructure (MICS1) [163]. Inhibitors of apoptosis proteins (IAPs) were first identified in viruses as suppressors of apoptosis that functionally interact and antagonize the enzymatic activity of mature caspases [164]. IAPs contain the baculoviral IAP repeat (BIR) domains which bind and inhibit both initiator and effector caspases. IAPs such as XIAP, cIAP1 and cIAP2 seem to exert their effect by the ubiquitination of caspases, through an additional domain the RING finger domain. This IAP-mediated inhibition may be released by proteins such as Smac/DIABLO and Omi/HtrA2, both of which are released from the mitochondria upon initiation of an apoptotic signal [165]. Ultimately, cytochrome c, apoptosis inducing factor (AIF), Smac/DIABLO, HtrA2/omi, and EndoG are released

from the mitochondria. Cytochrome c participates in the formation of the apoptosome and the activation of caspases, Smac/DIABLO and HtrA2/omi are inhibitors of IAPs and potentiate killing by caspases. AIF is cleaved at the mitochondria (tAIF) which makes it soluble and through its NLS translocate to the nucleus where it interacts with DNA and leads to chromatin condensation and DNA degradation. EndoG, once released from the mitochondria, translocate to the nucleus, where it nicks DNA and RNA [166]. Numerous other pathways are activated by intrinsic death signals; these include the tumour suppressor and transcription factor p53 [167] and calpains that are non-caspase cysteine proteases which are calcium-regulated and participate in neuronal cell death as a result of cytotoxic stress [142]. The calcium signaling pathway may also bring about crosstalk between mitochondria and the endoplasmic reticulum (ER), the major intracellular calcium store by interaction with the Bcl-2 family of proteins [168]. ER stress is coupled to the extrinsic and intrinsic pathways, as well as other independent pathways, that are yet controversial like the caspase-12 activation by IRE1, which recruit caspase-12 through the TNFR-associated factor 2 protein (TRAF2).

The role of apoptosis in PD

The implication of defective mitochondrial Complex I in the pathology of PD may arise from, or contribute to, increased cellular oxidative stress that may lead to DNA damage, apoptosis –related gene expression, and the breaching of mitochondrial OM, with the release of death-inducing molecules such as cytochrome c leading to caspase activation [169, 170]. Investigation of neuronal apoptosis in PD has found elevated levels of anti-apoptotic proteins, Bcl-2 and Par-4, in DA neurons [170, 171]. PD-associated gene

products have been determined to have a vital function in the maintenance of mitochondrial integrity and turnover, such as, *Pink1, parkin*, and *HtrA2* [37]. The association of α-synuclein with the mitochondria, especially Complex I, has been shown to increase apoptosis through caspase-dependent and caspase-independent pathways [120, 121]. The association of α-synuclein with the adenylate translocator, a component of the PTPC, leads to membrane depolarization and, mitochondrial morphological changes that result in translocation of AIF to the nucleus and increased neuronal apoptosis [172]. The blocking of the chaperone-mediated autophagy by α-synuclein results in cellular stress that activates neuronal apoptosis [173]. Mutations in parkin result in the accumulation of Pael-R, a substrate of parkin, that result in the accumulation of unfolded proteins in the ER and the activation of the ER-stress-induced cell death [174]. Pink1 and parkin have also been shown to play a role in neuronal apoptosis via the JNK signalling pathway and, p38/MAPK pathway by either the inhibition or promotion of apoptosis when overexpressed or suppressed [68]. Thus, neuronal apoptosis results chiefly by mitochondrial dysfunction that promote generation of ROS and leads to cellular oxidative stress, this potentially releases major death-inducing proteins from the mitochondria.

Endosomal-lysosomal system

The functioning of cellular clearance systems in neurons in an efficient and timely manner is crucial to survival as it aid in the removal of misfolded proteins and damaged organelles [39]. The clearance of aggregated and accumulated α-synuclein depends on the endosome-lysosome pathway and especially autophagy [175]. Evidence for the involvement of the endo-lysosomal system in the etiology of PD is the increased number

of autophagic vacuoles and presence of autophagy-related proteins in LBs, in addition to the down-regulation of lysosome-associated membrane protein type 2A (LAMP-2A) and heat shock cognate protein 70 (Hsc70) in the SNpc and cell lines [175, 176]. Briefly described below is autophagy and the specialized mitophagy in the context of PD and neuronal death.

Autophagy

This mechanism is responsible for the removal of soluble, aggregated and misfolded proteins as well as old and dysfunctional organelles through one of the two cellular proteolytic systems [177, 178]. It is mostly involved in the turnover of long lived proteins and organelles that are recycled into small peptides. The major autophagy subtypes are microautophagy, chaperone mediated autophagy (CMA) and macroautophagy which is commonly known as autophagy and form the wider autophagy-lysosome pathway [179, 180]. Microautophagy involves the engulfment of portions of the cytoplasm within lysosomes through invagination of lysosomal membranes; CMA involves degradation of target proteins mediated by HSC70 that recognizes amino acid motifs specified by KFERQ, this allows the target protein to bind LAMP2A and be translocated across lysosomal membranes; macroautophagy (commonly referred to as autophagy) is the most common and main subtype of autophagy, that involves the degradation of most cytosolic proteins and aged and damaged organelles [181]. Defective autophagy either by failure or excessive function can result in disease states as a result of neurodegeneration and neuronal death.

Mechanisms of Autophagy

The importance of this cellular process is exemplified by the conservation of autophagy-related genes in a wide variety of organisms, from yeast to mammals [182, 183]. Autophagy proceeds in four essential steps, namely, initiation or induction, expansion of the autophagosome, maturation of the autophagosome, and degradation (Figure 1.4). The initiation of autophagy is a tightly regulated step, with several complexes involved in the initiation or inhibition of this step, importantly the nutrient sensor; mammalian target of Rapamycin (mTOR) that negatively regulates the pathway by phosphorylation of ULK1, Atg13 and FIP200 that form part of the initiation complex; and AMP-activated protein kinase that negatively regulates mTOR to positively impact initiation of autophagy [183, 184]. The Bcl-2/ Bcl-X_L inhibition of the Beclin1/ class III PI3K complex forms another layer of regulation that includes the p53 tumour suppressor protein among others that respond to the absence or presence of nutrients, growth factors, and energy levels [177, 185]. The Beclin1 (Atg6)-interacting complex acts in a regulatory step, with Atg6-binding proteins like AMBRA1, UVRAG and Bif-1 promoting autophagy and Rubicon and Bcl-2/Bcl-X_L inhibiting autophagy [186].The formation of the phagophore marks the initiation of autophagy and the ULK1-Atg13-FIP200 complex is an important step, the origin is still unclear but the ER, Golgi complex, mitochondria or plasma membrane are possible sources [181]. The nucleation phase of initial phagophore formation requires the interaction of the ULK1 complex with the Atg6-interacting complex, composed of Atg6, class III PI3K, Vps15 and Atg14L. Stimulation of this complex generates phosphatidylinositol-3-phosphate that promotes autophagosomal membrane nucleation [185, 187, 188]. This is followed by autophagosomal elongation or expansion that

involves two ubiquitin-like conjugating systems, the Atg5/Atg12/Atg16L and MAP1LC3 conjugation to phosphatidylethanolamine (PE) that promote autophagosome elongation and membrane tethering [177, 181]. The maturation of the complete autophagosome involves fusion with either lysosomes or late stage endosomes to form autolysosomes or amphisomes [181]. Additional factors are required for the successful completion of these fusions, but the terminal step is the degradation of cytoplasmic components to recycle nutrients and molecules.

Endo-lysosomal aetiology of PD

Loss of function mutations in genes encoding proteins involved in the endo-lysosomal system, Glucocerebrosidase (*GBA1*), synaptojanin (*SYNJ1*), ATP13A2 (*ATP13A2/PARK9*), auxilin (*DNAJC6*), GAK (*PARK17*), Lrrk2 (*PARK8*), Vps35 (*VPS35*), RME-8 (*DNAJC13*), Rab7L1 (*RAB7L1*), parkin (*PARK2*) and Pink1 (*PARK6*) are directly associated with higher risk or susceptibility to PD [178, 189]. The accumulation and aggregation of α-synuclein impairs the endo-lysosomal system, and especially autophagy by inhibition of Rab1a and a reduction of autophagosome biogenesis [178, 180]. Furthermore, while wild type α-synuclein is degraded by the CMA, the two common PD mutations A30P and A53T, while they bind LAMP-2A, they are not internalized nor degraded but rather contribute to the accumulation of other substrates and dysfunction of CMA [177, 181]. In summary, mutations in; Lrrk2 are known to cause an impairment of the endo-lysosomal trafficking and Golgi apparatus sorting; Vps35 results in α-synuclein accumulation in endosomes due to disruption of cathepsin D trafficking; RME-8 and GAK results in PD-like neurodegeneration [178,

181]. Interestingly, the majority of the mutations seem to result in accumulation of α-synuclein, which in turn lead to further impairment of the endo-lysosomal system.

Mitophagy

Mitophagy, a special form of autophagy, maintains mitochondrial homeostasis by the selective degradation of damaged and dysfunctional mitochondria [190, 191]. Defective mitophagy result in the accumulation of impaired mitochondria that leads to oxidative stress, mitochondrial calcium overload and cell death via cytochrome c release – aspects that contribute to mitochondrial dysfunction. Two autosomal PD genes, *parkin* and *Pink1* mediates mitophagy and constitute a mitochondrial quality control function, Pink1 is the mitochondrial sensor [181, 190, 191].

In normal mitochondria, Pink1 is rapidly imported through the TOM complex of the OMM and into the TIM complex of IMM where it is cleaved by the mitochondrial processing peptidase (MPP). Pink1 is further cleaved by the rhomboid protease PARL, this cleavage releases an N-terminal deleted Pink1 into the cytosol where it is identified by the N-degron type 2 E3 ubiquitin ligases that target it to the proteasome [191]. In a defective mitochondrion (mitochondria) Pink1 is unable to translocate to the IMM through the disruption of the TIM complex by depolarizing agents, MPP and PARL are unable to cleave it and thus it accumulates in the OMM bound to the TOM complex [190, 191]. The presence of Pink1 on the OMM serve to recruit and phosphorylate parkin, a ubiquitin E3 ligase, which activates its ligase activity. In addition, Pink1 phosphorylates ubiquitin, which activates parkin, this potentiates the activation of parkin which can bind to ubiquitin or to chains of ubiquitin [55]. Once parkin is activated, it methodically

ubiquitinates at least 36 substrates of the OMM, that extracts them from the OMM and targets them to the proteasome [191]. In addition, this ubiquitination forms dense poly-ubiquitination chains that target the mitochondria to the forming autophagosome.
Other PD-associated gene products are implicated in mitophagy by disruption of the Pink1/ Parkin pathway, this include Fbxo7 that directly interacts with Pink1 and parkin to contribute to mitophagy [192]. DJ-1 translocate to depolarized mitochondria and induces mitophagy, its loss of function is associated with mitochondrial dysfunction [179]. The importance of mitophagy is mitochondrial homeostasis, by clearing damaged and dysfunctional mitochondria.

The ubiquitin-proteasome system

This is one of the two quality control systems for removal of misfolded proteins, the ubiquitin-proteasome system (UPS), is a proteolytic system that involves the conjugation of polyubiquitin chains to specific substrates to induce a selective ATP-dependent degradation [179, 193]. Briefly, the ubiquitination process is highly regulated and allow for the binding of a single or multiple ubiquitin (Ub) molecules to a lysine residue of the substrate or target protein, this is initiated through the ATP-dependent action of ubiquitin activating enzymes (E1) binding to a ubiquitin molecule [193, 194]. This ubiquitin is then transferred to a ubiquitin conjugating enzyme (E2), the E2 enzyme transfers the ubiquitin molecule to the target protein in cooperation with a ubiquitin ligase (E3), which is thought to confer substrate specificity. This last step is complex and involves a multitude of proteins depending on the E3 recruited that may include adaptor proteins, cofactors and scaffolding proteins, the marked substrate is then targeted to the proteasome for

degradation by the help of chaperone proteins that recognize and bind to poly-ubiquitin molecules [193]. Disease states can result from a failure of any of this steps including proteasome dysfunction, as such two mechanisms can be implicated, the UPS and the ubiquitin signalling system.

The presence of accumulated α-synuclein in Lewy bodies has been attributed to the breakdown of the UPS, and has been observed in the brains of patients with PD, furthermore, the inhibition of UPS causes protein inclusions and degeneration of the DA neurons [193, 195]. The expression of mutated *α-synuclein* induces the formation of filaments that directly interact with the 20S core of the proteasome to decrease its proteolytic function [196, 197]. More evidence for the involvement of the UPS in the pathology of PD is from mutations in *parkin*, which encodes a ubiquitin E3 ligase, that is thought to regulate the ubiquitination of α-synuclein [198, 199]. Several parkin substrates have significant functions in neurons, that include Pael-R, a G-protein-coupled transmembrane protein that appears to induce ER stress and cell death [200]; PARIS, a transcriptional repressor of the mitochondrial co-activator PGC-1α that is involved in mitochondrial biogenesis [201]. The association between parkin and Pink-1 is important in degradation and turnover of mitochondria [61]. Ubiquitination can be reversed by de-ubiquitination enzyme (DUB), *UCH-L1*, an early onset PD susceptibility gene that is involved in regulation of substrates that are implicated in a myriad of functions such as mRNA transcription, protein translation, cell survival and neuronal plasticity [193]. The self-dimerization of UCH-L1 results in an E3 ligase function, that ubiquitinates α-synuclein, resulting in PD pathology [202], and loss of DUB function leads to α-synuclein accumulation at the pre-synapse [203]. Another PD risk associated gene *FBXO7*, is

implicated in autosomal recessive PD when mutated [22]. It encodes an F-box protein, that constitutes one of the four subunits of the Skp1-Cullin-F-box (SCF) Ubiquitin ligase complex that is implicated in the UPS pathway [204]. Fbxo7 has been detected in Lewy bodies in brains of patients suffering from PD [205]. The number of PD genes involved in the UPS pathway probably shows the importance of this system in the pathology of PD.

Oxidative stress

Oxidative stress is one of the main molecular mechanisms underlying dopaminergic neurotoxicity and neuronal degeneration in PD [198]. Neuronal and glial sources are implicated in the generation of oxidative stress, with the main contributor being increased radical formation from mitochondria and ER. Neurons are particularly sensitive to oxidative stress due to their high oxygen consumption, high generation of reactive oxygen and nitrogen species from neurochemical reactions and the deposition of metal ions in the brain that comes with ageing [206]. Overall, oxidative stress in PD results from 1) changes in dopamine synthesis that results from high concentration of dopamine and intermediates from its catabolism, general metabolic failure in the SNpc, and low mitochondrial reserve of midbrain DA neurons, 2) oxygen metabolism in the brain that fluctuates in normal neurons to produce ROS, high consumption of oxygen, exposure to environmental toxins, low levels of antioxidants, and low levels of free radical scavenging enzymes, 3) changes in metal concentration such as increase in iron and copper, 4) calcium homeostasis with fluxes that overstimulate DA neurons due to pacemaker functions of L-type calcium channels and excitotoxicity by glutamate receptors, and 5) aberrant mutations in PD-linked genes that alter mitochondrial and the

endosomal-lysosomal system functions [207]. Oxidative stress results from the excessive accumulation of ROS and RNS and has a detrimental effect on DA neurons and PD. ROS result from the univalent metabolic reduction of oxygen to form short-lived chemically reactive and toxic species [206, 208]. Free radicals that are formed include superoxide anion radical, hydroxyl radical, hydroperoxyl radical, peroxyl radicals as well as non-radical species such as hydrogen peroxide, hypochlorous acid and peroxynitrite [209, 210]. Peroxynitrite results from the reaction between nitric oxide and superoxide, to form reactive nitrogen species (RNS) that also include nitrogen dioxide, dinitrogen trioxide and dinitrogen tetroxide [206]. Cellular sources of ROS formation can either be exogenous; from radiation, chemicals, drugs, environmental toxins and pollutants or endogenous; mainly from mitochondria electron transport chain (METC), ER, lysosomes and peroxisomes [206, 208-210]. Evidence for oxidative stress in PD is demonstrated by enhanced lipid peroxidation, protein oxidation, and DNA oxidation present in the SNpc [198]. The main mechanisms involved in oxidative stress in DA neurons are briefly discussed.

Dopamine synthesis

Dopamine is an important neurotransmitter and its synthesis has been implicated in the selective degeneration of DA neurons as it contributes to oxidative stress, mitochondrial dysfunction, defects in the proteasomal and lysosomal systems, and formation and stabilization of neurotoxic protofibrils of α-synuclein [38]. Dopamine is initially synthesized from tyrosine by tyrosine hydroxylase (TH) to L-dihydroxyphenylalanine (L-dopa) and requires oxygen, L-dopa is then decarboxylated to dopamine by aromatic

amino acid decarboxylase (AADC) that generate carbon dioxide. TH and AADC are coupled to the vesicular monoaminergic transporter 2 (VMAT-2), thus dopamine is not released into the cytosol but gets incorporated into monoaminergic synaptic vesicles that release it at the synaptic space [38, 208]. The oxidation of cytosolic dopamine results in ROS by formation of dopamine *o*-quinone, aminochrome and 5,6-indolequinone, excess cytosolic dopamine is degraded by monoamine oxidase (MAO) - found at the outer mitochondria membranes - by oxidative deamination to form 3,4-dihydroxyphenylacetaldehyde, ammonia and hydrogen peroxide [38, 208]. MAO is implicated in DA degeneration by the production of hydrogen peroxide, a key step in the formation of hydroxyl radicals.

Dopamine oxidation alters mitochondrial respiration and MPTP, the quinones formed through auto-oxidation can; modify PD-associated proteins by formation of adducts that include α-synuclein, parkin, DJ-1, SOD-2, and UCH-L1; inactivate dopamine transporter and TH enzyme; bring about mitochondria dysfunction of Complex I, III and V [38, 208]. The dopamine quinones can be oxidized to aminochrome, which generate superoxide radical and depletes NADPH, and forms neuromelanin that is found accumulated in the SNpc [38, 211]. Several factors can bring about dopamine oxidation, such as, manganese (III), copper (II), iron (III), xanthine oxidase, cytochrome P450, prostaglandin H synthase, lactoperoxidase and dopamine β-monooxygenase [38]. Neuromelanin appears to be associated with the development of PD, and accumulates in the SNpc and locus coeruleus, parts of the mid brain heavily affected by DA neurodegeneration [211]. As neurons die, they release the insoluble NM to the extra-cellular matrix, where it results in neuroinflammation and neuronal death.

Mitochondrial ROS generation

The mitochondria are the major intracellular source of ROS production via the respiratory chain complexes in the process of oxidative phosphorylation (Figure 1) to produce energy in the form of ATP [212]. The mitochondria electron transport chain (METC) is composed of five multi-subunit complexes; NADH: ubiquinone (UQ) oxidoreductase or NADH dehydrogenase (Complex I), succinate dehydrogenase (Complex II), cytochrome *bc1* complex (Complex III), cytochrome *c* oxidase (Complex IV), and ATP synthase (Complex V) [208, 212]. Mitochondrial Complex I and III are the main producers of ROS; Complex I produces superoxide in the matrix and Complex III in both the matrix and the intermembrane space. Briefly, mitochondria Complex I oxidizes NADH and reduces ubiquinone (UQ) to ubiquinol ($UQH_{2)}$ with translocation of protons from the matrix to the intermembrane space, during this process superoxide can be formed as a side process [212]. Complex II oxidizes succinate and reduces UQ to UQH_2 and is additionally known to produce low levels of superoxide, Complex III funnels electrons from the UQ pool to cytochrome c and is a major site for generation of superoxide [213]. Other sites of superoxide production in the mitochondria associate with the UQ pool, and are as a result of reverse electron transport by Complex I. Mitochondria are the sites of nitric oxide generation by the nitric oxide synthases (NOS) during the breakdown of arginine to citrulline; and though this process remains unclear in mitochondria [212], the presence of nitric oxide in the mitochondria interferes with respiration, mitochondria biogenesis, and mitochondrial homeostasis.

Defects of mitochondrial Complex I are observed in the SNpc of PD patients, in addition, α-ketoglutarate dehydrogenase; a Krebs cycle enzyme, is impaired [198]. Furthermore,

PD-linked gene mutations are associated with mitochondrial dysfunction that include altered protein localization, defects in mitochondrial morphology, and an impaired Complex I function [208-210, 212]. *Pink1* and *parkin* are involved in mitochondrial turnover, and mutations in these genes result in increased oxidative stress as a result of defective mitochondrial function [209]. Moreover, mutations in DJ-1; a key oxidative stress sensor, decreases respiration and membrane potential that increases ROS [208]. Changes in antioxidant systems have been reported in PD; low levels of GSH, high iron levels that may increase ROS by interacting with hydrogen peroxide to produce hydroxyl radicals [210]. Oxidative stress as a result of ROS production by the mitochondria appears to be a key component of DA neurodegeneration leading to PD.

Endoplasmic reticulum stress and ROS in PD

The endoplasmic reticulum (ER) is the primary organelle for secretory pathways; secretory and transmembrane proteins enter in the unfolded state and exit either folded or misfolded, and are directed to other organelles or targeted for destruction [214-217]. The functions of the ER are numerous and include lipid biosynthesis, protein folding, translocation, and post-translational modifications such as glycosylation, disulfide bond formation, and chaperone-mediated protein folding processes [217], most of the protein modifications occur at the Golgi apparatus after which they are translocated to their target location . The ER lumen contains enzymes and chaperones that are involved in protein folding; misfolded protein aggregates are degraded by the ER-associated degradation (ERAD) system via the ubiquitin-proteasome system or by autophagy [214, 215, 217]. Protein aggregates trigger ER stress, to restore ER homeostasis, an ER stress response is

activated that is known as the “unfolded protein response (UPR)” [216, 217]. Broadly the ER stress response consists of decreased protein synthesis, increased transcription of ER chaperones such as GRP78/BiP and ERAD genes, and finally the initiation of apoptosis.

Unfolded protein response pathway

The three main signaling pathways in UPR are: inositol-requiring enzyme 1α (IRE1α), protein kinase RNA (PKR)-like kinase (PERK), and activating transcription factor 6 (ATF6) [216-218] (Figure 4). These pathways work in parallel and employ unique signal transduction mechanisms to upregulate protein folding mechanisms and restore the ER balance.

IRE1α is a type I transmembrane serine/threonine receptor protein kinase and endoribonuclease activities that is able to trans-autophosphorylate after homo-oligomerization [216, 217]. Upon ER stress, IRE1α gets activated by homo-oligomerization which aligns the serine/threonine kinase on the cytosolic carboxyl terminal for trans-autophosphorylation, this activates the endoribonuclease domain. In animals, the RNAse cleaves a preexisting mRNA X-box binding protein 1 (XBP1) to produce spliced XBP1 that codes for a transcription factor which activates UPR target genes and ERAD [216]. Prolonged activation of IRE1α can activate apoptotic signaling kinase-1 (ASK-1) to activate downstream pathways such as JNK and p38 MAPK that promote ER stress mediated apoptosis. In addition, regulated IRE1α-dependent degradation (RIDD) attenuates the unfolded proteins by targeting the misfolded mRNAs for degradation [218]. Proteins that appear to interact with IRE1α influence the

restoration of ER homeostasis and tips the balance towards survival: the ER resident anti-apoptotic Bax inhibitor-1 (BI-1/TMBIM6) is known to block ER stress-induced cell death

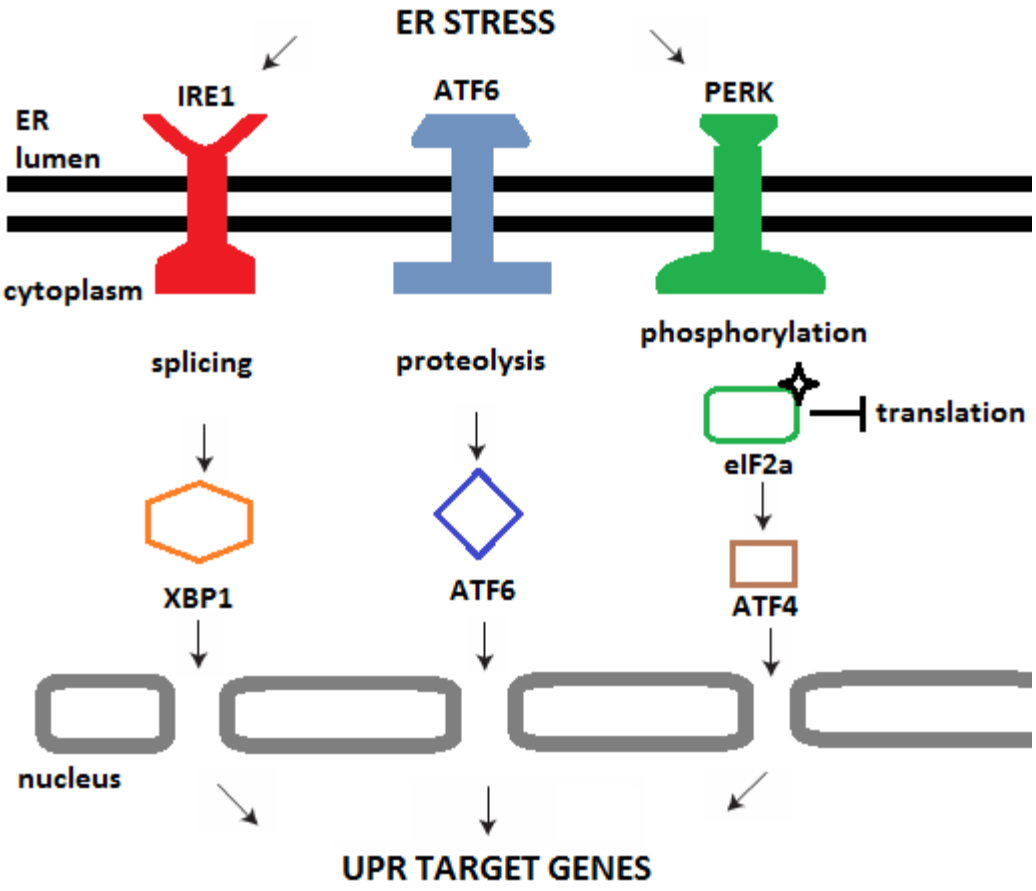

Figure 1.4 The unfolded protein response pathways

Upon ER stress, the IRE1, ATF6 or the PERK pathways can be induced to regulate the unfolded protein response. The ultimate goal is the upregulation of UPR target genes. A protracted activation of UPR leads to an apoptotic response.

by the regulation of ER calcium homeostasis and ER-generated ROS accumulation [219]. In addition, BI-1 has been suggested to specifically inhibit the IRE1α-dependent branch of UPR, though other studies show it has a general ER stress regulation function by its influence on the three main UPR signaling pathways. Of interest to note is that the IRE1α-dependent UPR pathway is the most conserved ER stress response mechanism [217], and the TMBIM family of antiapoptotic molecules are more conserved than the Bcl-2 family [145]. This evolutionary conservation of an ancient pathway, and its regulatory components may highlight a conserved system across diverse species.

PERK, a type I transmembrane serine/threonine kinase, is the translational arm of UPR that leads to the attenuation of mRNA translation under ER stress and thus preventing further protein synthesis and folding [216, 217]. Upon ER stress the cytosolic kinase domain trans-autophosphorylates and initiates translational arrest with phosphorylation of the eukaryotic initiation factor 2α (eIF2α) [218]. Phosphorylation of eIF2α stalls the 43S ternary complex and results in a general decline in translation of a majority of proteins, this reduces protein load on ER and causes cell cycle arrest and promotes survival [216]. Moreover, eIF2α causes the translational upregulation of transcription factors such as ATF4 and ATF5 that control many important genes involved in amino acid metabolism and transport, redox reactions, and ER stress-induced apoptosis. Under severe ER stress, ATF4 activates downstream UPR target genes; growth arrest and DNA damage-inducible 34 (*GADD34*) encodes a regulatory subunit of the protein phosphatase PP1C that dephosphorylates eIF2α and reverses translational attenuation, and transcription factor C/EBP homologous protein (*CHOP*) activates apoptotic genes [218]. Thus, PERK

initially mediates a survival response and on protracted ER stress it favours a proapoptotic response.

ATF6 is a type II transmembrane ER resident protein that is activated upon ER stress and translocate to the Golgi, where it undergoes sequential regulated intramembrane proteolysis (RIP) by the Golgi-resident site-1 and site-2 proteases (S1P and S2P) [216, 218]. The cleaved product is a transcription factor that binds to ER stress response element and activates UPR targets such as GRP78, CHOP and XBP1.

ER ROS production

Under normal cellular conditions, cells have a basal level of ROS for signaling and normal function, but under conditions of stress, the levels exponentially increase [214]. Nonetheless cellular mechanisms for clearing the excess ROS exist that depend on; enzymes such as SOD, glutathione peroxidase, catalase, and thioredoxin reductase as well as non-enzymatic molecules such as vitamins. The redox status within the lumen of the ER affects the proper folding and formation of disulfide bonds [217]. The ER lumen has a highly oxidized environment that facilitates disulfide bond formation with a high ratio of reduced glutathione to oxidized glutathione GSH/GSSG [214]. This oxidized environment prevents aggregation and accumulation of unfolded proteins.

In chaperone assisted protein oxidation, electron transfer from protein disulphide isomerase (PDI) to molecular oxygen and ER oxidorectin-1 (ERO1) depends on a FAD-dependent reaction [217]. This is suggested as the main generator of ROS in the ER, and especially proteins with multiple disulphide bonds are more vulnerable to high ROS production. Since the protein thiols are repaired by utilizing GSH, ROS can be generated

when GSH is depleted, additionally, accumulated unfolded proteins can elicit calcium leak from the ER to the cytosol thus increasing ROS production in the mitochondria [214, 217]. In addition, protein folding and refolding in the ER lumen are highly energy-dependent processes, the depletion of ATP can stimulate mitochondrial OXPHOS to increase ATP and consequently increase mitochondrial ROS generation. Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase 4 (Nox4) is implicated in ER ROS generation, due to its C-terminal special characteristics, electrons are constitutively transferred from NADPH to FAD [217]. Nox4-linked ROS generation is increased under conditions of stress and is known to trigger autophagic cell death. The microsomal monooxygenase (MMO) system is another major source of ROS in the ER and produces superoxide anion radicals and hydrogen peroxide; it oxygenates exogenous and endogenous substrates that are electron-accepting partners of NADPH-p450 reductase (NPR) [217]. The electron leakage between the coupling of NADPH to p450, and activation of p450 2E1 is a significant source of ROS in the ER. The previously discussed family of antiapoptotic molecules, TMBIM, of which BI-1 belongs regulates ER stress-mediated ROS accumulation by decreasing p450 2E1 activity and protein expression [219]. It is suggested that BI-1 decreases electron uncoupling between NPR and p450 2E1 by binding to one of them which reduces ER stress-initiated ROS production and cell death signaling.

ER stress link to PD

One of the main pieces of evidence linking ER stress to PD is the observation of upregulation of the ER stress sensors PERK and eIF2α in DA neurons of PD patients

[179, 216], and that several mutated PD-linked genes are involved in the regulation of ERS. Of the PD-associated genes, *α-synuclein* appears to be the main cause of ERS: it has a high propensity to misfold, aggregate, accumulate and induce normal folded species to misfold [179]. Wild type and mutated *α-synuclein* are able to elicit ERS and the upregulation of BiP/GRP78, XBP1, CHOP and ATF4 to induce cell death. Several studies implicate *α-synuclein* in the inhibition of the ER/Golgi transport, that leads to accumulation of immature proteins at the ER, or later steps in the biosynthetic secretory pathway that result in ER overload and ERS [39]. This appears to occur when α-synuclein enters the ER lumen, possibly through uptake by endocytosis followed by retrograde vesicular transport to the ER. In addition, α-synuclein has been reported to block ERAD which causes the buildup of excess unfolded proteins.

There is altered regulation of parkin, an E3 ubiquitin ligase, where ATF4 causes an upregulation and CHOP downregulates it, possibly as a mitochondrial protective response induced by ER stress [216]. Mutations in *parkin* may be responsible for a dysfunctional ERAD which relies on the UPS to degrade misfolded proteins. Moreover, mutations in *parkin* are implicated in the misfolding and accumulation of Pael-R that results in apoptosis through activation of CHOP. Lrrk2 is found localized in the ER lumen of DA neurons from PD patients [179], other PD-linked genes have been reported to abrogate the normal function of the ER, that leads to ERS.

ER-Mitochondrial interaction and calcium homeostasis

Cellular organelles exist in an intricate relationship with interaction and cooperation that facilitates rapid and efficient biological functions, such interactions are between the

nucleus and the ER and the ER and the mitochondria [214, 217]. The physical interaction between the mitochondria and the ER occurs at mitochondria-associated ER membranes (MAM) and bring the two organelles into close proximity [220]. Different protein interactions tether the ER and the mitochondria, such as the interaction between VDAC/Porin and the IP3 receptor, or the enrichment of Mitofusin 2 (MFN2) at the ER-mitochondrion interface where it can interact with another MFN2 or MFN1. This close interaction is the site for mitochondrial division, lipid transfer and metabolism, calcium exchange and signaling and the initiation of apoptosis [179, 220]. The ER is the main cellular calcium store, upon release, cytosolic calcium levels are restored by the mitochondria which takes up the calcium and utilizes it in oxidative phosphorylation in ATP production. A mitochondrial calcium overload is detrimental to cell survival and as such MAM allow for the exchange of calcium in a regulated manner [179].

DA neurons are maintained in an autonomously active state by the pacemaker action of voltage-dependent L-type calcium channels that elevate cytosolic calcium levels, this makes DA neurons vulnerable to mitochondria dysfunction as a result of the huge calcium buffer action [220]. Another consequence of calcium overload in the mitochondria is the opening of the mPTP that through the release of apoptogenic factors initiates cell death. The increase in mitochondria calcium uptake, that occurs during ER stress leads to an increase in oxidative phosphorylation, that in tandem increases ROS and when unchecked leads to cell death [214, 217]. This is a positive feedback as the ER continuously feeds the mitochondria more calcium which results in more ROS and leads to oxidative stress.

ER-induced oxidative stress and ROS production causes release of calcium through the IP3R that leads to the opening of the mPTP and accelerates mitochondrial ROS generation [214]. Early stages of ERS cause the release of calcium from ER, this calcium is mopped up by the mitochondria that results in elevated metabolism and increased ROS generation [217]. High mitochondrial calcium activates NOS that forms nitric oxide, NO can then interfere with both Complex I and IV and further enhance the production of ROS. Molecules that modulate ER calcium release influence cell survival, Bax inhibitor-1 and other members of the TMBIM family have been implicated in pro-survival signaling in the ER after ERS; BI-1 is proposed to alter ER calcium homeostasis by either, 1) possession of calcium channel properties, 2) sensitization of IP3R or 3) its Ca^{2+} /H^{+} antiporter action [145]. Therefore, the ER-mitochondrial calcium cycle is important for both ER-induced stress and ROS production in the mitochondria.

The presence of α-synuclein in the cytosol, the ER, the mitochondria and in the MAM implicates it in the regulation of calcium signaling and the ER-mitochondria tethering [179]. Mutant α-synuclein blocks protein localization at the ER-mitochondria interface that decreases the degree of tethering and impairs the integrity of the mitochondrial network. DJ-1 which is a PD-associated gene modulates mitochondrial calcium levels by promoting ER-mitochondria tethering [220]. *Pink1*, *GBA*, and *Lrrk2* are also implicated in altered ER-mitochondria by changes in calcium homeostasis and the representative neurodegeneration.

ER-Golgi axis and Golgi stress in PD

The Golgi complex is important in the secretory pathway and is responsible for the modification of proteins and lipids as well as correct delivery of target molecules to their destination [221]. The Golgi complex is closely positioned to the nucleus and is at the heart of the endomembrane trafficking system; it receives newly synthesized proteins from the ER; modifies them by glycosylation, sulfation, phosphorylation and proteolytic cleavage; and finally exports them to their target location. The disruption of this pathway is known to be causative factor for several disease states including PD.

Golgi fragmentation is observed in PD though it is yet unclear as to the functional and pathological significance of this disruption in disease aetiology [39]. The altered expression of *Rab1, Rab2, Rab8* and *Syntaxin-5,* early secretory machinery molecules, is implicated in PD-induced Golgi fragmentation [221]. The disruption of the ER-Golgi trafficking pathway is one of the mechanisms suggested for *α-synuclein*-induced neurotoxicity and may be through post ER budding when it interferes with Rab and SNARE-dependent COPII vesicle tethering and/or fusion [39]. The importance of this pathway is exemplified by the observation that α-synuclein toxicity blocks simultaneous steps of the biosynthetic secretory pathway.

Goals of study

The role of Bcl-2 proteins in *Drosophila melanogaster* is not well understood compared to their function in humans, and the involvement of mitochondrial dysfunction in the pathogenesis of PD and in cell death led us to investigate the effects of altered expression of Bcl-2 homologues in Parkinson disease models, and in particular those that have a

strong association to the mitochondria. Thus, we sought to investigate the protective and detrimental effects of the suppression and overexpression of *Buffy*, the sole pro-survival *Bcl-2* homologue in Drosophila (Chapter 2), and *Debcl*, the sole pro-death homologue in the *α-synuclein*-induced Drosophila model of PD (Chapter 3). Moreover, we sought to investigate the pro-survival roles of *Buffy* in different established models of PD that includes *parkin*, *Pink1* (Appendix 2), and *HtrA2* (Chapter 5), all which are strongly associated with mitochondrial dysfunction in the pathogenesis of PD and PD-like symptoms in Drosophila. We also characterized the phenotypes of loss of *Pdxk*, an increased risk to PD gene, in Drosophila DA neurons and attempted to rescue the *Pdxk*-induced phenotypes by overexpression of *Buffy* (Chapter 7). To further investigate the role of autophagy in PD, we suppressed *Atg6* also known as *Beclin-1*, and *Pi3K59F* also known as *Vps34* in DA neurons, and subsequently attempted to rescue the resultant phenotypes by overexpression of the pro-survival *Bcl-2* homologue *Buffy* (Chapter 4). To evaluate the role of the highly conserved anti-apoptotic family of TMBIM (Chapter 6), we suppressed *CG3814*, the Drosophila homologue of *Lifeguard/TMBIM2* in DA neurons and attempted to rescue its phenotypes with the pro-survival *Buffy*. We inhibited *CG2076*, the Drosophila homologue of *GHITM/TMBIM5* in DA neurons and additionally attempted to rescue the induced phenotypes through overexpression of *Buffy*. The last member of this family we investigated *BI-1/TMBIM6* that ties in the role of ER in stress-induced cell death, and additionally analysed the effects of the inhibition of *BI-1* along with expression of *α-synuclein* and the overexpression of *Buffy*. Interestingly the inhibition of these three members appeared to model PD in Drosophila. To further evaluate the role of the mitochondria in DA neurons, we inhibited porin – a mitochondrial

voltage-dependent anion channel (VDAC) protein (Chapter 8), and MICU1 – a mitochondrial calcium uptake channel protein (Appendix 3). The protective role of Buffy was evaluated in DA neurons with well-established PD models and the novel models.

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Co-Authorship Statement

The following is a co-authorship statement pertaining to the manuscript Chapters 2, 3, 4, 5, 6, 7, 8 and Appendices 1, 2 and 3 of this thesis laid out in accordance with the requirements of the School of Graduate Studies. My contribution in the completion of these manuscript chapters is addressed with respect to:

i) Design and identification of the research proposal

BES initiated the research project by setting the research goals to investigate the effects of altered expression of *Bcl-2* homologues in Drosophila models of Parkinson disease. Additional apoptotic and autophagic gene candidates were identified by PGM with critical review from BES. All experiments in Chapters 2, 3, 4, 5, 6, 7, 8 and Appendices 1, 2 and 3 were initiated, conceived and designed by PGM with critical review provided by BES.

ii) Practical aspects of the research

Bioinformatics, creation and testing of the derivative lines, and all experiments were carried out and performed by PGM.

iii) Data analysis

All data was collected and analyzed by PGM.

iv) Manuscript preparation

All the manuscripts contained in Chapters 2, 3, 4, 5, 6, 7, 8 and Appendices 1, 2 and 3 were prepared by PGM with critical review from BES.

Chapter 2 - The *Bcl-2* homologue *Buffy* rescues *α-synuclein*-induced Parkinson disease-like phenotypes in Drosophila

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Abstract

In contrast to the complexity found in mammals, only two *Bcl-2* family genes have been found in *Drosophila melanogaster* including the pro-cell survival, human *Bok*-related orthologue, *Buffy*. The directed expression of *α-synuclein*, the first gene identified to contribute to inherited forms of Parkinson disease (PD), in the dopaminergic neurons (DA) of flies has provided a robust and well-studied Drosophila model of PD complete with the loss of neurons and accompanying motor defects. To more fully understand the biological basis of *Bcl-2* genes in Parkinson disease (PD), we altered the expression of *Buffy* in the dopamine neurons with and without the expression of *α-synuclein*, and in the developing neuron-rich eye.

To alter the expression of *Buffy* in the dopaminergic neurons of Drosophila, the *Ddc-Gal4* transgene was used. The directed expression of *Buffy* in the dopamine producing neurons resulted in flies with increased climbing ability and enhanced survival, while the inhibition of *Buffy* in the dopaminergic neurons reduced climbing ability over time prematurely, similar to the phenotype observed in the *α-synuclein*-induced Drosophila model of PD. Subsequently, the expression of *Buffy* was altered in the *α-synuclein*-induced Drosophila model of PD. Analysis revealed that *Buffy* acted to rescue the associated loss of locomotor ability observed in the *α-synuclein*-induced model of PD, while Buffy RNA interference resulted in an enhanced *α-synuclein*-induced loss of climbing ability. In complementary experiments the overexpression of *Buffy* in the developing eye suppressed the mild rough eye phenotype that results from *Gal4* expression and from *α-synuclein* expression. When Buffy is inhibited the roughened eye phenotype is enhanced.

The inhibition of Buffy in DA neurons produces a novel model of PD in Drosophila. The directed expression of *Buffy* in DA neurons provide protection and counteracts the *α-synuclein*-induced Parkinson disease-like phenotypes. Taken all together this demonstrates a role for *Buffy*, a *Bcl-2* pro-cell survival gene, in neuroprotection.

Background

Parkinson disease (PD) is the most common human movement disorder and the second most common neurodegenerative disease; afflicting about 1 to 2% of the population over 50 years of age [1, 2]. PD is strongly associated with the selective and profound loss of dopaminergic (DA) neurons to result 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 [2]. The neuropathological hallmarks exhibited by PD patients include the presence of Lewy Bodies (LB) and Lewy Neurites (LN) in surviving neurons. This is due to the loss of DA neurons in the *substantia nigra pars compacta* (*SNpc*) region of the brain, coupled with the presence of eosinophilic, intracytoplasmic proteinaceous inclusions comprised of the α-synuclein and ubiquitin proteins, among others [2-4]. This unusual protein accumulation is believed to lead to cellular toxicity and, eventually, the PD pathogenesis. Other associated pathological mechanisms include aberrant protein aggregation and mitochondrial damage [5-7]. Although the majority of PD cases are considered to be sporadic, familial forms have been documented and much has been discovered through study of the associated gene loci in model organisms [8-10]. PARK1/4 was the first gene associated with PD to be identified [3], and it encodes for a small soluble protein of unknown function predominantly found in neural tissues [3, 8, 11]. The first Drosophila model of PD

utilized a human α-synuclein transgene to induce the PD-like symptoms [12]. The success of this model is its ability to recapitulate features of human PD such as 1) age-dependent loss in locomotor function 2) LB-like inclusions and 3) age-dependent loss of DA neurons; and therefore, has found wide use for studying the molecular basis of α-synuclein-induced neurodegeneration [12-19]. The utilization of the UAS/GAL4 spatio-temporal expression system [20], and the availability of a plethora of promoters or enhancers of which TH-Gal4, elav-Gal4 and Ddc-Gal4 are employed in modelling PD in flies, makes Drosophila a powerful model organism [12-19]. Mitochondrial dysfunction due to the accumulation of α-synuclein has been implicated as one of the mechanisms leading to PD [21-24]. The association of α-synuclein with components of the mitochondria is thought to lead to oxidative stress, apoptosis, autophagy and the eventual neurodegeneration.

The *Bcl-2* family of genes are key regulators of cell death and survival in animals and are functionally composed of proapoptotic and pro-cell survival (antiapoptotic) members [25-28]. The pro-survival proteins protect the mitochondria in part, from disruption by the proapoptotic proteins [26, 29-32]. In mammals, the antiapoptotic members possess four Bcl-2 homology (BH) domains - BH1, BH2, BH3 and BH4 – and include Bcl-2, $BclX_L$, Mcl-1 among others. The proapoptotic members, Bax, Bak and Bok, have three BH domains: BH1, BH2 and BH3. A BH3-only domain class of proapoptotic proteins is present and includes Bid, Bim, Bad, Bik, Hrk, Noxa and Puma [33-35]. The multi-domain proapoptotic proteins are required for mitochondrial outer membrane (MOM) permeabilization and the subsequent release of apoptogenic factors into the cytosol [36-

39]. As thus, the antiapoptotic members guard the mitochondria and stop the release of a plethora of death causing molecules that initiate apoptosis.

The *Bcl-2* family of 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 [40]. This delicate balance is maintained by the activity of the pro-survival and anti-survival members of the protein family. Many of the apoptotic pathway proteins that participate in the intrinsic and extrinsic cell death pathways have been identified in Drosophila [41-43]. The *Bcl-2* family member homologues in Drosophila are limited to the single pro-cell survival *Buffy* and the sole pro-cell death *Debcl* [44-48]. These two proteins share a strong similarity within their domains and with the mammalian pore-forming proapoptotic member Bok/Mtd.

In previous studies, the overexpression of *Buffy* has been shown to suppress *Pink1* mutant phenotypes [49] and suggest a role for this protein 1) in interacting with the Pink1 protein and other mitochondrial proteins or 2) in a pathway that regulates mitochondrial function and integrity. Studies show that *Buffy* has little involvement in cell death during development [50], though it has a role in regulating cell death that occurs in response to external stimuli and a role in the mitochondrial pathway for the activation of cell death during Drosophila oogenesis [51], all which point to an important role for this protein in aspects of cell death. Indeed, early studies have demonstrated that *Buffy* plays roles in both anti- and pro-survival [52, 53] depending upon 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 [51] and in the

Pink1 loss-of-function Parkinson disease model [49]. The predicted role of the mitochondria in PD pathogenesis makes the *α-synuclein*-induced model of PD [12] a very attractive model for the investigation of the role of Bcl-2 proteins. First, we examine the effects of increasing and decreasing Buffy activity in DA neurons and, secondly, we investigate the potential suppression of the *α-synuclein*-induced PD phenotypes by the overexpression of the pro-survival Bcl-2 homologue *Buffy*.

Materials and Methods

Drosophila media and culture

Stocks and crosses were maintained on a standard medium containing cornmeal, molasses, yeast, agar, water and treated with propionic acid and methylparaben. 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 re-culture. Stocks were kept at room temperature (22°C ± 2°C) while crosses and experiments were carried out at 25°C and 29°C.

Drosophila stocks and derivative lines

UAS-Buffy [52] was generously provided by Dr. Leonie Quinn (University of Melbourne), *UAS-α-synuclein* [12] was provided by Dr. M. Feany of Harvard Medical School and Dr. J. Hirsch (University of Virginia) provided *Ddc-Gal4* flies [54]. *UAS-Buffy-RNA$_i$* (w[*]; P{w[+mC]=UAS-Buffy.RNAi}3), *GMR-Gal4* [55] and *UAS-lacZ* flies were obtained from the Bloomington Drosophila Stock Center at Indiana University. The *UAS-α-synuclein/CyO; Ddc-Gal4/TM3* was generated using standard homologous recombination methods and was used to overexpress *α-synuclein* in the dopaminergic neurons using the *dopa decarboxylase* (*Ddc*) transgene. The *UAS-α-synuclein/CyO;*

GMR-Gal4 line was used to overexpress *α-synuclein* in the developing eye using the *Glass Multiple Reporter* (*GMR*) elements. PCR reactions and gel electrophoresis were used for analysis of recombination events.

Ageing assay

Several single vial matings of five females and three males of each genotype were made and a cohort of critical class male flies were collected upon eclosion. At least two hundred flies were aged per genotype at a density of 20 flies or fewer per vial on fresh media which was replenished every other day to avoid crowding. Flies were observed and scored every two days for the presence of deceased adults. Flies were considered dead when they did not display any movement upon agitation [56]. Longevity data was analysed using the GraphPad Prism version 5.04 and survival curves were compared using the log-rank (Mantel-Cox) test. Significance was determined at 95%, at a P-value less than or equal to 0.05 with Bonferroni correction.

Climbing assay

A cohort of critical class male flies was collected upon eclosion and scored for their ability to climb over their lifetime [57, 58]. 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 in ten repetitions. Analysis was performed using the GraphPad Prism version 5.04 and climbing curves were fitted using non-linear regression and compared using 95% confidence interval with a 0.05 P-value.

Scanning electron microscopy of the drosophila eye

Several single vial matings were made at 29°C and a cohort of adult male flies collected upon eclosion and aged for three days before being frozen at -80°C. Whole flies were

mounted on scanning electron microscope stubs, desiccated overnight and photographed with a FEI Mineral Liberation Analyzer 650F scanning electron microscope. For each cross at least 10 eye images were analysed using the National Institutes of Health (NIH) ImageJ software [59] and biometric analysis performed using GraphPad Prism version 5.04. The percent area of eye disruption was calculated as previously described [60]. But briefly, the ratio of disrupted eye area was calculated by dividing the total area of the eye with the total disrupted area of the eye. The total disrupted area of the eye was the sum of any two or more ommatidia that were fused together.

Results

Buffy is closely related to the human proapoptotic *Bok*

Bioinformatic analysis of the protein sequences encoded by the *Buffy* and *Bok* genes reveal 33% identity. The Buffy protein consists of 299 amino acids and reveals the presence of the BH1, BH2, BH3, BH4 and TM domains (Figure 1). The Eukaryotic Linear Motif (ELM) resource search for functional sites indicates the presence of a monopartite variant of a basically charged NLS between amino acids 101 and 106. There is a number of BH3-homology region binding sites in the central region of the protein. Bok has 212 amino acids and similarly shows the presence of the BH1, BH2, BH3, and BH4 domains. Although, the two proteins are determined to be antiapoptotic and proapoptotic respectively, both show the presence of a BH4 domain, the homology domain that is associated with pro-survival proteins.

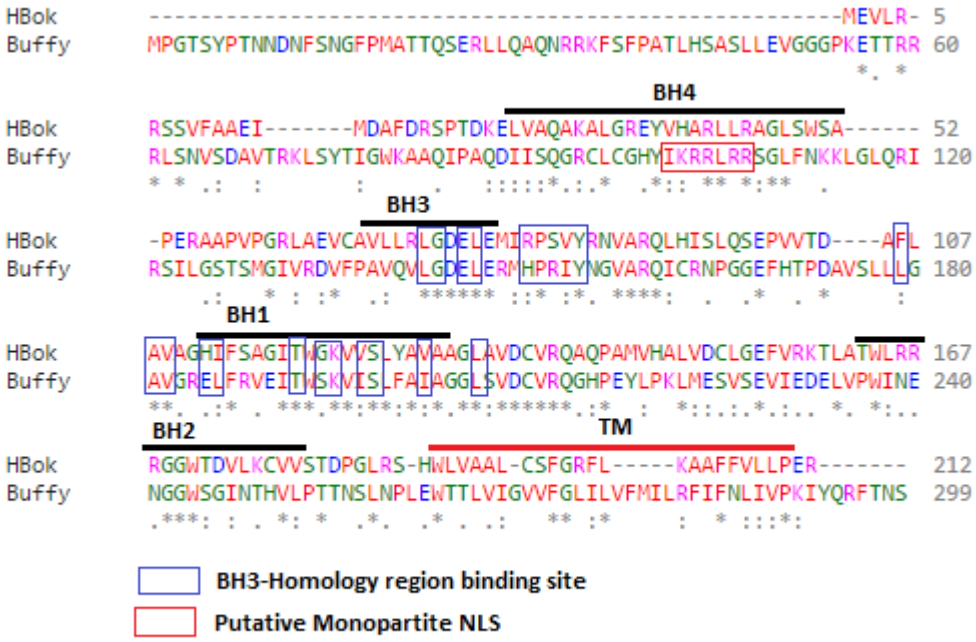


Figure 2.1 Buffy is closely related to human Bcl-2 ovarian killer (Bok)

When Buffy protein is aligned with human Bok, the Bcl-2 homology (BH) domains show strong conservation. Clustal Omega multiple sequence alignment [61, 62] of *Drosophila melanogaster* Buffy protein (*Drosophila melanogaster* NP_523702.1) with the human Bok (*Homo sapiens* NP_115904.1) showing the highlighted conserved BH domains, the BH3-homology region binding site, and the TM (trans-membrane) helices. Buffy possesses a monopartite basically charged nuclear localisation signal (NLS) region. The domains were identified using NCBI Conserved Domain Database Search (CDD) [63]. "*" indicate the residues that are identical, ":" indicate the conserved substitutions, "." indicate the semi-conserved substitutions. Colours show the chemical nature of amino acids: red is small hydrophobic (including aromatic), blue is acidic, magenta is basic, and green is basic with hydroxyl or amine groups.

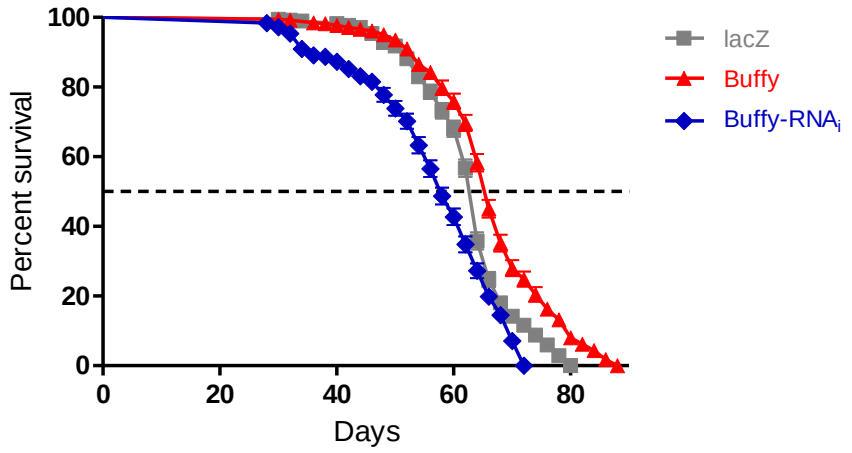
Loss of *Buffy* decreases lifespan and climbing ability

When *Buffy* is inhibited in the DA neurons by RNA interference, the resulting flies have a shortened lifespan and impaired climbing ability. The median lifespan for these flies is 58 days compared to 64 days for the controls (Figure 2A). The nonlinear fitting of the climbing curves resulted in a slope gradient that was significantly different at 95% confidence interval (Figure 2B). This suggests that Buffy is required for the normal functioning of DA neurons and inhibition in DA neurons confers a disadvantage by reducing lifespan and impairing the locomotor ability of these flies.

***Buffy* increases lifespan and climbing ability when overexpressed in DA neurons**

When *Buffy* is overexpressed in DA neurons, the survival parameters of these flies differ slightly (Figure 2A), with *Buffy*-overexpressing flies having a median lifespan of 68 days compared to 64 days for the controls. The overexpression of *Buffy* in DA neurons led to an increased climbing ability as indicated by the nonlinear fitting of the curve with 95% CI (Figure 2B). This suggests that *Buffy* improves longevity and markedly improves climbing ability in Drosophila when expressed in DA neurons to improve the general "healthspan" of these flies.

A.



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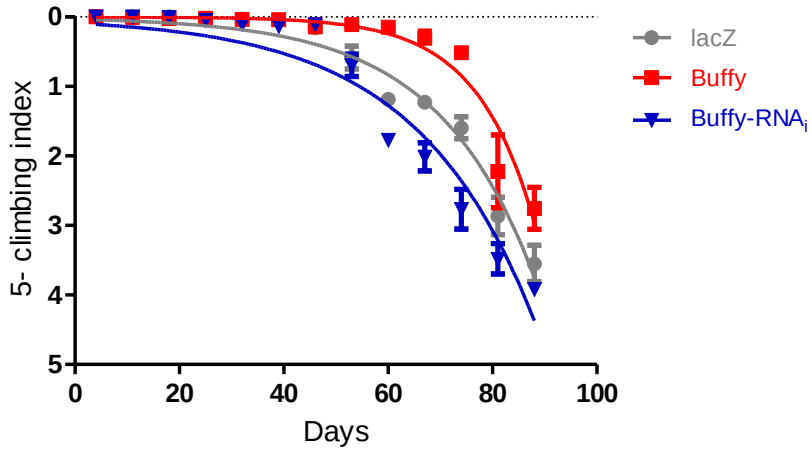


Figure 2.2 *Buffy* alters lifespan and climbing ability when mis-expressed in DA neurons

A) Directed expression of *UAS-Buffy* in the DA neurons driven by *Ddc-Gal4* results in increased survival compared to the controls overexpressing *UAS-lacZ,* while inhibition via *Buffy-RNAi* results in reduced survival. The genotypes are *UAS-lacZ/ Ddc-Gal4; UAS-Buffy/ Ddc-Gal4;* and *UAS-Buffy-RNAi/ Ddc-Gal4.* Longevity is shown as percent survival ($P < 0.01$, determined by log-rank and $n \geq 200$). B) Directed expression of *UAS-Buffy* results in increased climbing ability as determined by non-linear fitting of the climbing curves and comparing at 95% confidence intervals. Inhibition by *Buffy-RNAi* decreased the locomotor ability when expressed in dopaminergic neurons. The genotypes are *UAS-lacZ/ Ddc-Gal4; UAS-Buffy/ Ddc-Gal4;* and *UAS-Buffy-RNAi/ Ddc-Gal4.* Error bars indicate the standard error of the mean (SEM) and $n=50$.

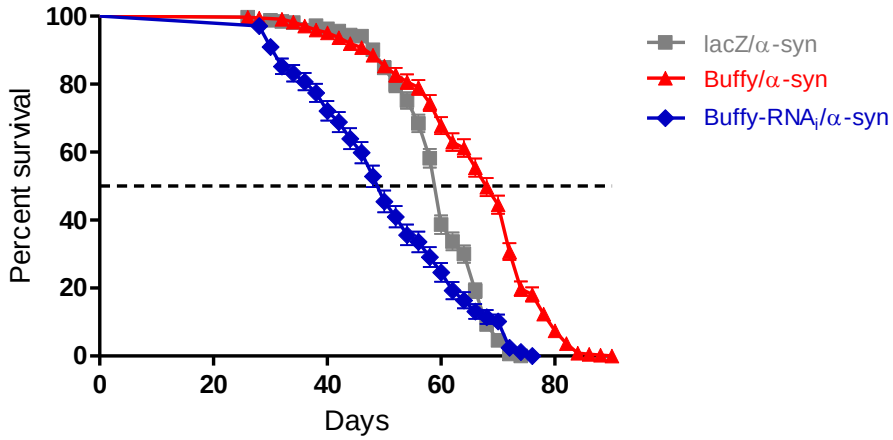
Inhibition of *Buffy* enhances the *α–synuclein*-induced phenotypes

The inhibition of *Buffy* by RNA interference when co-expressed with *α-synuclein*, under the directions of *Ddc-Gal4*, results in short-lived flies with strongly impaired locomotor function. The median lifespan of the *α-synuclein*-expressing control flies was 60 days, while that of those co-expressing the *Buffy-RNAi* inhibitory transgene along with *α-synuclein* was 50 days (Figure 3A). The climbing ability of these flies was more impaired than those expressing *α-synuclein* alone, as indicated by the nonlinear fitting of the climbing curves (Figure 3B).

Overexpression of *Buffy* in DA neurons rescues the *α–synuclein*-induced loss of climbing ability

The overexpression of *Buffy* in DA neurons expressing *α-synuclein* results in an increased median lifespan of 68 days, compared to 60 days for the control flies (Figure 3A). The climbing curves indicate that there was a significant improvement in the climbing ability of the flies when *Buffy* was overexpressed (Figure 3B) and thus, suppressing the phenotypes observed when *α-synuclein* is expressed in DA neurons. This suggests that the overexpression of *Buffy* confers protection to DA neurons, as a result of the expression of *α-synuclein*.

A.



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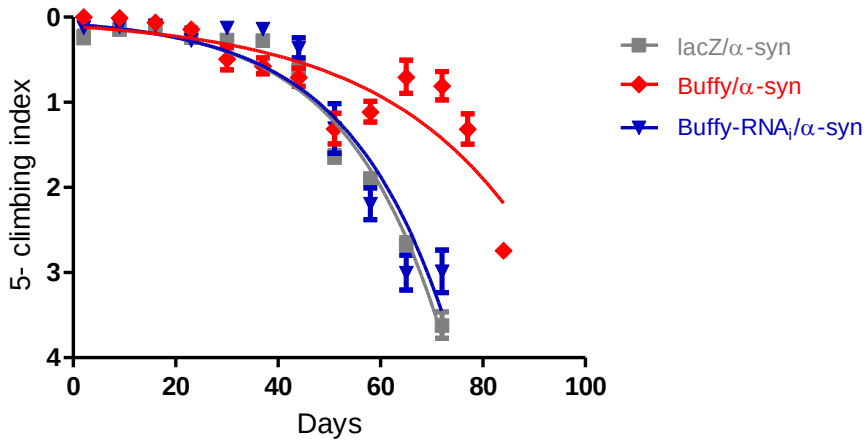


Figure 2.3 *Buffy* rescues the *α-synuclein*-induced phenotypes of decreased lifespan and climbing ability

A) Directed overexpression of *Buffy* in the DA neurons increase longevity whereas flies with *Buffy* loss-of-function show a decline in lifespan. Genotypes are *UAS-α-synuclein, Ddc-Gal4/UAS-lacZ; UAS-α-synuclein, Ddc-Gal4/ UAS-Buffy;* and *UAS-α-synuclein, Ddc-Gal4/ UAS-Buffy-RNAi.* Longevity is shown as percent survival ($P < 0.01$, determined by log-rank and $n \geq 200$). B) The co-expression of *Buffy* in the *α-synuclein* model of PD rescued the age-dependent loss in climbing ability. The directed overexpression of *Buffy* in the DA neurons remarkably increased the climbing ability over time compared to the control, while the suppression of *Buffy* resulted in flies that climbed similar to the control. The genotypes are *UAS-α-synuclein; Ddc-Gal4/UAS-lacZ, UAS-α-synuclein; Ddc-Gal4/ UAS-Buffy,* and *UAS-α-synuclein; Ddc-Gal4/ UAS-Buffy-RNAi.* Analysis of the climbing curves and significance was determined by comparing the 95% confidence intervals. Error bars indicate the SEM and $n=50$.

Overexpression of *Buffy* suppresses the *α-synuclein*-induced developmental defects in the eye

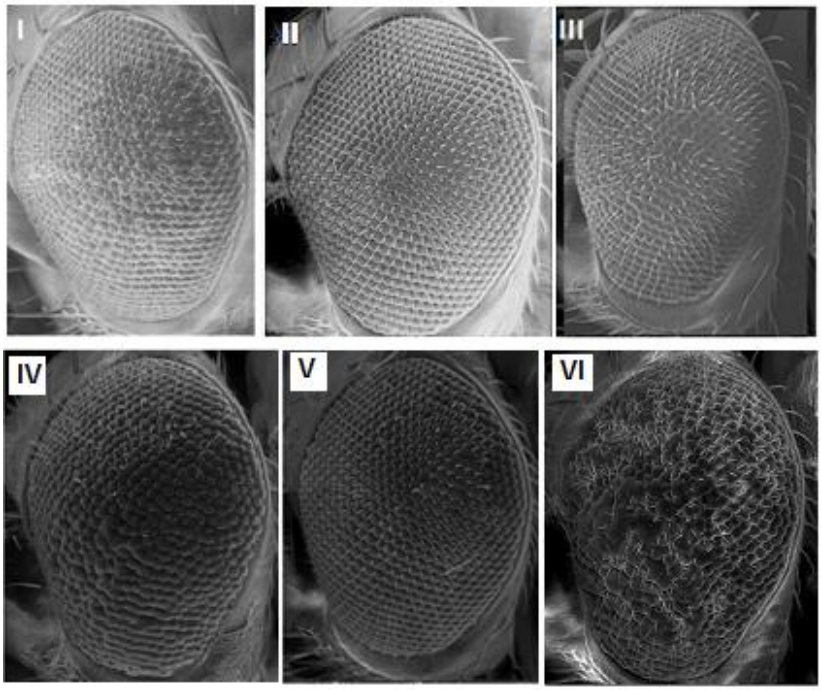
The expression of *α-synuclein* in the developing eye results in developmental defects. When *Buffy* is overexpressed in the developing eye, developmental defects resulting from *GMR-Gal4* (Figure 4B, I) and from *GMR-Gal4* and *α-synuclein* expression (Figure 4B, IV) are suppressed. The disruption of the ommatidial array is restored to control levels in both cases (Figure 4B). These results suggest that overexpression of *Buffy* can counteract the toxic effects of *α-synuclein* in the developing eye in addition to the effects of *GMR-Gal4*.

Discussion

The recapitulation of PD-like symptoms in *Drosophila melanogaster* and especially the age-dependent loss of climbing ability led to the investigation of gene products that could suppress this phenotype [12, 13, 64]. Mitochondrial dysfunction as a result of α-synuclein accumulation has been implicated in PD pathogenesis and, thus, we have investigated the consequences of the overexpression of the Drosophila *Bcl-2* homologue *Buffy*. The analysis of climbing over the lifespan of the flies has been applied to determine the role of the various gene products in rescuing the *α-synuclein*-induced phenotypes [12, 57, 64, 65]. 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.

The *α-synuclein*-expressing models of PD in Drosophila show little difference in lifespan between the control and wild type, A53T and A30P *α-synuclein* flies [12]. In our study, when *Buffy* is overexpressed in the DA neurons under the control of the *Ddc-Gal4* driver,

A.



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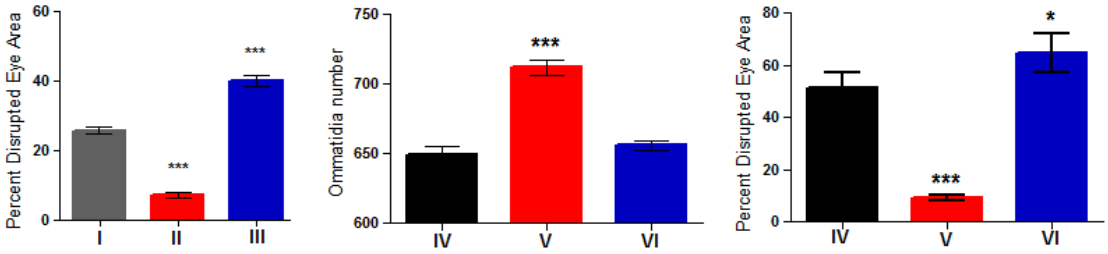

Figure 2.4 *Buffy* suppresses the *α-synuclein*-induced developmental defects in the eye

A) Scanning electron micrographs when *Buffy* is overexpressed or inhibited in the eye with the eye-specific *GMR-Gal4* transgene; (I) *GMR-Gal4/ UAS-lacZ;* (II) *GMR-Gal4/ UAS-Buffy;* (III) *GMR-Gal4/ UAS-Buffy-RNAi* and when co-expressed with *α-synuclein*; (IV) *UAS-α-synuclein; GMR-Gal4 / UAS-lacZ;* V) *UAS- α-synuclein; GMR-Gal4/ UAS-Buffy;* and VI) *UAS-α-synuclein; GMR-Gal4/ UAS-Buffy-RNAi.* B) Biometric analysis showed a significant difference in the disrupted area of the eye when *Buffy* was inhibited in the developing eye (I-III). Biometric analysis shows a marked difference when *Buffy* is inhibited in an *α-synuclein* expressing background (IV-VI) with decreased ommatidia number and highly degenerated ommatidial array whereas when *Buffy* is overexpressed in the *α-synuclein* background, there is a rescue of the *α-synuclein*-induced phenotypes as determined by one-way ANOVA and Dunnett's multiple comparison test ($P<0.05$ and 95% CI), error bars indicate the SEM, asterisks (*) represents statistical significance.

there is a significant increase in their longevity. This may be partly due to defects in mitochondrial Complex I function, the pro-survival *Buffy* likely plays a mitochondrial protective role to increase longevity. This was not verified experimentally and as such is one of our potential future investigations into the mechanistic properties of Buffy. The inhibition of *Buffy* in the DA neurons resulted in a marked decrease in survival. This inhibition of *Buffy* is sufficient to negate its protective role and thus promote cell death as has recently been shown by Clavier and colleagues [66]. Thus, the pro-survival properties of Buffy are evident.

Locomotor dysfunction is one of the associated symptoms of PD, the *α-synuclein*-expressing model demonstrated a clear age-dependent loss in climbing ability [12]. When we overexpressed *Buffy* in the DA neurons under the control of *Ddc-Gal4,* the flies produced a climbing index significantly different from that of control flies. The *Buffy* flies lost the climbing ability later than the control flies and is likely due to the protective role that Buffy confers to the mitochondria. In contrast, the inhibition of *Buffy* results in a highly-compromised climbing ability when compared to the controls. The degree of locomotor dysfunction seemed to be like that observed when *α-synuclein* is overexpressed in 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.

The inhibition of *Buffy* in the DA neurons of the *α-synuclein*-induced PD model significantly decreased lifespan, indicating that low levels of Buffy compromise the health of DA neurons. When *Buffy* was overexpressed along with *α-synuclein*, there was a marked improvement in the climbing ability of these flies. These results suggest that overexpressing *Buffy* in the DA neurons counteracts the *α-synuclein*-induced phenotype

of locomotor dysfunction over their lifespan. The *Buffy* loss-of-function flies displayed a reduced climbing ability compared to the control flies. Therefore, expression of the pro-survival *Buffy* can rescue the *α-synuclein*-dependent model of PD from climbing dysfunction.

Directed overexpression of *Buffy* in the developing eye rescues the roughened eye phenotypes caused by *Gal4* and *α-synuclein* expression, whereas the inhibition of *Buffy* results in a more disrupted ommatidial array. This indicates that elevated levels of *Buffy* in the developing eye offers protection from toxic protein insults to normalize cellular differentiation, neurogenesis and cell survival.

Conclusions

Buffy inhibition results in shortened lifespan and impaired locomotor function and represents a novel model of PD in *Drosophila melanogaster*. The overexpression of *Buffy* improves healthspan and counteracts the effects of *α-synuclein* expression to demonstrate its protective function. Further studies are required to fully elucidate how *Buffy* may interact with the other PD genes, and how these interactions fit into the regulation of mitochondrial integrity by the Bcl-2 proteins.

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Chapter 3 - The proapoptotic *Bcl-2* homologue *Debcl* enhances *α-synuclein*-induced Parkinson disease-like phenotypes in Drosophila

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Abstract

Parkinson disease (PD) is a debilitating movement disorder that afflicts 1 to 2% of the population over 50 years of age. The common hallmark for both sporadic and familial forms of PD is mitochondrial dysfunction. Mammals have at least twenty proapoptotic and antiapoptotic Bcl-2 family members, in contrast, only two *Bcl-2* family genes have been identified in *Drosophila melanogaster,* the proapoptotic mitochondrial localized *Debcl* and the antiapoptotic *Buffy.* The expression of *α-synuclein*, the first gene identified to contribute to inherited forms of PD, in the dopaminergic neurons (DA) of flies has provided a robust and well-studied Drosophila model of PD complete with the loss of neurons and accompanying motor defects. The altered expression of *Debcl* in the DA neurons and neuron-rich eye along with the expression of *α-synuclein* offers an opportunity to highlight the role of Debcl in mitochondrial-dependent neuronal degeneration and death. The directed overexpression of *Debcl* using the *Ddc-Gal4* transgene in the dopaminergic neurons of Drosophila resulted in flies with severely decreased survival and a premature age-dependent loss in climbing ability, phenotypes that are strongly associated with models of PD in Drosophila. The inhibition of *Debcl* resulted in enhanced survival and improved climbing ability whereas the overexpression of *Debcl* in the *α-synuclein*-induced Drosophila model of PD resulted in more severe phenotypes. In addition, the co-expression of *Debcl* along with *Buffy* partially counteracts the *Debcl*-induced phenotypes, to improve the lifespan and the associated loss of locomotor ability observed. In complementary experiments, the overexpression of *Debcl* along with the expression of *α-synuclein* in the eye enhanced the eye ablation that results from the overexpression of *Debcl*. The co-expression of *Buffy* along with *Debcl*

overexpression results in the rescue of the moderate developmental eye defects. The co-expression of *Buffy* along with inhibition of *Debcl* partially restores the eye to a roughened eye phenotype. Taken all together these results suggest a role for *Debcl* in neurodegenerative disorders.

Introduction

Parkinson disease (PD) is a human movement disorder that is strongly associated with the selective and profound degeneration and loss of dopaminergic (DA) neurons to result in a set of marked clinical features [1]. The neuropathological hallmarks exhibited by PD patients include the presence of Lewy Bodies (LB) which are intracytoplasmic proteinaceous inclusions composed of α-synuclein and ubiquitin among other proteins [1-3]. This atypical protein aggregation and accumulation is believed to lead to cellular toxicity and contribute to the pathogenesis of PD. Additional pathological mechanisms that are associated with PD include aberrant protein aggregation and mitochondrial damage [4-6]. Familial forms of PD have highlighted the genetic basis of PD and the study of the associated gene loci in model organisms offers great understanding of the disease aetiology and pathology [7-9]. The gene encoding α-synuclein, a small soluble protein of largely unknown function predominantly found in neural tissues, was first to be identified as responsible for inherited PD [3]. Mitochondrial dysfunction due to the accumulation of α-synuclein has been implicated as one of the mechanisms leading to PD [10-13]. The association of α-synuclein with components of the mitochondria is thought to lead to oxidative stress, apoptosis, autophagy and eventually, neurodegeneration. The first Drosophila model of PD utilized a human *α-synuclein* transgene to induce the PD-like symptoms [14]. This model system is very successful and widely applied, as it

displays the age-dependent loss of locomotor function, the degeneration of DA neurons and LB-like inclusions, features that are present in human PD [14-21]. Drosophila has available tissue specific gene enhancers such as *TH-Gal4*, *elav-Gal4* and *Ddc-Gal4*, which are used to model PD in flies in combination with the powerful bipartite UAS/Gal4 [22] system. Of importance is the correlation between DA neuron loss and the age-dependent loss of locomotor function [21, 23] which validates the implication that age-dependent loss of locomotor function is as a result of DA neuron degeneration.

The *Bcl-2* family of genes are crucial controllers of apoptosis in animals and are functionally composed of proapoptotic and antiapoptotic members [24-27]. In mammals, this multigene family has about 20 members, the antiapoptotic proteins protect the mitochondria from disruption by the proapoptotic proteins [25, 28-31]. The antiapoptotic members possess four Bcl-2 homology (BH) domains while the proapoptotic members have three to four BH domains. The proapoptotic proteins initiate apoptosis by the permeabilization of the outer mitochondrial membrane which results in the release of apoptogenic factors into the cytosol [32-35]. The antiapoptotic members protect the mitochondria from permeabilization by the proapoptotic members and block the release of apoptogenic factors such as cytochrome c, apoptosis inducing factor (AIF) among others from being released from the inner mitochondrial membrane into the cytosol.

Drosophila melanogaster possesses many of the apoptotic pathway proteins that participate in the intrinsic and extrinsic cell death pathways [36, 37]. The *Bcl-2* family member homologues in Drosophila are limited to the single antiapoptotic *Buffy* [38], and the sole proapoptotic *Death executioner Bcl-2 homologue, Debcl* [38-42]. Debcl has a

strong similarity with the mammalian mitochondria outer membrane permeabilization protein Bok/Mtd.

The importance of *Debcl* is perhaps demonstrated by the presence of 5' nuclear transcription factor Y (NF-Y) promoter region which has been shown to be important for gene promoter activity [43]. The tumour suppressor gene *Retinoblastoma* (*Rbf1* in Drosophila) induces a *Debcl*-and *Drp1*-dependent mitochondrial cell death [44]. *Rbf1* induces cell death by reducing the expression of the sole Debcl antagonist *Buffy* [45]. The *Rbf1*-induced apoptosis is dependent on *Debcl*-dependent mitochondrial ROS production and essentially Debcl is required downstream of Buffy for apoptosis to occur. The *Debcl*-induced ROS production appears to be through Glycerophosphate oxidase 1 participation to increase mitochondria ROS accumulation [46]. The organic solute carrier partner 1/ oxidored nitrodomain-containing protein 1 (OSCP1/NOR1), a known tumour suppressor induces apoptosis by the down-regulation of the *Buffy* gene and the up-regulation of the *Debcl* gene [47]. Debcl is not required for most developmental cell death, but has been shown to play a role in embryonic cell death [48] and stress-induced apoptosis [49]. Antiapoptotic Buffy antagonizes *Debcl*-induced apoptosis by physical interaction [38], probably at the mitochondria where Debcl localizes [50]. The presence of a mitochondrial outer membrane (MOM)-targeting motif in Debcl indicates it possibly has a role in mitochondrial cell death pathway.

The role of the mitochondria in PD pathogenesis makes the *α-synuclein*-induced model of PD [14] a very attractive model for the investigation of the role of Bcl-2 proteins. Here, we investigate the potential enhancement or suppression of the *α-synuclein*-induced PD

phenotypes by the inhibition and overexpression of the pro-apoptotic Bcl-2 homologue *Debcl*.

Materials & methods

Drosophila media and culture

Stocks and crosses were maintained on standard cornmeal/molasses/yeast/agar media treated with propionic acid and methylparaben. Stocks were sustained on solid media for two to three weeks before being transferred onto new media to re-culture. Stocks were kept at room temperature (22°C ± 2°C) while crosses and experiments were carried out at 25°C and 29°C.

Drosophila stocks and derivative lines

UAS-Buffy [38] was a gift from Dr. Leonie Quinn of University of Melbourne, *UAS-α-synuclein* [14] by Dr. M. Feany of Harvard Medical School and *Ddc-Gal4* [51] by Dr. J. Hirsch of University of Virginia. y^1 v^1*; P{y[+t7.7] v[+t1.8]=TRiP.JF02429}attP2* hereby referred to as *Debcl-RNAi*, *GMR-Gal4* [52] and *UAS-lacZ* were sourced from the Bloomington Drosophila Stock Center at Indiana University. The *UAS-α-synuclein/CyO; Ddc-Gal4/TM3; UAS-α-synuclein/CyO; GMR-Gal4; UAS-Buffy/CyO; Ddc-Gal4* and *UAS-Buffy/CyO; GMR-Gal4* derivative lines were generated using standard homologous recombination methods and were used for overexpression of either *α-synuclein* or *Buffy* in DA neurons using the *Ddc-Gal4* transgene or in the developing eye using the *GMR* response elements. PCR reaction was used 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. The 5’ to 3’ sequence of the

forward primer was GTGCCCAGTCATGACATTT, while that of the reverse primer was CCACAAAATCCACAGCACAC and were ordered from Invitrogen. The *Drosophila melanogaster* Buffy mRNA, NCBI reference sequence: NM_078978.2, was used to design a set of Buffy primers that would target both the endogenous and the overexpression transcripts. The 5' to 3' sequence of the forward primers were CACAGCGTTTATCCTGCTGA and CGGGTGGTGAGTTCCATACT, while that of the reverse primers were TCGCAGTGTGAAGATTCAGG and TTAATCCACGGAACCAGCTC, and were ordered from Eurofins MWG Operon. Gel electrophoresis was used for confirmation of recombination events via presence of the PCR product.

Ageing assay

Several single vial matings were made and a cohort of critical class male flies was collected upon eclosion. At least two hundred flies were aged per genotype at a density of 20 or fewer flies per vial to avoid crowding on fresh media which was replenished every other day. Flies were observed and scored every two days for the presence of deceased adults. Flies were considered dead when they did not display movement upon agitation [53]. Longevity data was analysed using the GraphPad Prism version 5.04 and survival curves were compared using the log-rank (Mantel-Cox) test. Significance was determined at 95%, at a P-value less than or equal to 0.05 with Bonferroni correction.

Climbing assay

A batch of male flies was collected upon eclosion and scored for their ability to climb [54]. 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 in 10 repetitions. Analysis was

performed using GraphPad Prism version 5.04 and climbing curves were fitted using non-linear regression and compared using 95% confidence interval with a 0.05 P-value.

Scanning electron microscopy of the Drosophila eye

Several single vial crosses were made at 29°C and adult male flies collected upon eclosion and aged for three days before being frozen at -80°C. Whole flies were mounted on scanning electron microscope stubs, desiccated overnight and photographed with a FEI Mineral Liberation Analyzer 650F scanning electron microscope. For each cross at least 10 eye images were analyzed using the National Institutes of Health (NIH) ImageJ software [55] and biometric analysis performed using GraphPad Prism version 5.04. The percent area of eye disruption was calculated as previously described [56]. Briefly, the ratio of disrupted eye area was calculated by dividing the total area of the eye with the total disrupted area of the eye which was the sum of any two or more ommatidia that were fused together. Comparisons for statistical analysis were performed using one-way ANOVA and Dunnett's multiple comparison test. Significance was determined at $p<0.05$.

Results

Debcl is similar to the human proapoptotic *Bcl-2 ovarian killer (Bok)*

Bioinformatic analysis of the protein sequences encoded by the *Debcl* and *Bok* genes reveal 37% identity and 55% similarity. The Debcl protein consists of 300 amino acids and indicates the existence of the BH1, BH2, BH3, BH4 and TM domains, similar to the 212 amino acids human Bok (Figure 1). An ELM resource search for functional sites [57] indicates the presence of a transmembrane domain (membrane anchor region), an inhibitor of apoptosis binding motif (IBM) at amino acids 1 to 5, a PDZ domain at amino acids 295 to 300, an ER retention motif at amino acids 109 to 115 and between amino

acids 258 to 262, an Atg8 binding motif at amino acids 36 to 42, a nuclear receptor box motif at amino acids 295 to 300, and a ubiquitination motif of the SPOP-binding consensus at amino acids 2 to 6 and another one at position 74 to 79. There is a number of BH3-homology region binding sites in the central region of the protein as determined by an NCBI conserved domain search [58]. Although the two proteins Bok and Debcl have been determined to be antiapoptotic, both show the presence of a BH4 domain, the homology domain that is most often associated with pro-survival proteins.

Directed misexpression of *Debcl* in DA neurons alters lifespan and locomotor ability

The inhibition of *Debcl* in the DA neurons by RNA interference results in a lifespan with a median survival of 64 days that is similar to 62 days for the controls expressing the benign *lacZ* transgene as determined by a Log-rank (Mantel-Cox) test (Figure 2A). The locomotor ability showed a slight improvement when nonlinear fitting of the climbing curves was performed with significant differences at 95% CI (Figure 2B). This suggests that the inhibition of the proapoptotic *Debcl* confers a small advantage for the normal functioning of DA neurons. When *Debcl* is overexpressed in DA neurons, the survival criteria of these flies differ greatly (Figure 2A), with *Debcl*-overexpressing flies having a median lifespan of 48 days compared to 62 days for the controls expressing the benign *lacZ* transgene as indicated by a Log-rank (Mantel-Cox) test. The overexpression of *Debcl* in DA neurons severely impairs climbing ability as determined by the nonlinear fitting of the curve with 95% CI (Figure 2B). This suggests that the overexpression of *Debcl* in DA neurons interferes with the normal functioning of these flies and results in compromised “healthspan”.

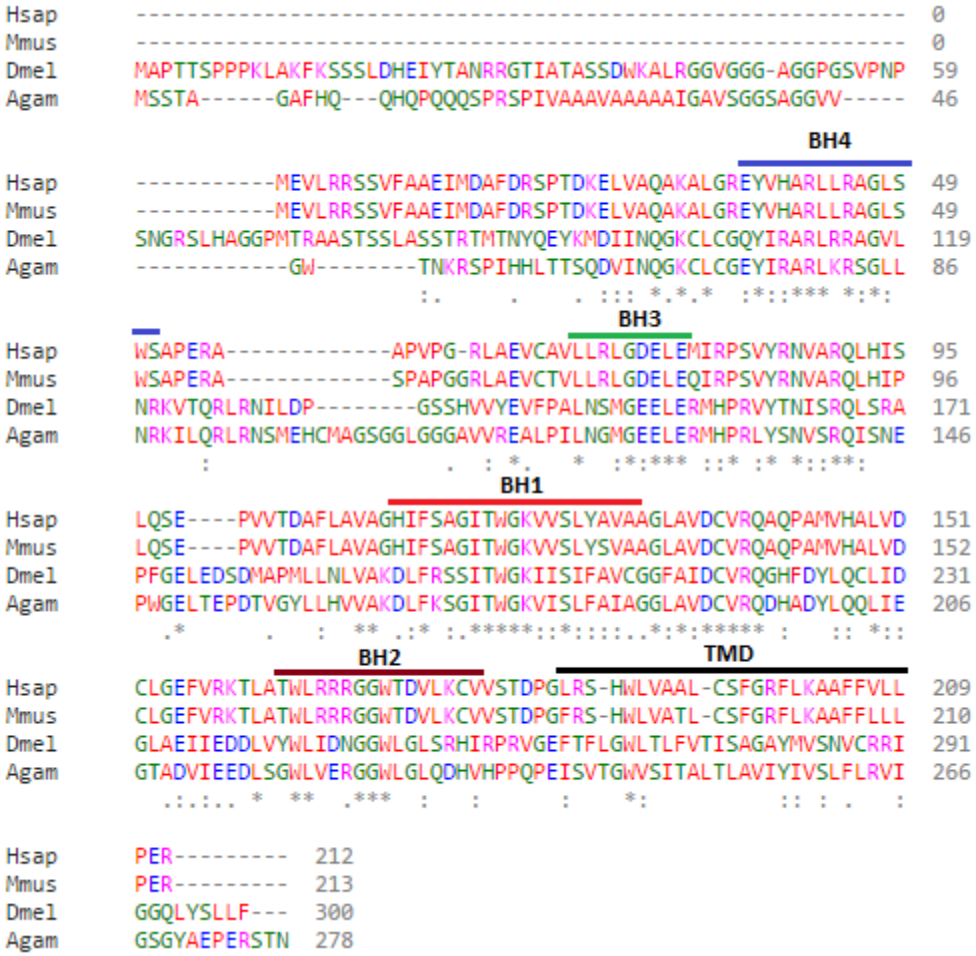
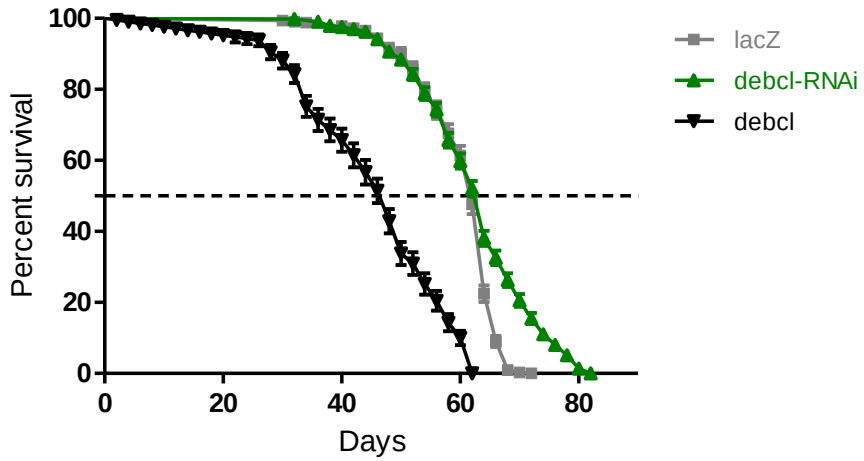


Figure 3.1 Debcl is related to human Bcl-2 ovarian killer (Bok)

(A). When Debcl protein is aligned with human Bok the Bcl-2 homology (BH) domains show strong conservation. Clustal Omega multiple sequence alignment [59, 60] of *Drosophila melanogaster* Debcl protein (Dmel is *Drosophila melanogaster* NP_788278.1) with the human Bok (Hsap is *Homo sapiens* NP_115904.1), mouse Bok (Mmus is *Mus musculus* NP_058058.1) and mosquito Bok (Agam is *Anopheles gambiae* NP_309956.4) showing the highlighted conserved BH domains and the TM helices. The domains were identified using NCBI Conserved Domain Database Search (CDD) [58] and ELM resource search for functional sites [57]. "*" indicate the residues that are identical, ":" indicate the conserved substitutions, "." indicate the semi-conserved substitutions. Colours show the chemical nature of amino acids. Red is small hydrophobic (including aromatic), Blue is acidic, Magenta is basic, and Green is basic with hydroxyl or amine groups.

A.



B.

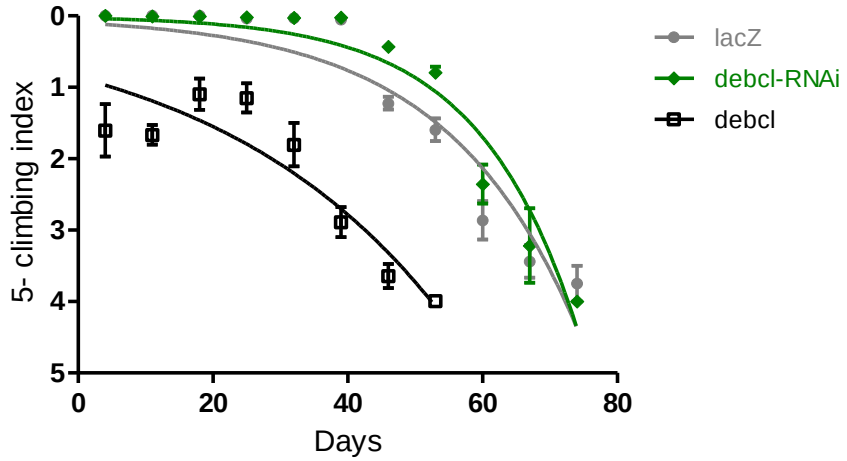


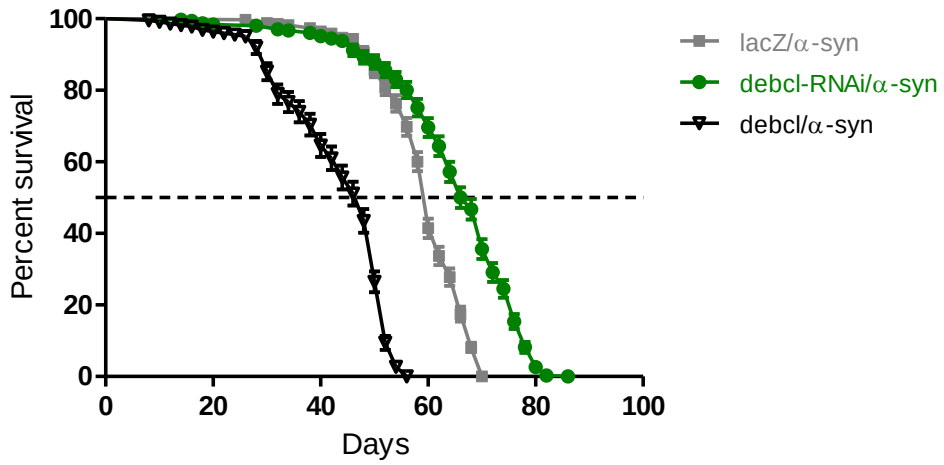
Figure 3.2 *Debcl* alters lifespan and climbing ability when expressed in DA neurons

A) The directed inhibition of *Debcl* in the DA neurons driven by *Ddc-Gal4* results in a slightly increased median survival compared to the control flies overexpressing *UAS-lacZ*, while the overexpression of *Debcl* results in severely reduced survival. The genotypes are *UAS-lacZ/ Ddc-Gal4; UAS-Debcl-RNAi/ Ddc-Gal4* and *UAS-Debcl/ Ddc-Gal4.* Longevity is shown as percent survival ($P < 0.01$, determined by log-rank and $n \geq 200$). B) The inhibition of *Debcl* results in improved climbing ability whereas the overexpression of *Debcl* results in a highly compromised climbing ability as determined by non-linear fitting of the climbing curves and comparing at 95% confidence intervals. The genotypes are *UAS-lacZ/ Ddc-Gal4; UAS-Debcl-RNAi/ Ddc-Gal4* and *UAS-Debcl/ Ddc-Gal4.* Error bars indicate the standard error of the mean (SEM) and $n=50$.

Altered expression of *Debcl* influences the *α–synuclein*-induced phenotypes

The inhibition of *Debcl* by RNAi along with the expression of *α-synuclein* under the direction of the *Ddc-Gal4* transgene results in increased lifespan and healthier climbing ability compared to the control (Figure 3). The *Debcl-RNAi* along with *α-synuclein*-expressing flies had a median lifespan of 67 days, while that of *α-synuclein*-expressing controls was 60 days as determined by a Log-rank (Mantel-Cox) test (Figure 3A). The climbing ability of these flies was slightly improved than of the *α-synuclein*-expressing controls as indicated by the nonlinear fitting of the climbing curves and compared the 95% CI (Figure 3B). These results show that the inhibition of the proapoptotic *Debcl* confers a significant advantage to flies under the influence of the neurotoxic effects of the human transgene *α-synuclein*. The overexpression of *Debcl* along with *α-synuclein* in DA neurons results in decreased median lifespan of 44 days, compared to 60 days for the control flies as determined by a Log-rank (Mantel-Cox) test (Figure 3A). The climbing curves indicate that there was a significant reduction in the climbing ability of the flies with overexpression of *Debcl* (Figure 3B) and thus, enhancing the phenotypes observed when *α-synuclein* is expressed in DA neurons. This suggests that the overexpression of *Debcl* further increases the toxic effects of the expression of *α-synuclein*.

A.



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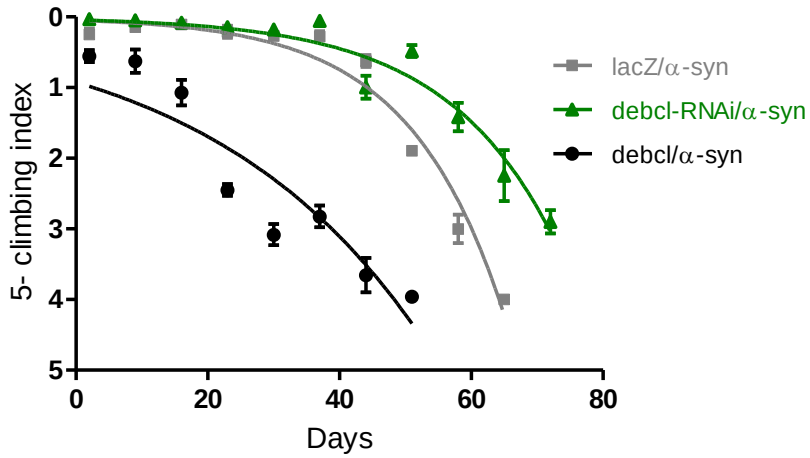


Figure 3.3 Overexpression of *Debcl* enhances the *α-synuclein*-induced phenotypes of decreased lifespan and climbing ability

A) Directed overexpression of *Debcl* in the DA neurons severely decreases longevity whereas its inhibition shows an incline in lifespan. Genotypes are *UAS-α-synuclein; Ddc-Gal4/UAS-lacZ; UAS-α-synuclein; Ddc-Gal4/ UAS-Debcl-RNAi;* and *UAS-α-synuclein; Ddc-Gal4/ UAS-Debcl.* Longevity is shown as percent survival ($P < 0.01$, determined by log-rank and $n \geq 200$). B) The co-expression of *Debcl* in the *α-synuclein* model of PD enhanced the age-dependent loss in climbing ability. The directed inhibition of *Debcl* in the DA neurons improved the climbing ability over time compared to the control. The genotypes are *UAS-α-synuclein; Ddc-Gal4/UAS-lacZ, UAS-α-synuclein; Ddc-Gal4/ UAS-Debcl-RNAi*, and *UAS-α-synuclein; Ddc-Gal4/ UAS-Debcl.* Analysis of the climbing curves and significance was determined by comparing the 95% confidence intervals. Error bars indicate the SEM and $n=50$.

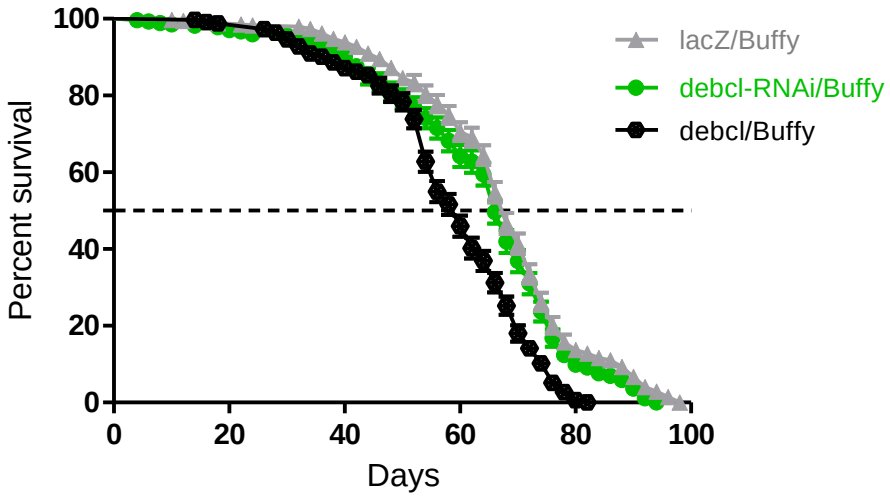
The overexpression of the pro-survival *Buffy* rescues the *Debcl*-induced phenotypes

The overexpression of *Buffy* and *Debcl* in DA neurons results in a longer lifespan and improved locomotor ability (Figure 4). The median lifespan of these flies was 62 days when compared to *Buffy* and *lacZ* overexpressing controls at 68 days. The median survival of *Debcl-RNAi* flies was 68 days as determined by a Log-rank (Mantel-Cox) test (Figure 4A). The climbing ability of these flies was also much improved as determined by comparing the climbing indices at 95% CI (Figure 4B). Taken together these results suggest that *Buffy* antagonizes the *Debcl*-induced phenotypes of shortened lifespan and poor climbing ability to markedly improve "healthspan".

Overexpression of *Debcl* enhances the *α-synuclein*-induced developmental eye defects

The overexpression of *Debcl* in the Drosophila eye results in severe ablation of the eye due to apoptosis [40, 41] while expression of *α-synuclein* in the eye results in developmental defects (Figure 5A, IV). When *Debcl* is overexpressed in the eye, developmental defects resulting from *Gal4* [61] (Figure 5A, I and 5B), inhibition of *Debcl* (Figure 5A, II and 4B), and overexpression of *Debcl* (Figure 5A, III and 5B) are enhanced. Biometric analysis of the ommatidia number and the percentage of eye disruption showed significant differences in the compared genotypes to the control that express the benign *lacZ* transgene (Figure 5B). The inhibition of *Debcl* along with *α-synuclein* expression (Figure 5A, V and 5C) and the co-expression of *Debcl* and *α-synuclein* (Figure 5A, VI and 5C) result in enhanced phenotypes. The disruption of the ommatidial array due to fusion of the ommatidia and smaller eye is severely enhanced by

A.



B.

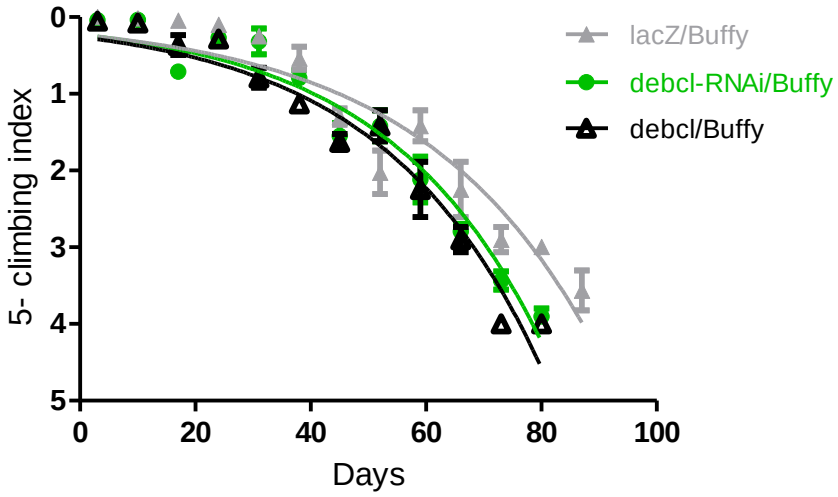
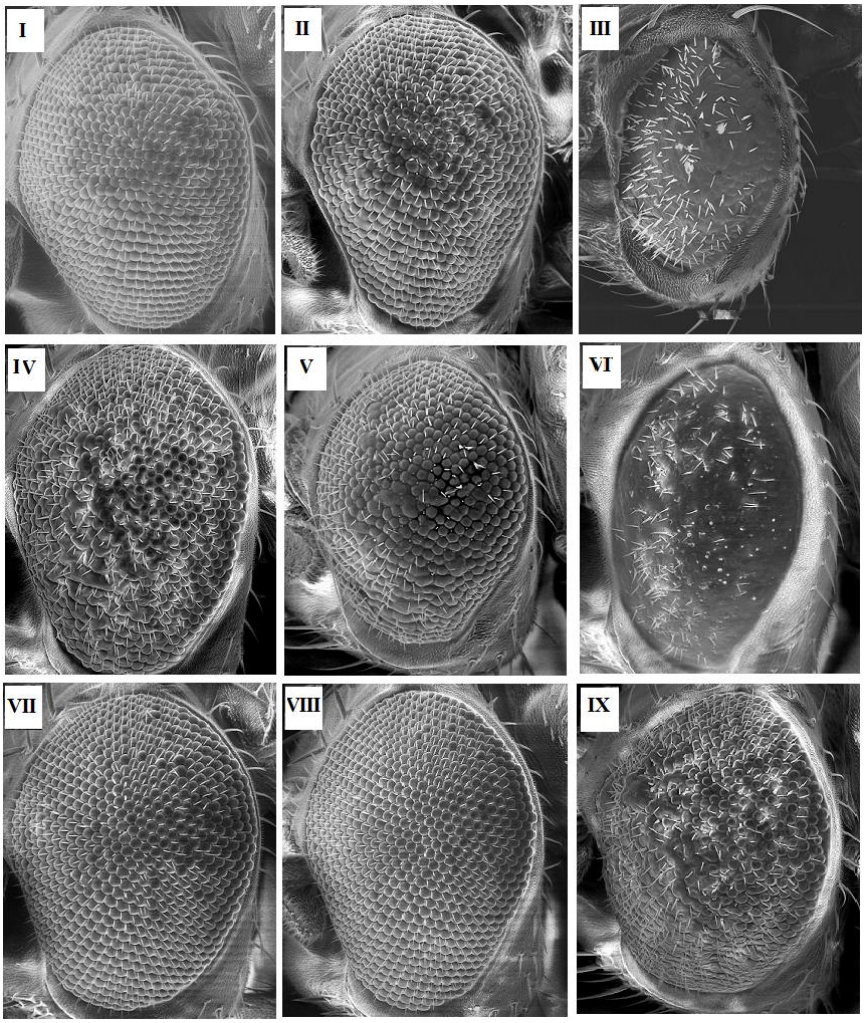


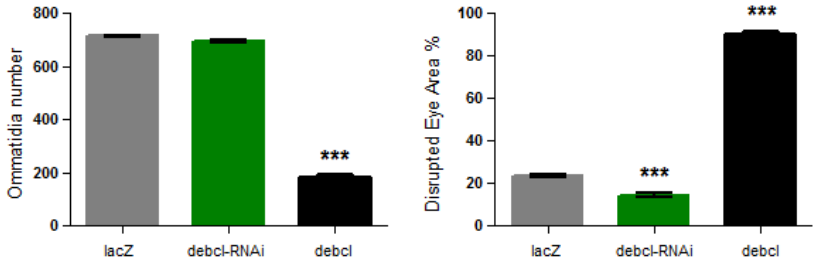
Figure 3.4 Overexpression of *Buffy* partially rescues the *Debcl*-induced phenotypes

A) The overexpression of *Buffy* along with the overexpression of *Debcl* or *Debcl-RNAi* restores lifespan and B) significantly improves the climbing ability of these flies. The genotypes are *UAS-Buffy; Ddc-Gal4/ UAS-lacZ, UAS-Buffy; Ddc-Gal4/ UAS-Debcl-RNAi* and *UAS-Buffy; Ddc-Gal4/ UAS-Debcl*. Longevity was determined by log-rank (Mantel-Cox) test and n≥200 while climbing ability curves were fitted non-linearly and compared with 95% CI.

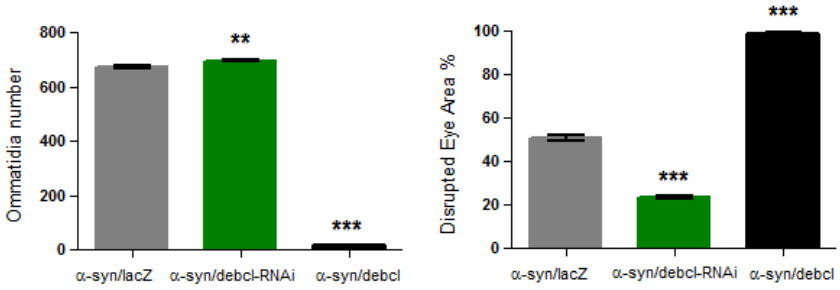
A.


I
II
III
IV
V
VI
VII
VIII
IX

B.


Ommatidia number
800
600
400
200
0
***
lacZ
debcl-RNAi
debcl
Disrupted Eye Area %
100
80
60
40
20
0
***
***
lacZ
debcl-RNAi
debcl

C.



D.

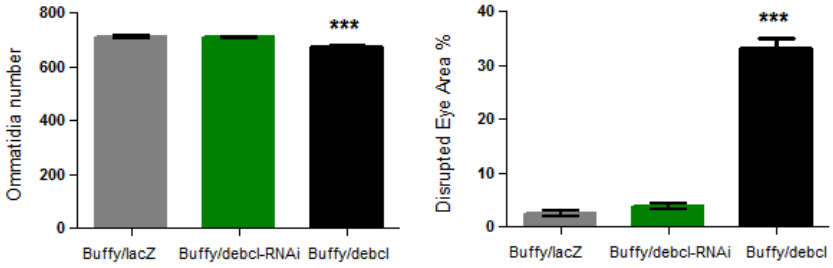


Figure 3.5 *Buffy* partially rescues the *Debcl*-induced developmental eye defects

A) Scanning electron micrographs when *Debcl* is overexpressed or inhibited in the eye with the eye-specific *GMR-Gal4* transgene; (I) *GMR-Gal4/ UAS-lacZ;* (II) *GMR-Gal4/ UAS-Debcl-RNAi;* (III) *GMR-Gal4/ UAS-Debcl,* when co-expressed with *α-synuclein*; (IV) *UAS-α-synuclein; GMR-Gal4 / UAS-lacZ;* V) *UAS-α-synuclein; GMR-Gal4 / UAS-Debcl-RNAi* VI) *UAS- α-synuclein; GMR-Gal4/ UAS-Debcl;* and when co-expressed with *Buffy* VII) *UAS-Buffy; GMR-Gal4/ UAS-lacZ* VIII) *UAS-Buffy; GMR-Gal4/ UAS-Debcl-RNAi* and IX) *UAS-Buffy; GMR-Gal4/ UAS-Debcl*. B) Biometric analysis showed a significant difference in the disrupted area of the eye when *Debcl* was inhibited in the developing eye and the depressed ommatidia number and high levels of disruption when *Debcl* is overexpressed. C) Biometric analysis shows a marked difference when *Debcl* is inhibited in an *α-synuclein* expressing background with increased ommatidia number and a less disrupted ommatidial array, whereas when *Debcl* is overexpressed along with the expression of *α-synuclein*, there is a dramatic decrease in ommatidia number coupled with severe disruption of the ommatidial array. D) The biometric analysis reveals the restoration of *Debcl*-induced phenotypes by overexpression of *Buffy*. The inhibition and overexpression of *Debcl* along with overexpression of *Buffy* results in increased ommatidia number together with improved disruption of the ommatidial array as determined by one-way ANOVA and Dunnett's multiple comparison test ($P<0.05$ and 95% CI), error bars indicate the SEM, asterisks (*) represents statistically significant result and n=10.

the overexpression of *Debcl* together with *α-synuclein* (Figure 4A, VI and 4C). The analysis of the ommatidia number and disruption of the eye revealed significant differences, the inhibition of *Debcl* yielded "healthier" eyes and its overexpression resulted in worsened phenotypes (Figure 5C). The ommatidial disarray that results from inhibition of *Debcl* are completely rescued by overexpression of the pro-survival *Buffy* (Figure 5A, VIII and 5D), while the ablated eye that result from *Debcl* overexpression is partially rescued upon *Buffy* overexpression, restoring the ablation to a mildly severe rough eye phenotype (Figure 5A, IX and 5D). Biometric analysis showed recouped ommatidia number and a lessened disruption of the eye, though they still were significantly different from the control (Figure 5D). These results suggest that overexpression of *Debcl* along with expression of *α-synuclein* enhances the *Debcl*-induced eye ablation, while the overexpression of *Debcl* together with *Buffy* partially rescues the eye phenotype.

Discussion

Since mitochondrial dysfunction is central to the pathology of both sporadic and familial forms of PD [62], it was important to highlight the role and consequences of the altered expression of the proapoptotic mitochondrial gene *Debcl* in this process. The overexpression of *Debcl* in Drosophila and other systems, including mammalian, has been demonstrated to lead to apoptosis [39-42, 48, 49, 63]. The recapitulation of PD-like symptoms in *Drosophila melanogaster,* especially the age-dependent loss of climbing ability, has led to investigation of genes that could suppress these phenotypes [14, 15, 64]. Our results show that the overexpression of *Debcl* results in a severely shortened lifespan followed by premature loss in climbing ability; phenotypes that are reminiscent of PD-

like symptoms in model organisms. Thus, our work shows the intricate balance between life and death decisions in the sensitive dopamine producing neurons. It seems that excess amounts of Debcl protein are sufficient to upset the survival mechanisms and lead to degeneration and death of DA neurons. The importance of *Debcl*-induced apoptosis is exhibited by the strict control in its gene product by the tumour suppressors *Rbf1* [44], *OSCP1/NOR1* [47], and *NF-Y* [43]. Furthermore, it has a motif for ubiquitination, probably by the *TrCP* homologue *slimb* that targets it for destruction by the proteasome [65]. The inhibition of *Debcl* had a converse result, with flies that had a longer lifespan and healthy climbing ability. It is possible that the suppression of *Debcl* tips the balance towards the survival pathways controlled by the antiapoptotic *Buffy*. Our results indicate that overexpression of *Debcl* appears to be a novel model of PD as a result of neuronal apoptosis.

The *α-synuclein*-induced model of PD in Drosophila shows little difference in lifespan between the control and wild type, A53T and A30P *α-synuclein* flies [14]. In our study, the overexpression of *Debcl* in the DA neurons resulted in a marked decrease in lifespan. This is in part due to toxic effects as a result of the expression of *α-synuclein*, and additionally, due to *Debcl*-induced apoptosis. The *Debcl*-induced apoptosis is mediated by other factors including; the mitochondrial fission protein *Drp1* [44] that interacts with Debcl to induce mitochondrial fragmentation; *Glycerophosphate oxidase-1* [46] that increases mitochondrial ROS accumulation; and possibly through the initiation of autophagy, since both *α-synuclein* expression [66] and *Debcl* [67] overexpression are implicated in this process. This worsening of phenotypes was also observed when *Debcl* was overexpressed with *α-synuclein* in the eye. The inhibition of *Debcl* in the DA neurons

resulted in a marked increase in survival and improved locomotor ability. This inhibition of *Debcl* is sufficient to negate its apoptotic role and thus promote cell survival through the opposing antiapoptotic Buffy.

Locomotor dysfunction is one of the major symptoms of PD. The demonstration of an age-dependent loss of climbing ability is pivotal to highlighting the effects of degeneration and death of DA neurons, ultimately as a consequence of altered gene expression as opposed to cellular senescence [68]. The overexpression of *Debcl* in the DA neurons produced a climbing index significantly different from that of control flies with the loss of climbing ability in an age-dependent manner and likely due to *Debcl*-induced neuronal degeneration. The degree of locomotor dysfunction seemed to be similar to that observed when *α-synuclein* is overexpressed in DA neurons. Taken together, these results would indicate a detrimental effect in overexpression of *Debcl* in DA neurons that result in a novel model of PD in flies.

In contrast, the inhibition of *Debcl* in the same neurons results in a remarkable improvement in climbing ability when compared to the controls. The inhibition of *Debcl* in the DA neurons of the *α-synuclein*-induced PD model significantly increased lifespan and climbing ability, indicating that reduced levels of *Debcl* are sufficient to alter the healthspan of DA neurons. The *Debcl*-induced apoptosis relies on downstream effectors that either induces ROS accumulation [46] or the fragmentation of the mitochondria [44]. As the down-regulation of *Buffy* or up-regulation of *Debcl* results in apoptosis [47], the cellular advantage of *Debcl* inhibition may be indirect through the de-repression of the *Buffy* gene product that confers survival advantages. The directed expression of *Buffy* along with *Debcl* results in an improved “healthspan” compared to the *Debcl*-induced

phenotypes and corroborate other studies that show the overexpression of the pro-survival Buffy confers survival advantages through increased survival and improved climbing ability under conditions of stress [69]. Our study suggests that the overexpression of *Buffy* is similar to an up-regulation that ultimately blocks *Debcl*-induced apoptosis, similar to results obtained when its regulation by Rbf1 or dE2F2 is altered to repress it transcriptionally [44, 45]. This suppression of *Buffy* is sufficient to induce *Debcl*-dependent apoptosis, in addition to the promotion of Debcl activity by dNF-Y [43]. The co-overexpression of *Debcl* and *Buffy* in the eye resulted in a partial rescue of the *Debcl*-induced phenotypes. Therefore, overexpression of the pro-survival *Buffy* suppresses the *Debcl*-dependent phenotypes.

Conclusions

Directed inhibition of *Debcl* results in improved survivorship and extended climbing ability whereas the directed overexpression of *Debcl* results in reduced lifespan and impaired locomotor function. These phenotypes are rescued upon co-expression with the pro-survival *Buffy*. The overexpression of *Debcl* enhances the effects of *α-synuclein* expression. *Buffy* counteracts *Debcl*-induced phenotypes, and represents a potential target to enhance neuronal survival in response to the detrimental effects of *Debcl*-induced apoptosis.

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Chapter 4 - The Inhibition of *Atg6* and *Pi3K59F* autophagy genes in dopaminergic neurons decreases lifespan and locomotor ability in *Drosophila melanogaster*

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Abstract

Autophagy is a cellular mechanism implicated in the pathology of Parkinson's disease. The proteins Atg6 (Beclin 1) and Pi3K59F are involved in autophagosome formation, a key step in the initiation of autophagy. We first used the *GMR-Gal4* driver to determine the effect of reducing the expression of the genes encoding these proteins on the developing *Drosophila melanogaster* eye. Subsequently, we inhibited their expression in *D. melanogaster* neurons under the direction of a *Dopa decarboxylase* (*Ddc*) transgene, and examined the effects on longevity and motor function. Decreased longevity coupled with an age-dependent loss of climbing ability was observed. In addition, we investigated the roles of these genes in the well-studied *α-synuclein*-induced Drosophila model of Parkinson's disease. In this context, lowered expression of *Atg6* or *Pi3K59F* in *Ddc-Gal4*-expressing neurons results in decreased longevity and associated age-dependent loss of locomotor ability. Inhibition of *Atg6* or *Pi3K59F* together with overexpression of the sole pro-survival *Bcl-2* Drosophila homolog *Buffy* in *Ddc-Gal4*-expressing neurons resulted in further decrease in the survival and climbing ability of *Atg6-RNAi* flies, whereas these measures were ameliorated in *Pi3K59F-RNAi* flies.

Introduction

Parkinson disease is the second most common human neurodegenerative disease and is characterized by the progressive degeneration and loss of the dopamine-producing neurons in the centrally located s*ubstantia nigra pars compacta* (*SNpc*) region of the brain. PD is marked by severe locomotor dysfunction as well as non-motoric symptoms such as autonomic, cognitive and psychiatric deficiencies [1, 2]. Although the majority of PD cases are sporadic, familial forms with a genetic link have been identified and studied

extensively in model organisms [3, 4]. The first gene to be identified associated with familial forms of PD encodes α-synuclein, a small soluble protein predominantly found in neural tissues [5-7]. The association of α-synuclein with components of the mitochondria is thought to lead to oxidative stress, apoptosis, autophagy and the eventual neurodegeneration [8-10]. The link between autophagic dysfunction and aberrant α-synuclein in the pathogenesis of both sporadic and familial forms of PD has been established [11]. The pathogenesis of PD must therefore involve several failed cellular mechanisms and the disruption of autophagic homeostasis contributes to the development of this neurodegenerative disorder.

Autophagy is a tightly regulated catabolic mechanism used by eukaryotic cells to degrade long-lived proteins and organelles. It is a multi-step pathway with key regulators TOR kinase, AMP-activated protein kinase, Bcl-2/Bcl-X_L inhibition of the Beclin1/class III PI3K complex, p53 tumour suppressor protein among others that respond to the absence or presence of nutrients, growth factors, and energy levels [12, 13]. The formation of the phagophore marks the initiation of autophagy and the ULK1-Atg13-FIP200 complex is pivotal. The nucleation phase of initial phagophore formation requires the interaction of the ULK1 complex with the Atg6-interacting complex, composed of Atg6, class III PI3K, Vps15 and Atg14L. Stimulation of this complex generates phosphatidylinositol-3-phosphate that promotes autophagosomal membrane nucleation [12, 14, 15]. This is followed by autophagosomal elongation and maturation. The final step is the fusion of the autophagosome to the acidic lysosome.

The Atg6-interacting complex acts in a regulatory step, with Atg6-binding proteins like AMBRA1, UVRAG and Bif-1 promoting autophagy and Rubicon and Bcl-2/Bcl-X_L

inhibiting autophagy. The inhibition of Atg6-dependent autophagy by the anti-apoptotic Bcl-2 proteins point to a regulatory role by this important protein family [16]. One way this inhibition is thought to occur is when anti-apoptotic Bcl-2 proteins bind to the Bcl-2 homology 3 (BH3) domain of Atg6 and thus interfering with the initiation of autophagy. Another group suggest Bcl-2 proteins affects autophagy only indirectly by the inhibition of the pro-apoptotic Bcl-2 proteins Bax and Bak [17]. The sequestration of Atg6 by Bcl-2 proteins can be reversed by phosphorylation of the Atg6 BH3 domain by the Death associated protein (DAP) kinase [18]. Atg6 binds class III PI3K within its Apg6 domain to effect its role in vacuolar protein sorting and autophagy [19]. Autophagy is highly conserved, with single orthologues of many of the components involved in this mechanism present in Drosophila [20, 21]. For example, the Atg6-interaction complex seems to be well conserved and is central to autophagy [22, 23]. Orthologues of Atg14L, UVRAG, Rubicon and Bcl-2 are present in the fly and may play the same role they do in mammals by forming different autophagy regulatory complexes.

PD is one of the diseases where the breakdown in autophagy has been implicated in the pathogenesis. The vesicular nature of Lewy Bodies, the dominant pathological features present in surviving neurons of PD patients, has led to the suggestion that the autophagic-lysosomal pathway contributes to the formation or dissolution of LBs [24, 25]. The implication of autophagy in human disease and especially in neurodegenerative disorders makes Drosophila an attractive model to study the consequences of altering gene products of the autophagy molecules, *Atg6* and the class III PI3K or *Pi3K59F*. The first fly model of PD utilized a human transgene of *α-synuclein* to induce the PD-like symptoms [26] and has become one of the most successful model systems to study PD due to recapitulation

of the degeneration and loss of DA neurons, age-dependent loss of locomotor ability, presence of LB-like inclusions and compromised survival [4]. We determined the effect of directed inhibition of *Atg6* or *Pi3K59F* via directed expression of RNAi transgenes in the DA neurons of Drosophila. Further *Atg6* or *Pi3K59F* inhibitory transgenes were co-expressed with *α-synuclein* to investigate their role in the first Drosophila model of PD. As well, loss of function of the autophagy genes were evaluated along with the only pro-survival *Bcl-2* homologue in Drosophila, *Buffy*.

Materials and Methods

Bioinformatic analysis

The protein sequences were sourced from National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/protein/) and the domains were identified using the NCBI Conserved Domain Database (CDD) (Marchler-Bauer et al. 2015) (http://www.ncbi.nlm.nih.gov/cdd) and the Eukaryotic Linear Motif (Dinkel et al. 2016) (http://elm.eu.org/) which focuses on annotation and detection of eukaryotic linear motifs (ELMs), also known as short linear motifs (SLiMs). A Clustal Omega multiple sequence alignment (http://www.ebi.ac.uk/Tools/msa/clustalo/) (Goujon et al. 2010; Sievers et al. 2011) was used to align sequences and show conservation of the domains in the selected organisms. The nuclear export signal (NES) was predicted using the NetNES 1.1 server (http://www.cbs.dtu.dk/services/NetNES/) (la Cour et al. 2004).

Drosophila media and culture

Stocks and crosses were maintained on a standard medium containing cornmeal, molasses, yeast, agar, and water and treated with propionic acid and methylparaben. Stocks were maintained on solid media for two to three weeks before transfer onto new

media to re-culture. Stocks were kept at room temperature (22°C ± 2°C) while crosses and experiments were carried out at 25°C and 29°C.

Drosophila stocks and derivative lines

UAS-α-synuclein [26] was generously provided by Dr. M. Feany of Harvard Medical School, *UAS-Buffy* [27] by Dr. Leonie Quinn of University of Melbourne, and *Ddc-Gal4* [28] by Dr. J. Hirsch of University of Virginia. *Atg6-RNAi* (*w^{1118}; P{GD11647}v22122*), and *Pi3K59F-RNAi* (*P{KK107602}VIE-260B*) were obtained from Vienna Drosophila Resource Center. *GMR-Gal4* [29] and *UAS-lacZ* flies were obtained from the Bloomington Drosophila Stock Center at Indiana University. The *UAS-α-synuclein/CyO; Ddc-Gal4/TM3* and *UAS-α-synuclein/CyO; GMR-Gal4* were generated using standard homologous recombination methods and were used to overexpress *α-synuclein* in the DA neurons or developing eye under the direction of *Ddc-Gal4* or *GMR-Gal4*. The *UAS-Buffy/CyO; Ddc-Gal4* and *UAS-Buffy/CyO; GMR-Gal4* lines were used to overexpress *Buffy* in the DA neurons and in the developing eye respectively. PCR reaction was used 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. The 5' to 3' sequence of the forward primer was GTGCCCAGTCATGACATTT, while that of the reverse primer was CCACAAAATCCACAGCACAC and were ordered from Invitrogen. The *Drosophila melanogaster* Buffy mRNA, NCBI reference sequence: NM_078978.2, was used to design a set of Buffy primers that would target both the endogenous and the overexpression transcripts. The 5' to 3' sequence of the forward primers were

CACAGCGTTTATCCTGCTGA and CGGGTGGTGAGTTCCATACT, while that of the reverse primers were TCGCAGTGTGAAGATTCAGG and TTAATCCACGGAACCAGCTC and were ordered from Eurofins MWG Operon. Gel electrophoresis was used for confirmation of recombination events via presence of the corresponding PCR product.

Ageing assay

Several crosses between five females and three males were made of each genotype and a cohort of each adult critical class male flies was collected upon eclosion. At least two hundred flies were aged per genotype, at a density of ≤ 20 flies per vial to avoid overcrowding. Flies were observed and scored every two days for presence of deceased adults [30] and survivors transferred to fresh media. Longevity data was analysed using GraphPad Prism version 5.04 and survival curves were compared using the log-rank (Mantel-Cox) test. Significance was determined at 95%, at a P-value less than or equal to 0.05 with Bonferroni correction.

Climbing assay

A cohort of the adult critical class male flies from each genotype was collected upon eclosion and scored for their ability to climb over time [31, 32]. Climbing analysis was performed using GraphPad Prism version 5.04, climbing curves were fitted using non-linear regression and compared using 95% confidence intervals at a 0.05 P-value.

Scanning electron microscopy of the drosophila eye

Several single vial matings of five females and three males were made of each genotype at 29°C and a group of adult male flies collected upon eclosion and aged for three days before being frozen at -80°C. Whole flies were mounted on scanning electron microscope

stubs, desiccated overnight and photographed with a FEI Mineral Liberation Analyzer 650F scanning electron microscope. For each cross at least 10 eye images were analysed using the National Institutes of Health (NIH) ImageJ software [33] and biometric analysis performed using GraphPad Prism version 5.04. The ratio of disruption of the eye was calculated as previously described [34]. Comparisons for statistical analysis were performed using one-way ANOVA and Dunnett's multiple comparison test. Significance was determined at $p<0.05$. But briefly, the ratio of disrupted eye area was calculated by dividing the total area of the eye with the total disrupted area of the eye. The total disrupted area of the eye was the sum of any two or more ommatidia that were fused together.

Results

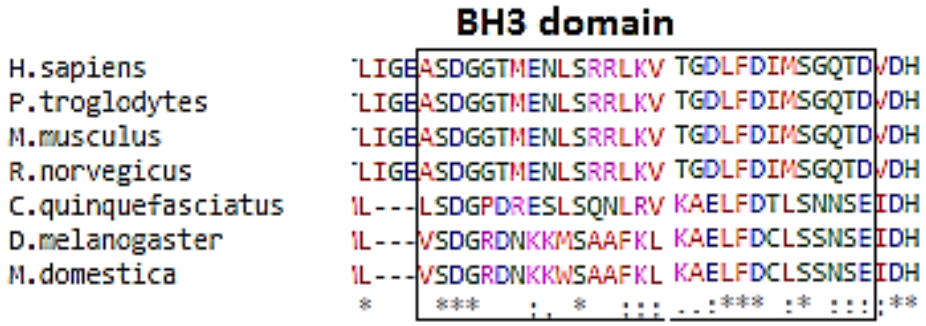
Both Atg6 and Pi3K59F are evolutionarily conserved

Bioinformatic analysis of the protein sequences of Atg6 (also known as Beclin 1) and Pi3K59F revealed domains that are remarkably conserved among diverse species, signifying the importance of these two autophagy proteins (Figure 1). The Drosophila Atg6 protein is comprised of 422 amino acids, and when aligned with human Beclin 1 which is composed of 450 amino acids, it has 50% identity and 67% similarity and the APG6/Vps30 domain is conserved as well as a NES (Figure 1A). A putative non-canonical BH3 domain is possibly present in Drosophila that could function in inhibiting Atg6 as occurs in mammals through sequestration by the pro-survival Bcl-2 proteins (Figure 1B). A multiple sequence alignment of the Drosophila Pi3K59F protein with the human homologue shows evolutionary conservation in the class III PI3K genes with a 64% identity and 79% similarity (Figure 1C), with both members possessing the C2, PIK

A.


APG6 DOMAIN
NES

B.


BH3 domain
H.sapiens
P.troglodytes
M.musculus
R.norvegicus
C.quinquefasciatus
D.melanogaster
M.domestica

C.


C2 DOMAIN
PIK ACCESSORY DOMAIN
CATALYTIC DOMAIN
ATP BINDING CATALYTIC LOOP
ATP BINDING ACTIVATION LOOP

Figure 4.1 The autophagy proteins Atg6 and Pi3K59F are evolutionarily conserved

(A). Multiple alignment of the *Drosophila melanogaster* homologue and the human Atg6 shows conservation of the APG6/Vps30 domain that is important for the initiation of autophagy and presence of a NES (Hsap is *Homo sapiens* NP_003757.1 and Dmel is *Drosophila melanogaster* NP_523702.1). (B) Drosophila possess a putative BH3 domain which is conserved with a few amino acid substitutions as Clustal Omega multiple sequence alignment [43, 44] of Drosophila Atg6 protein with that of mammalian and insect homologues shows (H.sapiens is *Homo sapiens* NP_003757.1, P.troglodytes is *Pan troglodytes* XP_511522.2, M.musculus is *Mus musculus* NP_062530.2, R.norvegicus is *Rattus norvegicus* NP_001029289.1, C.quinquefasciatus is *Culex quinquefacsiatus* XP_001861442.1, D.melanogaster is *Drosophila melanogaster* NP_523702.1 and M.domestica is *Musca domestica* XP_005189450.1). (C) Pi3K59F is highly conserved, with both organisms containing the C2, the PIK accessory domain and the kinase catalytic domains (Hsap is *Homo sapiens* NP_002638.2 and Dmel is *Drosophila melanogaster* NP_477133.1). The domains were identified using the NCBI Conserved Domain Database Search (CDD) [45]. "*" indicate the residues that are identical, ":" indicate the conserved substitutions, "." indicate the semi-conserved substitutions. Colours show the chemical nature of amino acids. Red is small hydrophobic (including aromatic), Blue is acidic, Magenta is basic, and Green is basic with hydroxyl or amine groups.

and the kinase catalytic domains. The Drosophila sequence shows the presence of a NES, NLS, several Atg8 binding motifs and a di-Arginine ER retention motif as determined by the ELM database [35].

The conditional expression of *Atg6* or *Pi3K59F* in the Drosophila developing eye results in differing eye phenotypes

The directed inhibition of *Atg6* or *Pi3K59F* in the developing eye results in a significant disruption of the ommatidial array and while the inhibition of *Atg6* results in a higher mean ommatidia number, that of *Pi3K59F* result in a lower mean ommatidia number (Figure 2A, I-III). The suppression of these two autophagy genes in the developing eye result in highly disrupted ommatidia (Figure 2B) and points to a role of these autophagy proteins in the normal development of the eye.

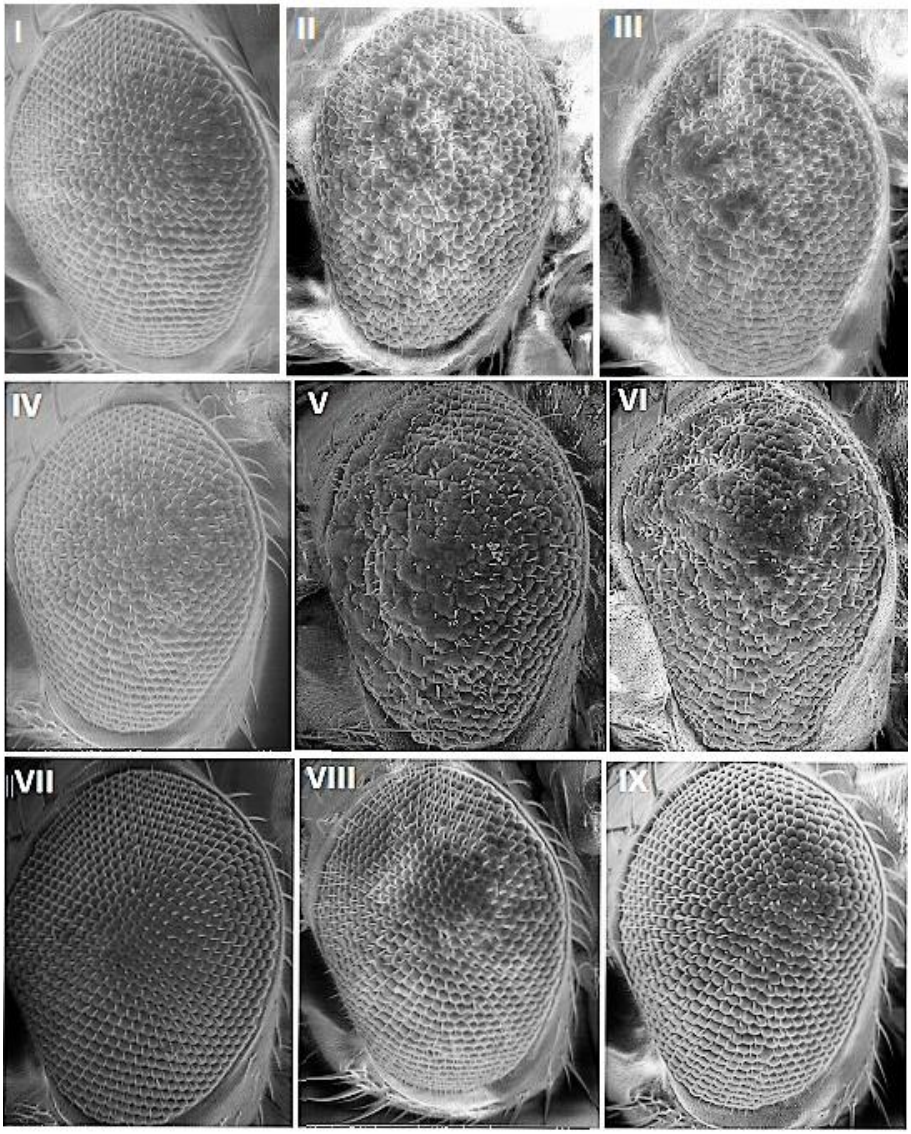
The co-expression of *Atg6-RNAi* or *Pi3K59F-RNAi* with *α-synuclein*-expression in the developing eye enhanced the developmental eye defects (Figure 2A, IV-VI). The disruption of the ommatidial array is pronounced and the mean number of ommatidia is reduced (Figure 2C). These results suggest that the inhibition of *Atg6* or *Pi3K59F* coupled with the toxic effects of α-synuclein in the developing eye enhances the developmental eye defects.

Similarly, the co-expression of *Atg6* or *Pi3K59F* with *Buffy* in the developing eye results in a slight increase in the number of ommatidia and a marked decrease in the disruption of the ommatidial array (Figure 2A, VII-IX and 2D).

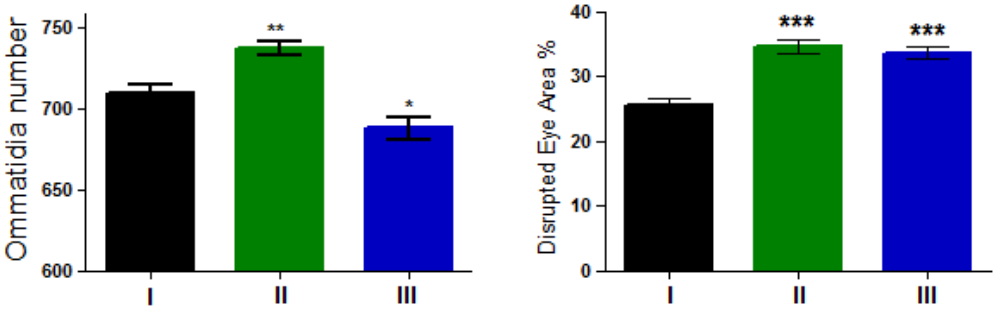
Inhibition of *Atg6* or *Pi3K59F* in the DA neurons phenocopies PD phenotypes

The inhibition of *Atg6* or *Pi3K59F* in DA neurons by RNA interference under the control of *Ddc-Gal4* results in flies with decreased survival (Figure 3A). The median lifespan of

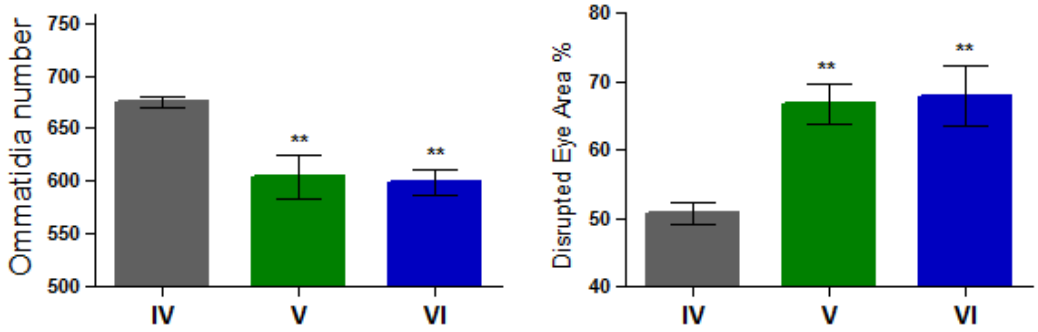
A.


I
II
III
IV
V
VI
VII
VIII
IX

B.


Ommatidia number
750
700
650
600
**
*
I
II
III
Disrupted Eye Area %
40
30
20
10
0
***
***
I
II
III

C.



D.

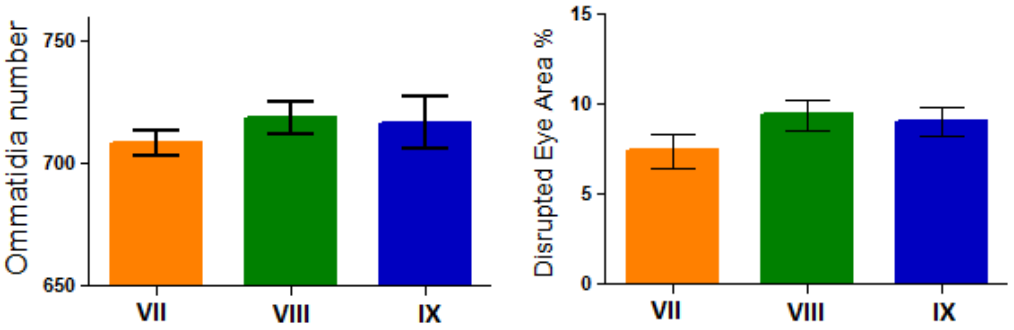


Figure 4.2 The conditional expression of *Atg6* or *Pi3K59F* in the Drosophila developing eye results in differing eye phenotypes

A) Scanning electron micrographs when *Atg6* or *Pi3K59F* are suppressed in the developing eye; (I) *GMR-GAL4/ UAS-lacZ*, (II) *GMR-GAL4/ Atg6-RNAi* and (III) *GMR-GAL4/ Pi3K59F-RNAi*, or when inhibited along with *α-synuclein*-expression; IV) *UAS-α-synuclein; GMR-Gal4/ UAS-lacZ*, V) *UAS-α-synuclein; GMR-Gal4/ Atg6-RNAi*, and VI) *UAS-α-synuclein; GMR-Gal4/ Pi3K59F-RNAi*, and when co-expressed with *Buffy*; VII) *UAS-Buffy; GMR-Gal4/ UAS-lacZ*, VIII) *UAS-Buffy; GMR-Gal4/ Atg6-RNAi* and IX) *UAS-Buffy; GMR-Gal4/ Pi3K59F-RNAi*. B) Biometric analysis shows a significant difference in the number of ommatidia and percent disruption of the ommatidial array, with *Atg6* flies having a higher mean number of ommatidia whereas *Pi3K59F* flies had a lower mean number of ommatidia compared to the control flies. C) The inhibition of *Atg6* or *Pi3K59F* with *α-synuclein* results in lower ommatidial count and a higher percentage of eye disruption than the control. D) When *Buffy* is co-expressed in the eye with *Atg6* or *Pi3K59F*, there is an increase in the mean number of ommatidia and the percentage of disrupted area of the eye declines compared to the control. Comparisons were determined by one-way ANOVA and Dunnett's multiple comparison test ($P<0.05$), error bars are SEM, $n=10$ and asterisks (*) represent statistical significance.

Atg6-RNAi flies was 58 days and 64 days for *Pi3K59F-RNAi* flies compared to 73 days for the control. The directed inhibition of *Atg6* or *Pi3K59F* in DA neurons resulted in impaired climbing ability as indicated by the comparison of the climbing curves at 95% CI (Figure 3B). This suggests that the inhibition of *Atg6* or *Pi3K59F* decreases longevity and climbing ability in Drosophila when expressed in DA neurons and thus, normal levels of these proteins are required for the normal functioning of DA neurons.

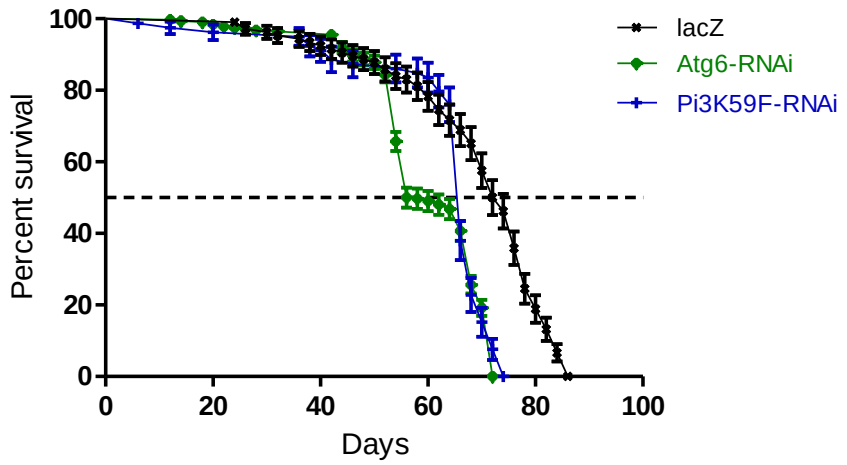
Inhibition of *Atg6* or *Pi3K59F* in the *α–synuclein*-induced PD model alters survival and climbing ability

The overexpression of *α-synuclein* in DA neurons results in flies that have impaired climbing ability and a shortened lifespan, the inhibition of *Atg6* or *Pi3K59F* in DA neurons of flies expressing *α-synuclein* slightly altered lifespan and locomotor ability (Figure 4A and 4B). *Atg6-RNAi* flies had a median lifespan of 56 days same as *Pi3K59F-RNAi* flies compared to the controls at 62 days. The nonlinear fitting of the curves with 95% CI determined that the rate of climbing was not statistically different between the *Atg6-RNAi* or *Pi3K59F-RNAi* flies when compared to the control, though the climbing indices were slightly lower than the ones obtained from the controls. This suggests that inhibition of the autophagy genes appears to slightly modify the phenotypes present when *α-synuclein* is expressed in the DA neurons of Drosophila.

Co-expression of *Atg6* or *Pi3K59F* with *Buffy* suppresses the *Pi3K59F*–induced phenotypes

The co-expression of *Atg6-RNAi* or *Pi3K59F-RNAi* with the pro-survival *Bcl-2* homologue in Drosophila, *Buffy*, results in an enhanced loss of *Atg6*-induced phenotypes and a suppression of the loss of *Pi3K59F*-induced phenotypes (Figure 5). The overexpression of *Buffy* with *Atg6-RNAi* resulted in reduced survival with a median

A.



B.

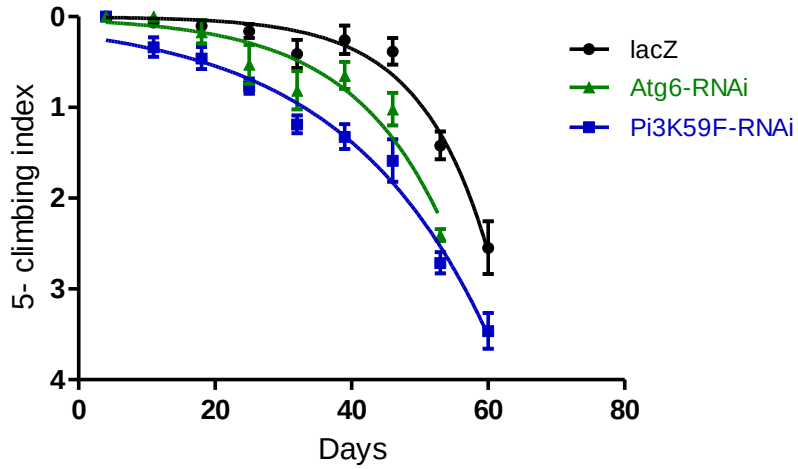
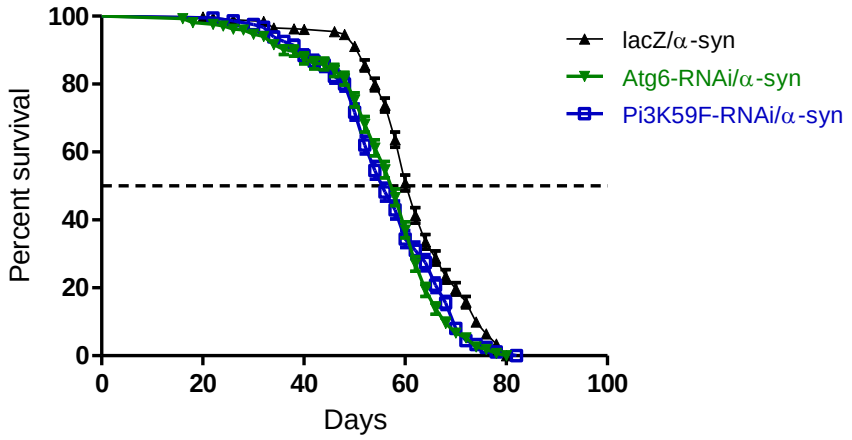


Figure 4.3 Inhibition of *Atg6* or *Pi3K59F* in the DA neurons phenocopies PD-like phenotypes

A) Directed inhibition of *Atg6* or *Pi3K59F* in the DA neurons directed by *Ddc-Gal4* results in a decrease in survival compared to the control flies overexpressing *UAS-lacZ.* The genotypes are *Ddc-GAL4/ UAS-lacZ, Ddc-GAL4/ Atg6-RNAi* and *Ddc-GAL4/ Pi3K59F-RNAi.* Longevity is shown as percent survival $P < 0.05$, determined by the log-rank (Mantel-Cox) test and $n \geq 200$). B) The inhibition of *Atg6* or *Pi3K59F* in the DA neurons resulted in a significant decrease in climbing ability as determined by non-linear fitting of the climbing curves and comparing 95% confidence intervals. The genotypes are *Ddc-Gal4/ UAS-lacZ, Ddc-Gal4/ Atg6-RNAi* and *Ddc-Gal4/Pi3K59F-RNAi.* Error bars indicate SEM and $n=50$.

A.



B.

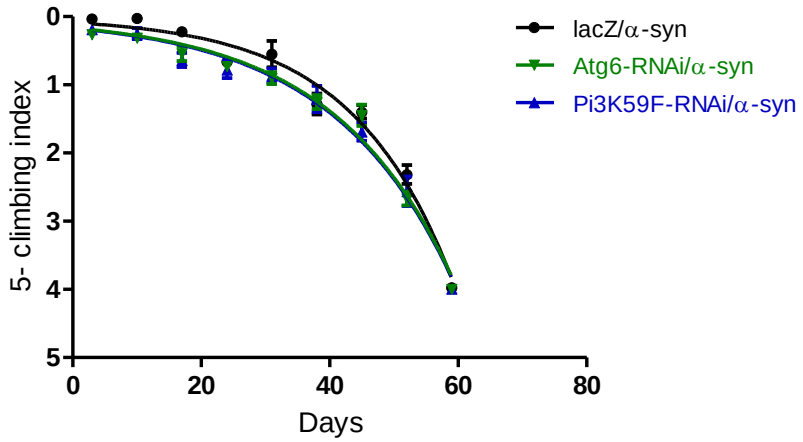


Figure 4.4 Inhibition of *Atg6* or *Pi3K59F* in the *α–synuclein*-induced PD model does not alter impaired survival and climbing ability

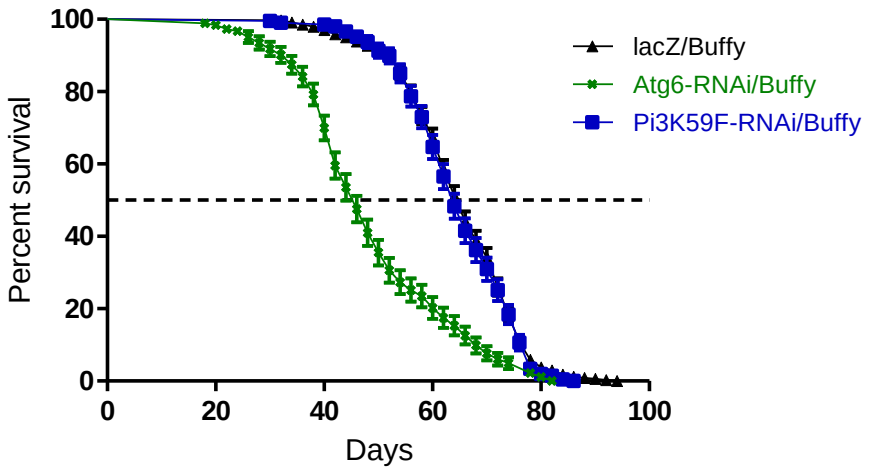
A) The inhibition of *Atg6* or *Pi3K59F* along with *α-synuclein*-expression in the DA neurons did not alter median lifespan when compared to the controls. Genotypes are *UAS-α-synuclein; Ddc-Gal4/ UAS-lacZ, UAS-α-synuclein; Ddc-Gal4/ Atg6-RNAi* and *UAS-α-synuclein; Ddc-Gal4/ Pi3K59F-RNAi.* Longevity is shown as percent survival, $P < 0.05$, determined by log-rank (Mantel-Cox) test with $n \leq 200$. B) The co-expression of *Atg6-RNA$_i$* or *Pi3K59F-RNAi* in the *α-synuclein*-induced model of PD did not result in any significant age-dependent loss in climbing ability compared to the control. The genotypes are *UAS-α-synuclein; Ddc-Gal4/ UAS-lacZ, UAS-α-synuclein; Ddc-Gal4/ Atg6-RNAi* and *UAS-α-synuclein; Ddc-Gal4/ Pi3K59F-RNAi.* Error bars indicate SEM and n=50.

lifespan of 46 days compared to 66 days for the control (Figure 5A). The impaired climbing ability was enhanced in these flies as determined by the nonlinear fitting of the climbing curves and compared at 95% CI (Figure 5B). This is possibly due to the interaction of Buffy with the BH3 domain of Atg6 and further restricting the activity of Atg6 in the initiation of autophagy. Interestingly, the co-expression of *Pi3K59F* with *Buffy* results in a suppression of the phenotypes observed of reduced survival and climbing ability. The median lifespan of these flies was 66 days compared to 66 days in the controls (Figure 5A). The climbing ability of these flies improved to normal levels when the climbing curves were compared with the control (Figure 5B).

Discussion

The biometric analysis of the Drosophila eye when *Atg6* or *Pi3K59F* was inhibited shows significant differences in the eye phenotypes. The inhibition of *Atg6* results in a significant increase in the number of ommatidia and the disrupted area of the eye. Alternatively, when *Pi3K59F* is inhibited, there is a reduction in the number of ommatidia but with a high degree of degeneration of the ommatidial array. Though these two autophagy proteins are known to form the same complex, it is possible that when exclusively conditionally inhibited, they may have differing effects on the developing eye. Altered *Atg6* or *Pi3K59F* expression in the *α-synuclein*-expressing developmental eye defects model resulted in an enhanced phenotype. Both genes resulted in a depressed ommatidia number and highly degenerated ommatidial array. It is interesting that the effect of inhibiting *Atg6* or *Pi3K59F* in an α-synuclein background is the worsening of the phenotype in the developing eye, a trend that we did not observe in the DA neurons.

A.



B.

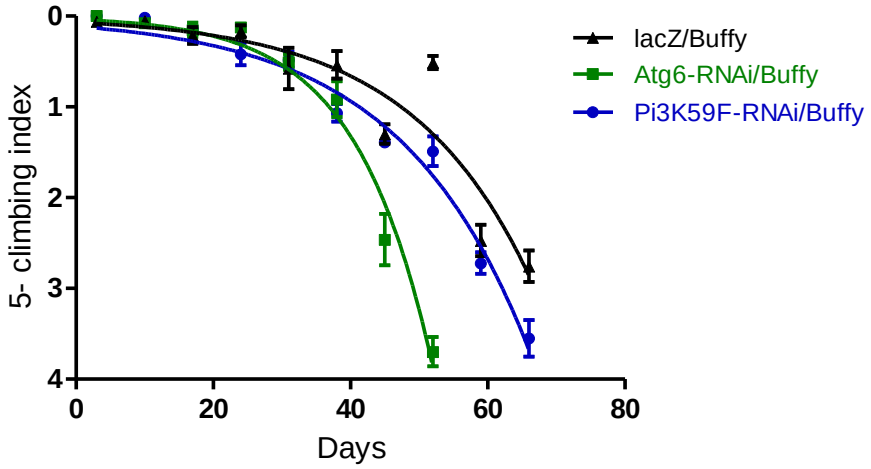


Figure 4.5 Co-expression of *Atg6* or *Pi3K59F* with *Buffy* suppresses the loss of *Pi3K59F*-induced phenotypes and enhances the loss of *Atg6*-induced phenotypes

A) The co-expression of *Buffy* with *Atg6-RNAi* enhanced the suppression of *Atg6*-induced decrease in survival whereas the co-expression of *Buffy* with *Pi3K59F-RNAi* resulted in suppression of reduced median survival. Genotypes are *UAS-Buffy; Ddc-Gal4/UAS-lacZ, UAS-Buffy; Ddc-Gal4/ Atg6-RNAi,* and *UAS-Buffy; Ddc-Gal4/ Pi3K59F-RNAi.* B) The co-expression of *Atg6-RNAi* with *Buffy* in the DA neurons result in an enhancement of the age-dependent loss in climbing ability. Alternatively, the co-expression of *Pi3K59F-RNAi* with *Buffy* result in the suppression of the age-dependent loss in climbing ability when compared to the control. The genotypes are *UAS-Buffy; Ddc-Gal4/UAS-lacZ, UAS-Buffy; Ddc-Gal4/ Atg6-RNAi,* and *UAS-Buffy; Ddc-Gal4/ Pi3K59F-RNAi.* Error bars indicate SEM and *n*=50.

The toxic effects of α-synuclein in the developing eye are well documented [26], and shows that the toxic effects of α-synuclein in the eye is additive with a worsening eye phenotype with additional assaults [34]. It seems therefore, that the inhibition of the autophagy genes *Atg6* or *Pi3K59F* in tandem with α-synuclein expression produces severe eye degeneration. The co-expression of the Bcl-2 protein *Buffy* with *Atg6-RNAi* or *Pi3K59F-RNAi* resulted in increased ommatidia number and a reduction in disruption of the eye. Interestingly, the inhibition of *Pi3K59F* in the developing eye results in reduced ommatidia number, contrary to when co-expressed with *Buffy,* the number of ommatidia increases but the degeneration of the eye worsens tremendously. Similarly, the inhibition of *Atg6* in the developing eye result in increased ommatidia number and co-expression with *Buffy* results in a similar ommatidia count and percent disruption of the eye.

The conditional expression of *Atg6* or *Pi3K59F* in the DA neurons of *Drosophila melanogaster* result in reduced survival and an age-dependent loss in climbing ability, phenotypes associated with Drosophila models of PD. The reduction of Atg6 and Pi3K59F activity in the dopamine producing neurons may be detrimental to the health of these flies that manifest PD-like symptoms. Atg6 and Pi3K59F are involved in the nucleation phase of autophagy in the formation of the autophagosome [12]. The inhibition of these two important autophagy proteins may lead to decreased autophagic activity and further degeneration of the DA neurons.

When *α-synuclein* is overexpressed in the DA neurons, it leads to locomotor impaired flies. In previous studies, the accumulation of α-synuclein in Lewy Bodies and Lewy Neurites has been attributed to failure of the UPS degradation system [36] but recent studies show α-synuclein could be degraded by the lysosomal pathway and especially

autophagy and chaperone-mediated autophagy (CMA) [13, 15, 37]. The co-expression of *Atg6* or *Pi3K59F* with *α-synuclein* does not alter longevity and climbing ability when compared to *α-synuclein*-expressing flies. The inhibition of autophagy leads to the accumulation of α-synuclein demonstrating an important role in normal α-synuclein turnover [38]. The post-translational modifications of α-synuclein interferes with its degradation by CMA, the degradation of other products by CMA [39] and inhibition of an early point in autophagosome formation [40]. The evidence obtained point to an already compromised autophagy system and as such the toxicity of α-synuclein and the *α-synuclein*-induced phenotypes are not worsened by reducing the activity of the autophagy system.

The *Bcl-2* family of proteins bind Atg6, a BH3-only protein, in a multimeric complex that is involved in vesicle nucleation stage of the autophagosome formation. The discovery of the BH3 domain, a binding site for interaction between the antiapoptotic Bcl-2 proteins and required for inhibition, in Atg6 showed that not only do Bcl-2 proteins regulate apoptosis, but function as anti-autophagic proteins [41]. Bcl-2 proteins seem to play a crucial role in maintaining autophagic homeostasis since phosphorylation of Atg6 that weakens this interaction promote autophagy [18]. Atg6 has a binding site for Pi3K59F and it is this interaction that leads to the production of phosphoinositol-3-phosphate that is pivotal to the nucleation process [19]. The overexpression of the Bcl-2 homologue *Buffy* with *Atg6-RNA*$_i$ resulted in the worsening of survival and climbing ability in *Atg6* flies but interestingly rescues the inhibition of *Pi3K59F*-induced phenotypes. Taken together, these results suggest enhanced *Atg6*-induced phenotypes and suppression of the *Pi3K59F*-induced phenotypes by *Buffy*. Since Bcl-2 proteins have been shown to block

Atg6-dependent autophagy by inhibiting the formation of the Atg6/Pi3K59F complex [42], we are inclined to theorize that Buffy plays a similar role in *Drosophila melanogaster*. Excessive levels of autophagy lead to cell death, and as such Buffy may act to balance the levels of autophagy via the Atg6 pathway to remove dysfunctional mitochondria, counteracting the PD-like symptoms in Drosophila. The accumulation of α-synuclein promotes excessive levels of autophagy leading to removal of mitochondria, and it is possible that once most neurons are depleted of mitochondria, synaptic function is interrupted.

Conclusions

The inhibition of either *Atg6* or *Pi3K59F* result in reduced longevity and impaired locomotor function, possibly a novel model of PD. It will be important to establish the function of these proteins other than in autophagy so as to further understand their function. Buffy, like most pro-survival Bcl-2 proteins, shows anti-autophagic tendencies by worsening the phenotypes of the inhibition of *Atg6* or *Pi3K59F*. It is yet unclear how Buffy effects this response, but it might interact with the putative BH3 domain of Atg6, disrupting the formation of the Atg6/Pi3K59F complex that is key to the initiation of autophagy. Further studies are required to elucidate the roles of Atg6, Pi3K59F, and Buffy in the autophagy–lysosome pathway.

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Chapter 5 - The *HtrA2* Drosophila model of Parkinson Disease is suppressed by the pro-survival Bcl-2 *Buffy*

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Abstract

Mutations in *High temperature requirement A2 (HtrA2),* also designated *PARK13,* which lead to the loss of its protease activity have been associated with Parkinson disease (PD). HtrA2 is a mitochondrial protease that translocates to the cytosol upon the initiation of apoptosis where it participates in the abrogation of inhibitors of apoptosis (IAP) inhibition of caspases. Here we demonstrate that the loss of the *HtrA2* function in the dopaminergic neurons of *Drosophila melanogaster* results in PD-like phenotypes and we attempt to restore the age-dependent loss in locomotor ability by co-expressing the sole pro-survival *Bcl-2* homologue *Buffy*. The inhibition of *HtrA2* in the dopaminergic neurons of Drosophila resulted in shortened lifespan and impaired climbing ability and the overexpression of *Buffy* rescued the reduction in lifespan and the age-dependent loss of locomotor ability. In supportive experiments, the inhibition of *HtrA2* in the Drosophila eye results in eye defects, marked by reduction in ommatidia number and increased disruption of the ommatidial array; phenotypes that are suppressed by the overexpression of *Buffy*.

Introduction

Loss of function mutations in *High temperature requirement A2 (HtrA2;* also known as *Omi),* a mitochondrial localized serine protease, are linked to Parkinson disease (PD) [1]. *HtrA2* encodes a nuclear protein with a mitochondrial targeting signal at the amino-terminus, that appears to be related to the Drosophila Reaper, Hid and Grim pro-apoptotic proteins due to the presence of the inhibitor of apoptosis (IAP) inhibitory Reaper-like motif [2]. The HtrA2 protein is released into the cytosol from the mitochondria during

apoptosis to bind IAPs and thus block the inhibition of caspases [3]. The binding of HtrA2 to IAPs activates the protease function.

A common facet of Parkinson disease (PD) is mitochondrial dysfunction and the loss of mitochondrial Complex I function in the midbrain of PD patients highlights the importance of the mitochondria in the pathology [4-6]. A role for *HtrA2* in neurodegeneration has been established through an association with the autosomal dominant early onset Alzheimer disease gene *presenilin* [7]. As well, the amyloid β protein interacts with the HtrA2 protein [8]. The motor neuron degeneration (*mnd2*) disorder in mice is caused by a loss-of-function mutation in the protease-domain encoding region of *HtrA2* [9]. Further studies in mice showed a link between altered expression of *HtrA2* and PD-like neurodegeneration [10]. This protein seems to play an important role in cellular protection in response to stress.

In *Drosophila melanogaster*, the HtrA2 serine protease has been shown to function in apoptosis through the cleavage of IAP1 in the vicinity of the mitochondria [11-13]. Modelling of PD in Drosophila is robust and has been insightful in understanding the role of several PD-linked genes in disease pathology [14-16]. The links between HtrA2 and other PD-linked genes has been demonstrated in both mammals and the fly, including the Pink1 kinase [17, 18] and the E3 ubiquitin ligase, parkin [18, 19]. The link between HtrA2 and the Pink1/Parkin pathway has been challenged by an *in vivo* study that relied on a loss of function [20]. Whether HtrA2 functions downstream of the Pink1/Parkin pathway or if it affects mitochondrial homeostasis, the loss of its protease activity is known to lead to neuronal degeneration.

We demonstrate that an age-dependent loss in locomotor function, accompanied by a reduction in lifespan, results when *HtrA2* is inhibited in the DA neurons of Drosophila, which corroborates previous studies [19]. In addition, the overexpression of the anti-apoptotic Bcl-2 homologue *Buffy* has been shown to restore healthspan in a disease model [21]. Here, we report inhibition of *HtrA2* along with overexpression of the sole pro-survival *Bcl-2* homologue *Buffy*.

Materials and methods

Drosophila media and culture

Stocks and crosses were maintained on standard cornmeal/molasses/yeast/agar media treated with propionic acid and methylparaben. Stocks were maintained on solid media and kept at room temperature (22°C ± 2°C) while crosses and experiments were carried out at 25°C and 29°C.

Drosophila stocks and derivative lines

UAS-Buffy [22] was provided by Dr. Leonie Quinn (University of Melbourne) and *Ddc-Gal4* [23] by Dr. J. Hirsch of University of Virginia. w^{1118}*; P{GD13932}cv24104* referred to as *HtrA2-RNAi* was obtained from Vienna Drosophila Resource Center, *GMR-Gal4* [24] and *UAS-lacZ* flies were acquired from the Bloomington Drosophila Stock Center at Indiana University. The *GMR-Gal4; UAS-Buffy/CyO; Ddc-Gal4* and *UAS-Buffy/CyO; GMR-Gal4* were generated using standard homologous recombination methods and were used for overexpression of *Buffy* in DA neurons using the *Ddc-Gal4* transgene or in the developing eye using the *GMR* response elements. PCR reactions and gel electrophoresis were used for analysis of recombination events.

Ageing assay

Several single-vial matings were carried out to produce a cohort of critical class male flies that were collected upon eclosion. At least two hundred flies were aged per genotype at a density of 20 or fewer flies per vial to avoid crowding on fresh media replenished every other day. Flies were observed and scored every two days for the presence of deceased adults. Flies were considered dead when they did not display movement upon agitation [25]. Longevity data was analysed using the GraphPad Prism version 5.04 and survival curves were compared using the log-rank (Mantel-Cox) test. Significance was determined at 95%, at a P-value less than or equal to 0.05 with Bonferroni correction.

Climbing assay

Cohort of critical class male flies was collected upon eclosion and scored for their ability to climb during their lifetime [26, 27]. 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 in ten repetitions. Analysis was performed using the GraphPad Prism version 5.04 and climbing curves were fitted using non-linear regression and compared using 95% confidence interval with a 0.05 P-value.

Scanning electron microscopy of the drosophila eye

Several single-vial matings were made at 29^{o} C, a cohort of adult male flies collected upon eclosion and aged for three days before being frozen at -80^{o} C. Whole flies were mounted on scanning electron microscope stubs, desiccated overnight and photographed with a FEI Mineral Liberation Analyzer 650F scanning electron microscope. For each cross at least 10 eye images were analysed using the National Institutes of Health (NIH) ImageJ software [28] and biometric analysis performed using GraphPad Prism version

5.04. The percent area of eye disruption was calculated as previously described [29]. The ratio of disrupted eye area was calculated by dividing the total area of the eye with the total disrupted area of the eye. The total disrupted area of the eye was the sum of any two or more ommatidia that were fused together. Statistical analyses were performed using one-way ANOVA and Dunnett's multiple comparison test at $p<0.05$.

Results

The HtrA2 protease and PDZ domains are highly conserved

The Drosophila and human protein sequences have a 52% identity and 70% similarity along their full length as determined by NCBI BLAST comparison, the two domains, Trypsin and PDZ are highly conserved as determined by an NCBI conserved domain search [30] (Figure 1). An ELM resource search for functional sites [31] indicates the presence of an inhibitor of apoptosis binding motif (IBM) that function in the abrogation of caspase inhibition by IAPs, three different di-Arginine ER retention motifs, an Atg8 binding motif and a possible NLS. TargetP [32] shows a pre-sequence cleavage site at the 17^{th} amino acid in Drosophila and the 45^{th} amino acid in the human version.

Inhibition of *HtrA2* results in an age-dependent loss in locomotor ability

The inhibition of *HtrA2* in the DA neurons results in shortened lifespan and impaired climbing ability with a median survival of 58 days compared to 75 days for the *lacZ* controls as determined by Log-rank (Mantel-Cox) test (Figure 2A). The directed inhibition of *HtrA2* in the DA neurons produces flies with significantly impaired climbing ability as determined by the nonlinear fitting of the climbing curves (Figure 2B). These results suggest an important role for HtrA2 in the normal functioning of DA neurons in Drosophila.

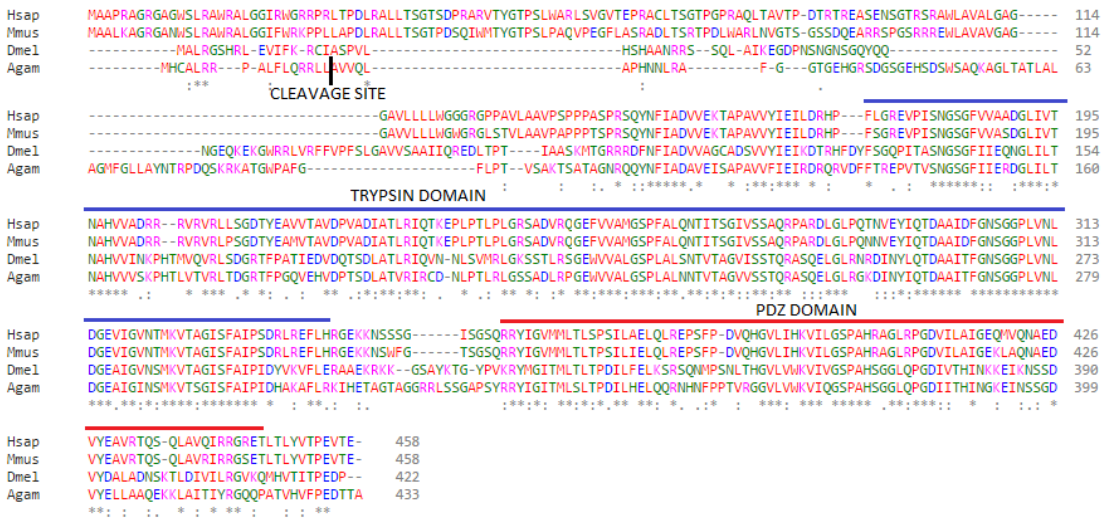
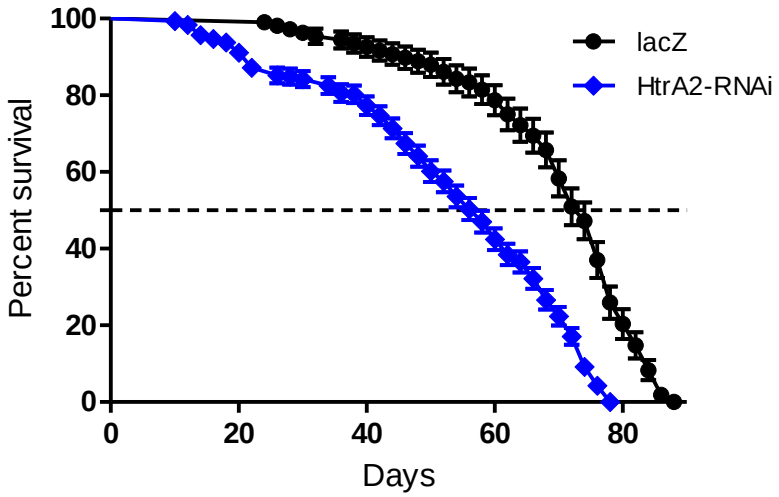


Figure 5.1 The protease and PDZ domains are conserved in human and Drosophila HtrA2

The *Drosophila melanogaster HtrA2 isoform B* gene encodes a protein comprised of 422 amino acids and contains a presequence cleavage site, trypsin and PDZ domains. Domains are highly conserved in the organisms compared as determined by the NCBI Conserved Domain Database Search (CDD) [30] and the Eukaryotic Linear Motif resource search [33]. A Clustal Omega multiple sequence alignment [34, 35] show conservation of the trypsin and PDZ domains (Hsap is *Homo sapiens* NP_037379.1, Mmus is *Mus musculus* NP_062726.3, Dmel is *Drosophila melanogaster* NP_001262565.1 and Agam is *Anopheles gambiae* XP_310886.5). "*" indicate the residues that are identical, ":" indicate the conserved substitutions, "." indicate the semi-conserved substitutions. Colours show the chemical nature of amino acids. Red is small hydrophobic (including aromatic), Blue is acidic, Magenta is basic, and Green is basic with hydroxyl or amine groups.

A.



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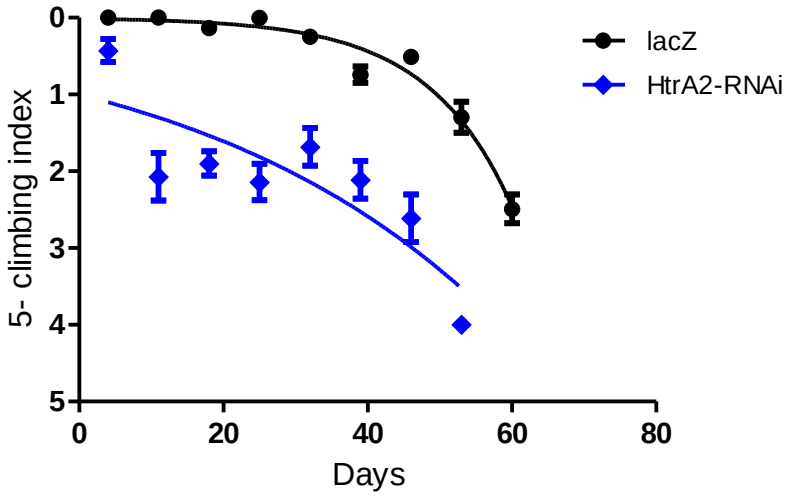


Figure 5.2 Loss of *HtrA2* activity in DA neurons shortens lifespan and retards climbing ability

A) The inhibition of *HtrA2* in DA neurons using the *Ddc-Gal4* transgene results in decreased median lifespan when compared to control flies expressing *UAS-lacZ*. The genotypes are *Ddc-Gal4/ UAS-lacZ* and *Ddc-Gal4/ UAS-HtrA2-RNAi.* Longevity is shown as percent survival ($P < 0.05$, determined by the log-rank (Mantel-Cox) test and $n \geq 200$). B) The inhibition of *HtrA2* in the DA neurons resulted in a significant decline in climbing ability as determined by nonlinear fitting of the climbing curves and comparing 95% CI. The genotypes are *Ddc-Gal4/ UAS-lacZ* and *Ddc-Gal4/ UAS-HtrA2-RNAi.* Error bars indicate SEM and $n=50$.

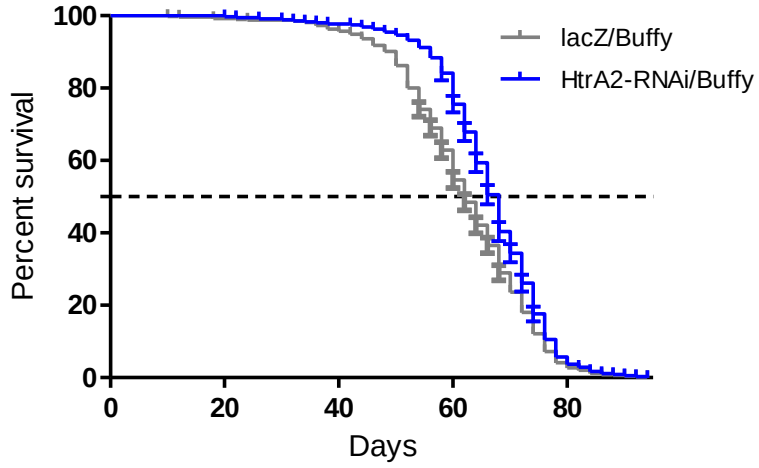
HtrA2 loss of function phenotypes are rescued by overexpression of pro-survival *Buffy*

The overexpression of the pro-survival *Bcl-2* homologue *Buffy* along with the suppression of *HtrA2* result in increased lifespan and improved climbing ability (Figure 3A and 3B). The co-expression of *Buffy* with *HtrA2-RNAi* resulted in increased median survival of 68 days when compared to *Buffy* control flies with median survival of 65 days as determined by Log-rank test (Figure 3A). The climbing ability of the *HtrA2-RNAi* flies was improved as determined by comparison of the climbing curves at 95% CI (Figure 3B). These results suggest a pro-survival role for *Buffy;* by increasing the general healthspan of *HtrA2-RNAi* flies.

Inhibition of *HtrA2* in the eye decreases ommatidia number and increases degeneration, phenotypes that are rescued upon *Buffy* overexpression

The inhibition of *HtrA2* in the eye under the control of the *GMR-Gal4* transgene, results in decreased ommatidia number and significant disruption of the ommatidial array (Figure 4A, II and 4B, I) as determined by an unpaired T-test $p<0.0001$. The overexpression of *Buffy* along with the inhibition of *HtrA2* restored the number of ommatidia and the percentage disruption to control levels as determined by an unpaired T-test, $p>0.50$ (Figure 4A, III and 4B, II). Taken together, these results suggest that loss of *HtrA2* activity is detrimental to normal development of the Drosophila eye and that *Buffy* suppresses the developmental eye defects that result from this inhibition.

A.



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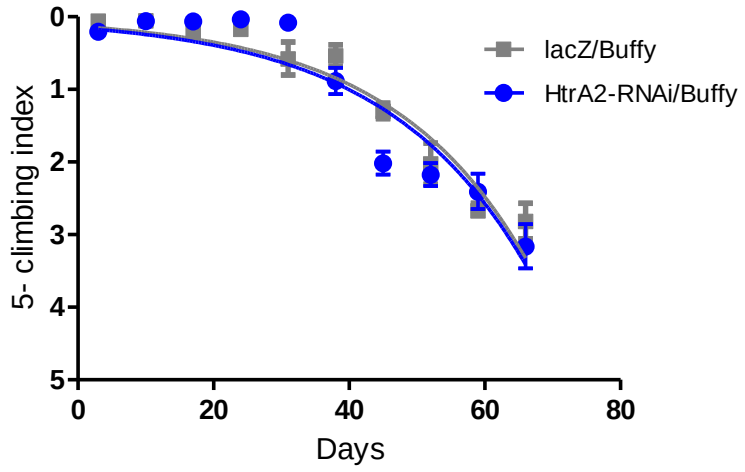
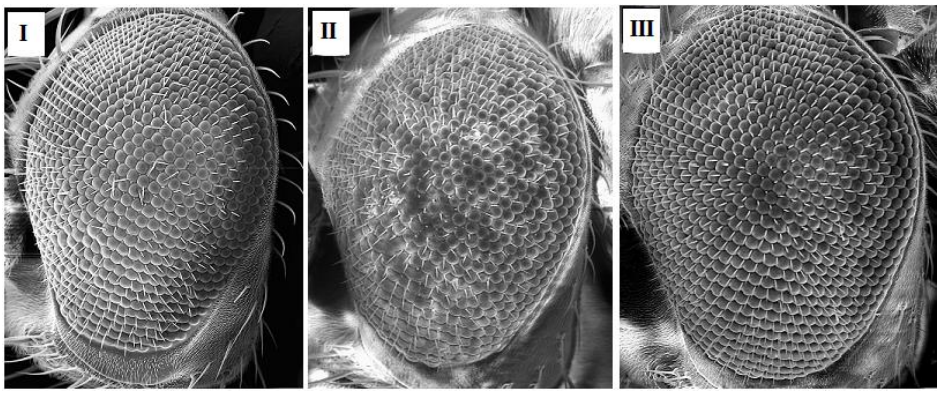


Figure 5.3 *HtrA2* loss of function phenotypes are suppressed by overexpression of *Buffy*

A) The co-expression of *Buffy* with *HtrA2-RNAi* result in the suppression of the observed phenotype of decreased survival when compared to the control. Genotypes are *Ddc-Gal4 UAS-Buffy/ UAS-lacZ* and *Ddc-Gal4 UAS-Buffy/ UAS-HtrA2-RNAi.* Longevity is shown as percent survival ($P < 0.05$, determined by log-rank (Mantel-Cox) test with $n \leq 200$). B) The inhibition of *HtrA2* along with the overexpression of *Buffy* in the DA neurons results in the suppression of the age-dependent loss in climbing ability. The genotypes are *Ddc-Gal4 UAS-Buffy/ UAS-lacZ* and *Ddc-Gal4 UAS-Buffy/ UAS-HtrA2-RNAi.* Analysis was done by nonlinear fitting of the climbing curves and significance was determined by comparing the 95% CI. Error bars indicate SEM and $n=50$.

A.



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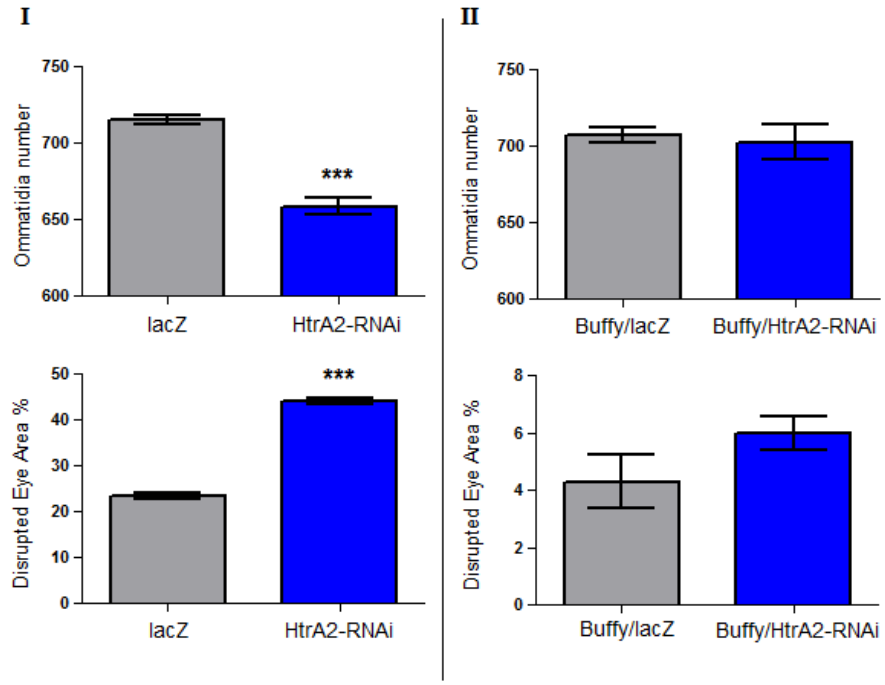


Figure 5.4 *HtrA2*-induced eye phenotypes are suppressed upon *Buffy* overexpression

A) Scanning electron micrographs when *HtrA2* is inhibited in the eye and co-expressed along with either *α-synuclein* or *Buffy*. The genotypes are (I) *GMR-Gal4/ UAS-lacZ;* (II) *GMR-Gal4/ UAS-HtrA2-RNAi;* and (III) *UAS-Buffy; GMR-Gal4/ UAS-HtrA2-RNAi*. B) Biometric analysis when I) *HtrA2* is inhibited in the eye indicated decreased ommatidia number and higher percentage of ommatidial disruption when compared to the control. II) The overexpression of *Buffy* results in restoration of the number of ommatidia and degree of ommatidial disruption to control levels. The genotypes are *UAS-Buffy; GMR-Gal4/ lacZ* and *UAS-Buffy; GMR-Gal4/ UAS-HtrA2-RNAi* Comparisons were determined by unpaired two-tailed T-test ($P<0.05$), error bars are SEM, asterisks (*) represent statistical significance and $n=10$.

Discussion

The loss of protease function [1] or mutations in the PDZ domain [36] of PARK13/ HtrA2 confers susceptibility to PD, and disruption of HtrA2 was found to cause neurodegeneration and PD-like phenotypes in mice [10]. In corroborative experiments [19], when we inhibited *HtrA2* in DA neurons of Drosophila using the *dopa decarboxylase* transgene, it resulted in a marked decline in survival and led to premature loss in locomotor function. HtrA2 is involved in cell death, the deletion of this gene in mice did not alter cell death rates, instead, suffered loss of a population of neurons in the striatum leading to neurodegeneration [10]. Studies suggest that the primary role of HtrA2 in neurons is protection against stress [19, 36]. The inhibition of *HtrA2* in DA neurons resulted in flies with poor healthspan, they had shortened lifespans and lost their climbing ability prematurely. HtrA2 seems to have a special neuroprotective function since loss in its protease or PDZ domain activity, or its deletion or inhibition seems to lead to neurodegeneration. Interestingly, the inhibition of *HtrA2* in the eye resulted in fewer number of ommatidia and worsening of the rough eye phenotype. It seems the neuroprotective functions of this protein are not limited to DA neurons specifically, but general neuroprotection.

The functional HtrA2 acts in the vicinity of the mitochondria in Drosophila [11], further showing the importance of the mitochondria in cell death and aetiology of PD. In mammals HtrA2 is involved in a *p53*-dependent cell death pathway [37], but its role in Drosophila is an elusive one. In our experiments, the overexpression of the sole

pro-survival *Bcl-2* homologue in Drosophila, *Buffy* [22], along with the inhibition of *HtrA2* counteracted the loss of *HtrA2*-induced phenotypes. These flies had an increase in lifespan that was augmented by improved locomotor ability suggesting a protective role for Buffy. The *HtrA2* null mutants in Drosophila do not exhibit mitochondrial morphological defects [20], though this does not exclude mitochondrial dysfunction. The study also did not find DA neuronal loss or muscle degeneration. Though the loss of DA neurons in Drosophila has remained a contentious issue as regards models of Parkinson disease [14, 16], there is consensus on PD-like phenotypes such as loss in locomotor ability. A lack of loss in DA neurons does not exclude degeneration and the progression of PD-like phenotypes in Drosophila. We have previously shown that the overexpression of Buffy suppresses the *α-synuclein*-induced PD-like phenotypes [21]. Therefore, the alleviation of *HtrA2*-induced phenotypes by overexpression of the pro-survival *Buffy* may be a general survival pathway signal or may be a specific *HtrA2*-dependent response. We obtained similar results when *Buffy* was overexpressed in the eye along with *HtrA2-RNAi*, with restored ommatidia number and decreased disruption of the ommatidial array. It seems that this serine protease functions in survival since its inhibition results in compromised healthspan, and the overexpression of the pro-survival *Buffy* rescues the phenotypes.

In conclusion, the overexpression of the pro-survival Bcl-2 homologue Buffy along with the inhibition of HtrA2 suppresses the HtrA2-induced phenotypes of shortened lifespan, locomotor dysfunction and small roughened eye.

Supplementary results

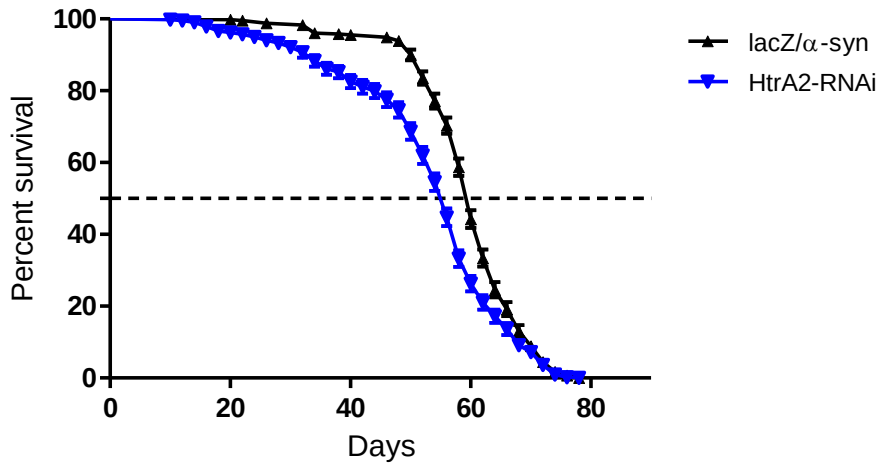
The co-expression of *HtrA2-RNAi* along with *α-synuclein* shortens lifespan and slightly enhances loss in locomotor ability

The suppression of HtrA2 along with the expression of α-synuclein in DA neurons results in shortened lifespan but does not worsen the compromised climbing ability observed (Figure 5). These flies had a median survival of 56 days when compared to the *α-synuclein* expression controls at 60 days as determined by log-rank test (Figure 5A). A non-linear fit of the climbing curves indicates no significance difference when the CI are compared at 95% (Figure 5B). These results show that the combined effects of the pro-apoptotic *HtrA2* and the expression of *α-synuclein* in DA neurons compromises lifespan.

The expression of *α-synuclein* along with the inhibition of *HtrA2* enhances the eye phenotypes

The expression of *α-synuclein* along with the inhibition of *HtrA2* in the developing eye results in further depression of the eye phenotypes, with a reduction in ommatidia number and an increase in ommatidial disarray as determined by an unpaired T-test (Figure 6A and 6B).

A.



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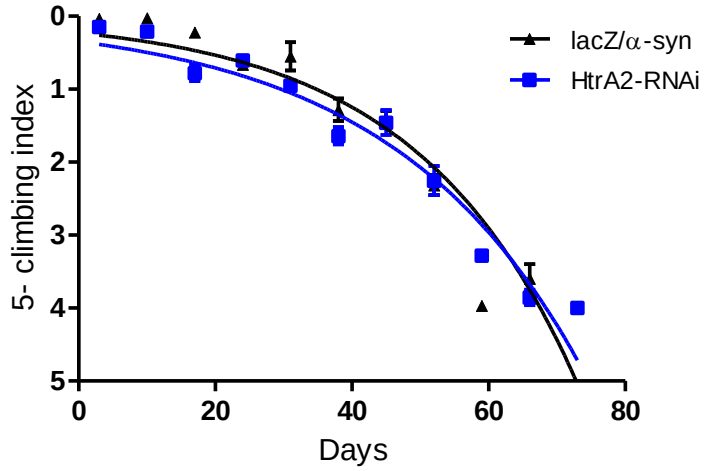
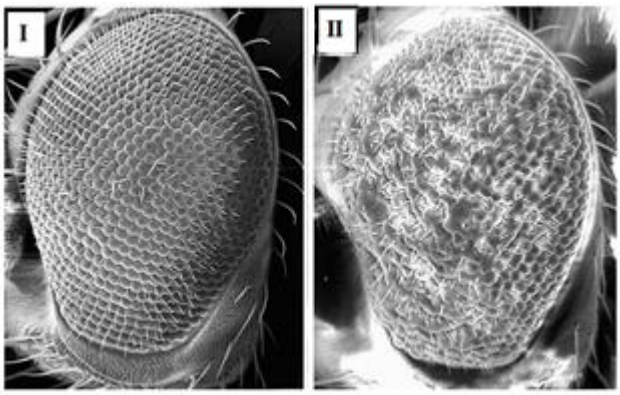


Figure 5-5 The co-expression of *HtrA2-RNAi* along with *α-synuclein* shortens lifespan but does not worsen locomotor ability

A) The directed inhibition of *HtrA2* along with *α-synuclein* expression in the DA neurons shortened lifespan when compared to the control. Genotypes are *UAS-α-synuclein; Ddc-Gal4/ UAS-lacZ* and *UAS-α-synuclein; Ddc-Gal4/ UAS-HtrA2-RNAi.* Longevity is shown as percent survival ($P < 0.05$, determined by log-rank (Mantel-Cox) test with $n \leq 200$). B) The co-expression of *HtrA2-RNAi* with *α-synuclein* resulted in a slight decrease in age-dependent loss in climbing ability compared to the control. The genotypes are *UAS-α-synuclein; Ddc-Gal4/ UAS-lacZ* and *UAS-α-synuclein; Ddc-Gal4/ UAS-HtrA2-RNAi.* Analysis was done by nonlinear fitting of the climbing curves and significance was determined by comparing the 95% CI. Error bars indicate SEM and $n=50$.

A.



B.

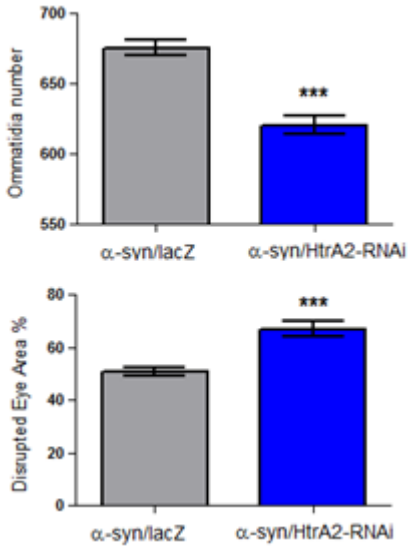


Figure 5-6 *HtrA2*-induced eye phenotypes are enhanced upon *α-synuclein* expression

A) Scanning electron micrographs when *HtrA2* is inhibited in the eye and co-expressed along with either *α-synuclein* or *Buffy*. The genotypes are (I) *GMR-Gal4/ UAS-lacZ;* and (II) *UAS-α-synuclein; GMR-Gal4/ UAS-HtrA2-RNAi*. B) Biometric analysis of the inhibition of *HtrA2* along with *α-synuclein* expression result in worsened eye phenotypes when compared to controls. The genotypes are *UAS-α-synuclein; GMR-Gal4/ UAS-lacZ* and *UAS-α-synuclein; GMR-Gal4/ UAS-HtrA2-RNAi*. Comparisons were determined by unpaired two-tailed T-test ($P<0.05$), error bars are SEM, asterisks (*) represent statistical significance and $n=10$.

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Chapter 6 - The anti-apoptotic *Bax-inhibitor-1* family in *Drosophila melanogaster*

Overview

The Bax inhibitor-1 family or the transmembrane Bax inhibitor-1 motif containing (TMBIM) family members are key regulators of cell death. It consists of at least six members that are found in a wide range of organisms, from bacteria, yeast, plants and animals. They contain at least six membrane-spanning domains and are found in a variety of cellular organelles such as the ER membrane localized Bax inhibitor-1 (Chapter 6A), the integral membrane Lifeguard (Chapter 6B), and the mitochondrial localized Growth hormone-inducible transmembrane protein (GHITM) (Chapter 6C). We sought to determine the effect of the inhibition of these three Drosophila homologues in the dopaminergic and other neurons, and investigated whether the pro-survival *Bcl-2* homologue *Buffy* could counteract the induced phenotypes. In the case of Lifeguard and GHITM, we sought to additionally, via bioinformatics, to characterize the Drosophila homologues. Our findings are discussed in the following three subsections of the chapter.

Bax-inhibitor-1 knockdown phenotypes are suppressed by *Buffy* and exacerbate degeneration in a *Drosophila* model of Parkinson disease

A version of this chapter has been accepted for publication in PeerJ (M'Angale and Staveley, 2016).

Abstract

Bax inhibitor-1 (BI-1) is an integral transmembrane protein that acts as a suppressor of *Bax*-induced apoptosis by regulation of endoplasmic reticulum (ER) stress-induced cell death. The gene is evolutionarily conserved, being found in diverse organisms that include yeast, Arabidopsis, Drosophila, mouse and humans. BI-1 is implicated in the regulation of calcium levels, reactive oxygen species, apoptosis, autophagy and ER stress signalling pathways. BI-1 is cytoprotective and its dysregulation result in disruption of cellular homeostasis and disease. We inhibited *BI-1* in the sensitive *dopa decarboxylase* (*Ddc*) expressing neurons of *Drosophila melanogaster* to investigate its neuroprotective functions. We used organismal assays to assess longevity of the flies to determine the effect of the altered expression of *BI-1* in the *Ddc-Gal4*-expressing neurons by employing two RNAi transgenic fly lines. We measured the locomotor ability of these RNAi lines by computing the climbing indices of the climbing ability and compared them to a control line that expresses the *lacZ* transgene. Finally, we performed biometric analysis of the developing eye, where we counted the number of ommatidia and calculated the area of ommatidial disruption. The inhibition of *BI-1* in these neurons was achieved under the direction of the *Ddc-Gal4* transgene and resulted in shortened lifespan and precocious loss of locomotor ability, phenotypes that appear to be strongly associated with the degenerative loss of dopaminergic neurons. BI-1 interacts with anti-apoptotic *Bcl-2* family members and, co-expression of *Buffy*, which encodes the sole anti-apoptotic Bcl-2 protein in Drosophila, with *BI-1-RNAi* results in suppression of the reduced lifespan and impaired climbing ability. Expression of human *α-synuclein* in Drosophila dopaminergic neurons results in neuronal degeneration, accompanied by the age-dependent loss in

climbing ability. We exploited this neurotoxic system to investigate possible BI-1 neuroprotective function. The co-expression of *α-synuclein* with *BI-1-RNAi* results in a slight decrease in lifespan coupled with an impairment in climbing ability. In supportive experiments, we employed the neuron-rich Drosophila compound eye to investigate subtle phenotypes that result from altered gene expression. The inhibition of *BI-1* in the Drosophila developing eye under the direction of the *GMR-Gal4* transgene results in reduced ommatidia number and increased disruption of the ommatidial array. Similarly, the co-expression of *BI-1-RNAi* with *Buffy* results in the suppression of the eye phenotypes. The expression of *α-synuclein* along with the inhibition of *BI-1* resulted in reduction of ommatidia number and a higher degree of disruption of the ommatidial array. Inhibition of *BI-1* in the dopaminergic neurons of Drosophila results in a shortened lifespan and premature loss in climbing ability, phenotypes that appear to be strongly associated with models of Parkinson disease in Drosophila, and which are suppressed upon overexpression of *Buffy* and worsened by co-expression with *α-synuclein.* This suggests that *BI-1* is neuroprotective and its inhibition can be counteracted by the overexpression of the pro-survival *Bcl-2* homologue.

Introduction

Bax inhibitor-1 (BI-1) belongs to a diverse group of proteins, known as Transmembrane Bax Inhibitor-1 Motif-containing (TMBIM) family [1-4], that have been determined to be regulators of cell death. A different nomenclature categorizes these proteins into the *LFG* family, adopted from the family member *Lifeguard* [5], which consists of at least six highly conserved members in a wide range of organisms [2, 6, 7]. These regulators of cell death, accomplish this role by the regulation of the death receptor, modulation of the

endoplasmic reticulum (ER) calcium homeostasis, ER stress signalling pathways, autophagy, reactive oxygen species (ROS) production, cytosolic acidification and, other cellular activities [3, 4]. The founding member of this group is *BI-1*, or *TMBIM6* also known as *testis enhanced gene transcript* (*TEGT*), and has been demonstrated to inhibit the effect of *Bax*-induced cell death [8, 9]. Members of this protein family possess a BI-1-like domain with six to seven transmembrane-spanning regions that are strongly associated with the ER membranes [9-11]. *BI-1* is highly conserved across diverse species with eukaryotic homologues of *BI-1* able to block *Bax*-induced cell death when expressed in yeast [7], thus implying it regulates an evolutionarily conserved cytoprotective pathway.

This protein though not structurally related to the *B c*ell *l*ymphoma *2* family of proteins, forms a complex with the pro-survival members Bcl-2 and Bcl-X_L but not with Bax or Bak [9, 12]. Therefore, it is likely the anti-apoptotic activity of *BI-1/TMBIM6* is mediated by interaction with pro-survival members of the *Bcl-2* family and acts downstream of Bcl-X_L [13]. *BI-1* deficient cells, that include neurons, are more sensitive to apoptosis induced by ER stress and has been linked to the modulation of ER calcium homeostasis [10, 14]. This implicates BI-1 in a variety of human diseases that include numerous cancers, obesity, liver diseases, autoimmune response, and diabetes [3, 4, 15-17]. Neuroprotective roles include, protection from oxygen-glucose deprivation, promotion of neuronal proliferation and differentiation, and stress-induced protection [14, 18-20]. It regulates ROS production by; modulation of unfolded protein response (UPR) induction in the ER [21], suppression of mitochondria-mediated ROS production [22], reduction of cytochrome P450 2E1 activity and regulation of the ER membrane lipid peroxidation

[23]. BI-1 undoubtedly has significant cytoprotective roles and their abrogation lead to cellular homeostatic dysfunction and disease.

Drosophila melanogaster appear to possess most of the TMBIM protein family homologues with TMBIM6/BI-1 represented by *BI-1/CG7188* [4, 5, 24]. Drosophila has been used as a model organism in the study of gene expression and in human disease models, albeit with very promising results [25]. Several studies have used Drosophila to elucidate the importance of this protein in cellular homeostasis; including functional conservation of this protein in evolutionarily diverse organisms [7], BI-1 as a negative regulator of the ER stress sensor IRE1α and its role in the UPR [12], and its modulation of autophagy [26]. Expression in the *Ddc-Gal4*-expressing neurons is the focus of our studies as they are very sensitive to subtle differences in gene products and can be used to study ROS, ER stress, apoptosis, autophagy and many other cellular processes. This is mainly because they degenerate in an age-dependent manner and this degeneration manifests as deficiency in locomotor function [25, 27-29]. The key elements of the Drosophila model of Parkinson disease that utilizes the expression of a human *α-synuclein* transgene to induce the PD-like symptoms [27]; is its ability to recapitulate some features of human PD that include, age-dependent loss of DA neurons that manifest in age-dependent loss in locomotor function [25, 27, 29-34]. The spatio-temporal *UAS/GAL4* expression system [35], and the availability of a plethora of promoters or enhancers of which *TH-Gal4*, *elav-Gal4* and *Ddc-Gal4* are employed to model PD in flies [25, 27, 29-34].

The *Bcl-2* family member homologues in Drosophila are limited to the single anti-apoptotic *Buffy* and the pro-apoptotic *Debcl* [36]. In previous studies, the overexpression

of *Buffy* has been shown to confer survival advantages specifically in response to external stimuli and in conditions of cellular stress [37-40]. This point to an important role for this protein in aspects of cell death. We investigated the outcome of the inhibition of *BI-1* in Drosophila neurons, and further determined whether there is an interaction with the anti-apoptotic Bcl-2 protein Buffy. We employed two different RNAi lines to determine the specificity of the effects of inhibition of this gene and compared them to a control line. We further co-expressed *BI-1* in DA neurons along with *α-synuclein* to investigate whether it possesses neuroprotective functions. Lastly, in supportive experiments we attempted to establish a role for BI-1 in the Drosophila developing eye.

Materials and Methods

Bioinformatic analysis

The protein sequences were obtained from the National Center for Biotechnology Information (NCBI; http://www.ncbi.nlm.nih.gov/protein/) and the domains were identified using the NCBI Conserved Domain Database (CDD; http://www.ncbi.nlm.nih.gov/cdd) [41] and the Eukaryotic Linear Motif (ELM; http://elm.eu.org/) [42] which focuses on annotation and detection of eukaryotic linear motifs (ELMs), also known as short linear motifs (SLiMs). A multiple sequence alignment was done using Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/) [43, 44] to show conservation of the domains in the selected organisms. The prediction of the nuclear export signal (NES) was by NetNES (http://www.cbs.dtu.dk/services/NetNES/) [45]. Further analysis of protein sequences was performed with Phyre2 [46], a web portal for protein modelling, prediction and analysis (http://www.sbg.bio.ic.ac.uk/phyre2/html/page.cgi?id=index). The sub-cellular

localisation was performed by MultiLoc2 [47] (https://abi.inf.uni-tuebingen.de/Services/MultiLoc2). Transmembrane domains were further investigated and identified using TMpred [48], a program based on statistical analysis of TMbase (http://www.ch.embnet.org/software/TMPRED_form.html).

Drosophila media, stocks and derivative lines

Stocks and crosses were maintained on standard cornmeal/molasses/yeast/agar media treated with propionic acid and methylparaben to inhibit fungal growth. Stocks were kept at room temperature while crosses and experiments for analysis of ageing and climbing ability were carried out at 25° C while those for the eye analysis were performed at 29° C. The *P{KK100983VIE-260B* stock hereby referred to as *UAS-BI-1-RNAi (1)* (http://stockcenter.vdrc.at/control/product/~VIEW_INDEX=0/~VIEW_SIZE=100/~product_id=110358) and w^{1118}*; P{GD1660}v37108* hereby referred to as *UAS-BI-1-RNAi (2)* (http://stockcenter.vdrc.at/control/product/~VIEW_INDEX=0/~VIEW_SIZE=100/~product_id=37108) were obtained from Vienna Drosophila Resource Center. Additional information on the RNAi constructs http://www.flyrnai.org/up-torr/GetSummaryByGene?organism=Fly. The *UAS-Buffy* [36] was provided by Dr. L. Quinn (University of Melbourne), *Ddc-Gal4* flies [49] by Dr. J. Hirsch (University of Virginia) and *UAS-α-synuclein* [27] by Dr. M. Feany (Harvard Medical School). *GMR-Gal4* [50] and *UAS-lacZ* flies were obtained from the Bloomington Drosophila Stock Center.

The *UAS-α-synuclein/CyO; Ddc-Gal4/TM3; UAS-α-synuclein/CyO; GMR-Gal4; UAS-Buffy/CyO; Ddc-Gal4* and *UAS-Buffy/CyO; GMR-Gal4* complex lines were used to overexpress *α-synuclein* or *Buffy* in neurons and the developing eye and were produced

employing standard homologous recombination and marker selection methods as previously described [51, 52]. Gel electrophoresis was used to detect the presence of PCR products.

Ageing assay

Several single vial crosses of each genotype were performed and a cohort of male flies collected upon eclosion and assessed using a protocol previously described [40, 53]. For each genotype at least 200 flies were aged and scored every 2 days for the presence of deceased adults [54]. Survival data was analysed using GraphPad Prism version 5.04, and curves were compared using the Log-rank (Mantel-Cox) test with statistical significance determined at 95%, at a $P \leq 0.05$ with application of a Bonferroni correction to the familywise P value.

Climbing assay

A cohort of the critical class male flies was collected upon eclosion and scored for their ability to climb using a method that was previously described [55]. Climbing analysis was performed using the GraphPad Prism version 5.04 and climbing curves were fitted using non-linear regression and compared using 95% confidence interval with a P-value of 0.05 or less being statistically significant.

Scanning electron microscopy of the Drosophila eye

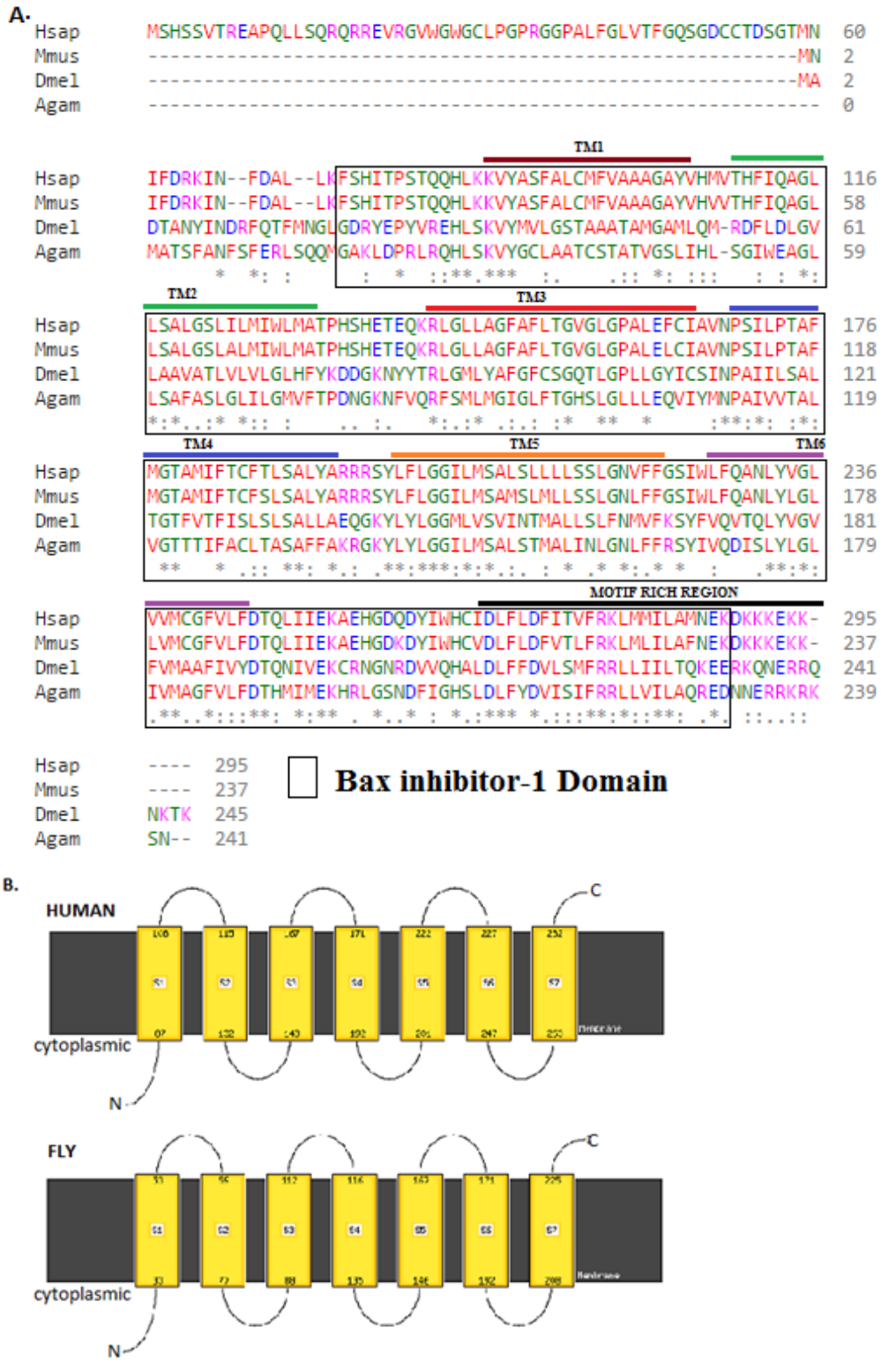
Male flies were collected upon eclosion and aged for up to five days and then prepared for scanning electron microscopy using a standard protocol as previously described [40]. For each cross at least 10 different eye images were analysed using the National Institutes of Health (NIH) ImageJ software [56] and biometric analysis performed using GraphPad Prism version 5.04. Disruption area of the eye was calculated as has been previously

described [57]. Statistical comparisons comprised one-way analyses of variance (ANOVA) and Dunnett's multiple comparison tests. P-values less than 0.05 were considered significant.

Results

Drosophila *BI-1* is closely related to the human homologue

The 245 amino acids Drosophila BI-1 isoform A has a 42% identity and 68% similarity to the 295 amino acids human isoform B. The Drosophila homologue has a BI-1 domain between amino acids 21 – 223 and the human version at 74 – 286 (Figure 1) as determined by the NCBI Conserved Domain Database [41]. An alignment of the protein sequences using Clustal Omega [43, 44] shows high conservation of the BI-1-like domain in the organisms analysed (Figure 1A). Six transmembrane (TM) domains in both Drosophila and human BI-1, that are numbered TM1 to TM6 (Figure 1A) were identified using both Eukaryotic linear motif (ELM) [58] and TMpred [48]. An analysis of membrane-spanning domains by Phyre2 [46] reveals seven TM domains (Figure 1B) in both sequences that are highly identical in the cytoplasmic to intracellular orientation. An inhibitor of apoptosis binding motif (IBM) at amino acids 1-5, an endoplasmic reticulum (ER) retention motif at position 221-224, and binding motifs for Atg8 at position 212-224 and calmodulin at amino acids 226-242 were identified by ELM. The presence of nuclear export signal (NES) was detected in both Drosophila and human BI-1 using NetNES [45] and only in Drosophila using the ELM. The 3D modelling of these proteins using Phyre2 (Figure 1C) shows a close similarity in the structure and the orientation of the transmembrane domains with the image coloured by rainbow from the N → C terminus.


A.
Hsap MSHSSVTREAPQLLSQRQRREVRGVWGWGCLPGPRGGPALFGLVTFGQSGDCCTDSGTMN 60
Mmus ----------MN 2
Dmel ----------MA 2
Agam ---------- 0
TM1
Hsap IFDRKIN--FDAL--LKFSHITPSTQQHLKKVYASFALCMFVAAAGAYVHMVTHFIQAGL 116
Mmus IFDRKIN--FDAL--LKFSHITPSTQQHLKKVYASFALCMFVAAAGAYVHVVTHFIQAGL 58
Dmel DTANYINDRFQTFMNGLGDRYEPYVREHLSKVYMVLGSTAAATAMGAMLQM-RDFLDLGV 61
Agam MATSFANFSFERLSQQMGAKLDPRLRQHLSKVYGCLAATCSTATVGSLIHL-SGIWEAGL 59
TM2
TM3
Hsap LSALGSLILMIWLMATPHSHETEQKRLGLLAGFAFLTGVGLGPALEFCIAVNPSILPTAF 176
Mmus LSALGSLALMIWLMATPHSHETEQKRLGLLAGFAFLTGVGLGPALELCIAVNPSILPTAF 118
Dmel LAAVATLVLVLGLHFYKDDGKNYYTRLGMLYAFGFCSGQTLGPLLGYICSINPAIILSAL 121
Agam LSAFASLGLILGMVFTPDNGKNFVQRFSMLMGIGLFTGHSLGLLLEQVIYMNPAIVVTAL 119
TM4
TM5
TM6
Hsap MGTAMIFTCFTLSALYARRRSYLFLGGILMSALSLLLLSSLGNVFFGSIWLFQANLYVGL 236
Mmus MGTAMIFTCFSLSALYARRRSYLFLGGILMSAMSLMLLSSLGNLFFGSIWLFQANLYLGL 178
Dmel TGTFVTFISLSLSALLAEQGKYLYLGGMLVSVINTMALLSLFNMVFKSYFVQVTQLYVGV 181
Agam VGTTTIFACLTASAFFAKRGKYLYLGGILMSALSTMALINLGNLFFRSYIVQDISLYLGL 179
MOTIF RICH REGION
Hsap VVMCGFVLFDTQLIIEKAEHGDQDYIWHCIDLFLDFITVFRKLMMILAMNEKDKKKEKK- 295
Mmus LVMCGFVLFDTQLIIEKAEHGDKDYIWHCVDLFLDFVTLFRKLMLILAFNEKDKKKEKK- 237
Dmel FVMAAFIVYDTQNIVEKCRNGNRDVVQHALDLFFDVLSMFRRLLIILTQKEERKQNERRQ 241
Agam IVMAGFVLFDTHMIMEKHRLGSNDFIGHSLDLFYDVISIFRRLLVILAQREDNNERRKRK 239
Hsap ---- 295
Mmus ---- 237
Dmel NKTK 245
Agam SN-- 241
Bax inhibitor-1 Domain
B.
HUMAN
C
cytoplasmic
N
FLY
C
cytoplasmic
N

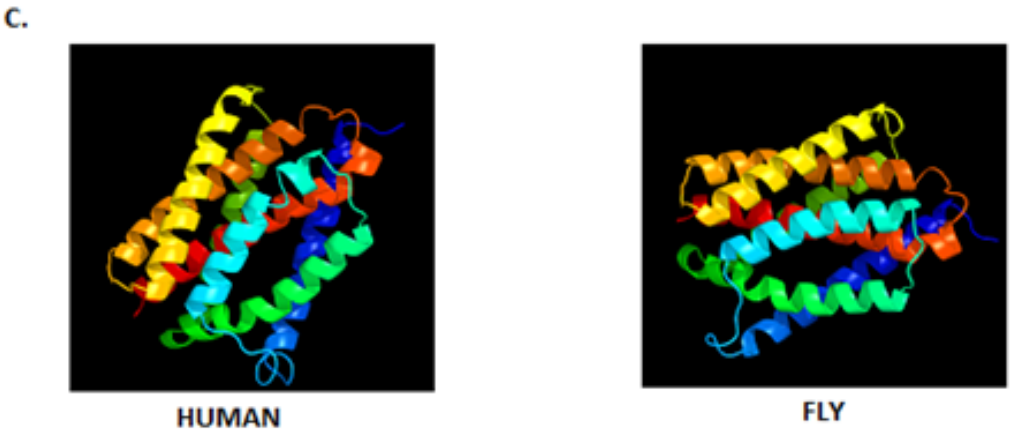


Figure 6.1 Drosophila BI-1 has six TM domains that are evolutionarily conserved

A) The Drosophila, human, mouse and mosquito homologues contain a BI-1 domain with the Drosophila version situated between amino acids 21 – 223 and the human version at 74 – 286 as determined by the NCBI Conserved Domain Database [41]. They have six transmembrane-spanning regions as predicted by the Eukaryotic Linear Motif (ELM) [58] and TMpred [48]. It shows presence of a motif rich region, that contains a NES, ER retention motif, Atg8 and calmodulin binding motifs as identified using ELM. Sequence alignment was performed by Clustal Omega [43, 44] and showed high conservation of the Bax inhibitor-1 domain (Hsap is *Homo sapiens* NP_001092046.1, Mmus is *Mus musculus* NP_001164506.1, Dmel is *Drosophila melanogaster* NP_648205.1 and Agam is *Anopheles gambiae* XP_315790.3). "*" indicate the residues that are identical, ":" indicate the conserved substitutions, "." indicate the semi-conserved substitutions. Colours show the chemical nature of amino acids. Red is small hydrophobic (including aromatic), Blue is acidic, Magenta is basic, and Green is basic with hydroxyl or amine groups. B) Additional protein analysis performed using Phyre2 [46] revealed the presence of seven transmembrane domains in both the Drosophila and human sequences (Image cartoons are obtained from Phyre2). C) The 3D modelling of the Drosophila and human proteins using Phyre2 shows a close similarity in the structure and the orientation of the transmembrane domains with the image coloured by rainbow from the N → C terminus (Image cartoons are obtained from Phyre2).

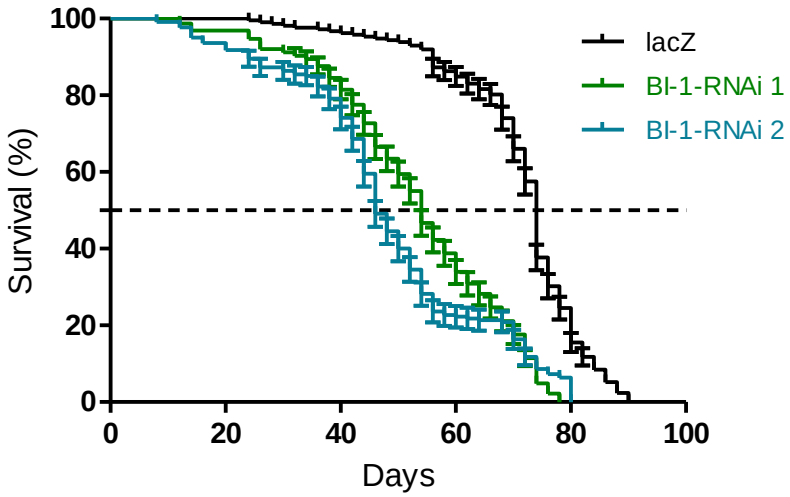
Inhibition of *BI-1* in DA neurons decreases lifespan and severely impairs locomotor function

The expression of both *BI-1-RNAi* lines in the *Ddc-Gal4*-expressing neurons results in decreased lifespan and impaired locomotor function. The median lifespan for these flies was 54 days for *BI-1-RNAi (1)* and 46 days for *BI-1-RNAi (2)* when compared to 70 days for the controls that express the *lacZ* transgene as determined by the Log-rank (Mantel-Cox) test (Figure 2A). When *BI-1* is suppressed in these neurons, the flies develop an early onset impairment of locomotor ability as determined by the nonlinear fitting of the climbing curves (Figure 2B). The 95% CI for the slope were 0.033 to 0.050 and 0.0175 to 0.0355 for the two RNAi lines respectively when compared to 0.070 to 0.0975 for the *lacZ* control flies. These results appear to suggest a role for *BI-1* in the protection of neurons in Drosophila.

***Buffy* suppresses the loss of *BI-1*-induced phenotypes**

The directed overexpression of the pro-survival Bcl-2 homologue *Buffy* results in increased lifespan and improved climbing ability. When *Buffy* is co-expressed with both *BI-1-RNAi* lines in the *Ddc-Gal4*-expressing neurons, the results indicate an increased median lifespan of 70 days and 72 days respectively when compared to 72 days for *Buffy* co-expressed with *lacZ* control flies, as determined by Log-rank test (Figure 3A). The climbing ability of the *BI-1-RNAi* flies was not significantly different from the *Buffy* co-expressed with *lacZ* controls as determined by comparison of the *BI-1-RNAi* climbing curves (Figure 3B) with the control curve. The 95% CI for the slope of *BI-1-RNAi (1)* was 0.0340 to 0.057 and that of *BI-1-RNAi (2)* was 0.040 to 0.061 when compared to 0.035 to 0.050 for the controls. Taken together these results suggest a pro-survival role for *BI-1;* as

A.



B.

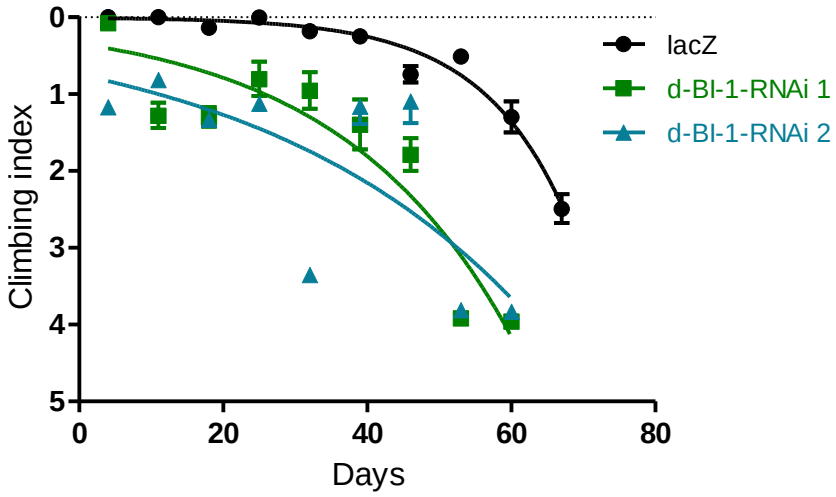
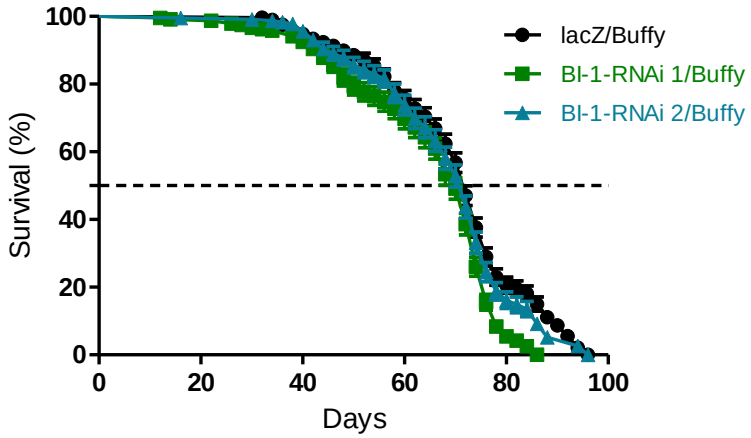


Figure 6.2 Loss of *BI-1* activity decreases survival and impairs climbing ability

A) The inhibition of *BI-1* in the *Ddc-Gal4*-expressing neurons results in reduced lifespan when compared to control flies expressing *UAS-lacZ*. The genotypes are *Ddc-Gal4/ UAS-lacZ, Ddc-Gal4/ UAS-BI-1-RNAi (1)* and *Ddc-Gal4/ UAS-BI-1-RNAi (2).* Longevity is shown as percent survival ($P < 0.05$, determined by the log-rank (Mantel-Cox) test and $N \geq 200$). B) The inhibition of *BI-1* in these neurons resulted in a significant decrease in climbing ability as determined by nonlinear fitting of the climbing curves and comparing the 95% CI. The genotypes are *Ddc-Gal4/ UAS-lacZ, Ddc-Gal4/ UAS-BI-1-RNAi (1)* and *Ddc-Gal4/ UAS-BI-1-RNAi (2).* Error bars indicate standard error of the mean and $N=50$.

A.



B.

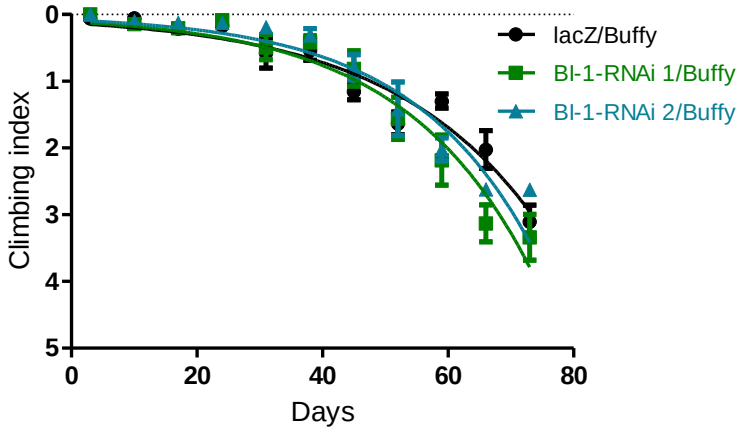


Figure 6.3 The *BI-1*-induced phenotypes can be suppressed by the overexpression of *Buffy*

A) The co-expression of *Buffy* with *BI-1-RNAi* in the Ddc-Gal4-expressing neurons result in the rescue of the observed phenotype of decreased survival when compared to the control. Genotypes are *UAS-Buffy; Ddc-Gal4/UAS-lacZ, UAS-Buffy; Ddc-Gal4/ UAS-BI-1-RNAi (1),* and *UAS-Buffy; Ddc-Gal4/ UAS-BI-1-RNAi (2).* Longevity is shown as percent survival ($P < 0.05$, determined by log-rank (Mantel-Cox) test with $N \leq 200$). B) The inhibition of *BI-1* along with the overexpression of *Buffy* in these neurons results in the suppression of the age-dependent loss in climbing ability. The genotypes are *UAS-Buffy; Ddc-Gal4/UAS-lacZ, UAS-Buffy; Ddc-Gal4/ UAS-BI-1-RNAi (1),* and *UAS-Buffy; Ddc-Gal4/ UAS-BI-1-RNAi (2).* Analysis was done by nonlinear fitting of the climbing curves and significance was determined by comparing the 95% CI. Error bars indicate standard error of the mean and $N=50$.

the phenotypes induced by its inhibition are significantly counteracted by the pro-survival *Bcl-2* homologue *Buffy*.

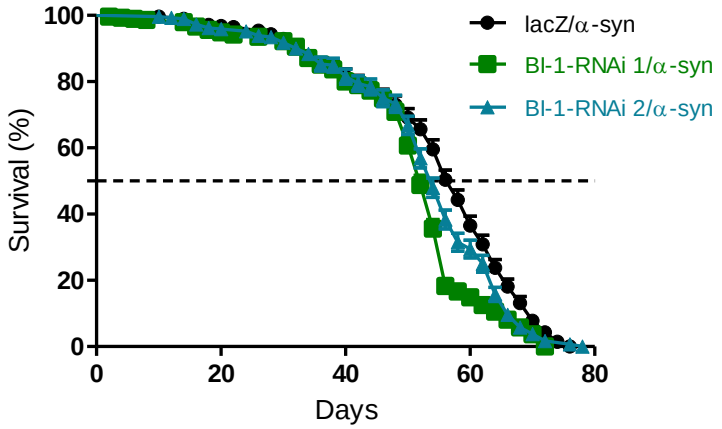
Inhibition of *BI-1* with the expression of *α-synuclein* slightly alters phenotypes

The expression of *α-synuclein* in dopaminergic neurons results in impaired locomotor function that is attributed to cellular toxicity. The co-expression of *BI-1-RNAi* along with *α-synuclein* in the *Ddc-Gal4*-expressing neurons, slightly exacerbated the reduced survival and the loss in climbing ability observed with the expression of *α-synuclein*. The median lifespan was 52 days and 54 days for flies that express *BI-1-RNAi* along with *α-synuclein* compared to 58 days for controls that co-express *α-synuclein* along with the *lacZ* transgene (Figure 4A) as determined by Log rank test with $p<0.001$. A comparison of the climbing curves by nonlinear fitting at 95% CI revealed they were significantly different (Figure 4B), with a CI of 0.038 to 0.049 for *BI-1-RNAi (1)* and 0.025 to 0.033 for *BI-1-RNAi (2)* co-expressed along with *α-synuclein* and compared to 0.052 to 0.069 for the *α-synuclein* co-expressed with *lacZ* control flies. This implies that the inhibition of *BI-1* in the *Ddc-Gal4*-expressing neurons abrogates its cytoprotective function and enhances the *α-synuclein*-induced phenotypes.

Inhibition of *BI-1* in the eye decreases ommatidia number and increases degeneration, phenotypes that are rescued upon *Buffy* overexpression

The directed inhibition of *BI-1* in the Drosophila developing eye using the *GMR-Gal4* transgene resulted in eyes with decreased number of ommatidia and a higher disruption of the ommatidial array in both the RNAi lines that were tested (Figure 4A – C and 4J) as determined by a one-way analysis of variance with a p value less than 0.0001. Co-expression of both *BI-1-RNAi* lines with *Buffy* restored the mean number of ommatidia

A.



B.

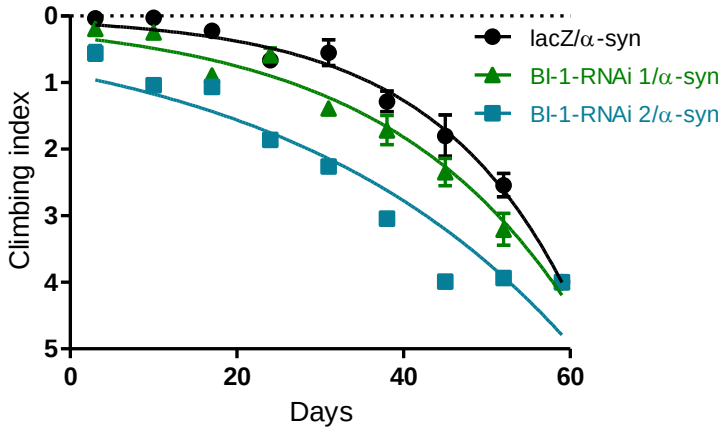
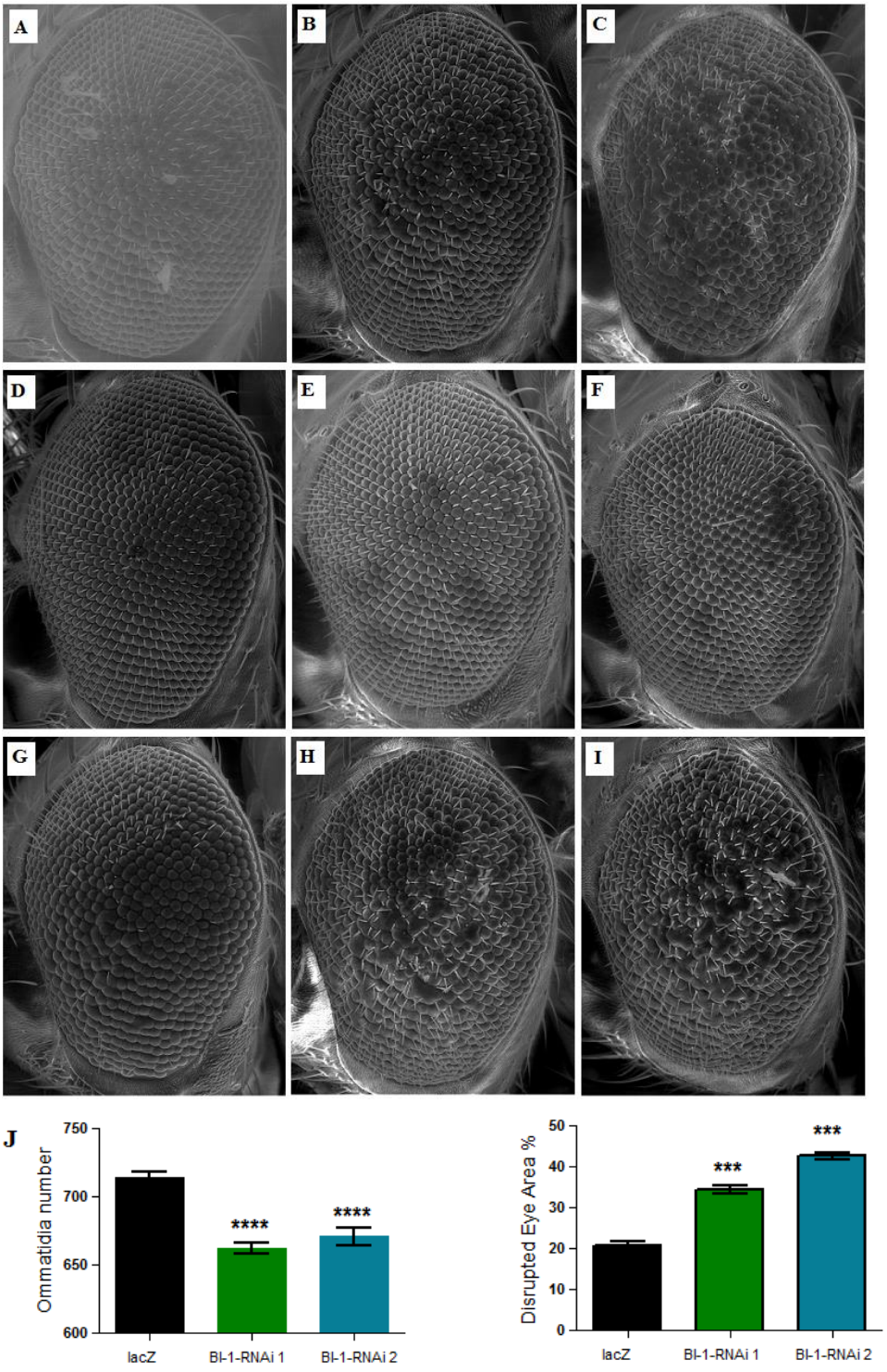


Figure 6.4 Loss of *BI-1* in neurons enhances the *α-synuclein*-induced phenotypes

A) The directed inhibition of *BI-1* along with *α-synuclein* expression in the Ddc-Gal4-expressing neurons resulted in a shortened lifespan when compared to the control. Genotypes are *UAS-α-synuclein; Ddc-Gal4/ UAS-lacZ, UAS-α-synuclein; Ddc-Gal4/ UAS-BI-1-RNAi (1)* and *UAS-α-synuclein; Ddc-Gal4/ UAS-BI-1-RNAi (2).* Longevity is shown as percent survival ($P < 0.05$, determined by log-rank (Mantel-Cox) test with $N \leq 200$). B) The co-expression of *BI-1-RNAi* with *α-synuclein* resulted in a slight but significant decrease in the age-dependent loss in climbing ability when compared to the control. The genotypes are *UAS-α-synuclein; Ddc-Gal4/ UAS-lacZ, UAS-α-synuclein; Ddc-Gal4/ UAS-BI-1-RNAi (1)* and *UAS-α-synuclein; Ddc-Gal4/ UAS-BI-1-RNAi (2).* Analysis was done by nonlinear fitting of the climbing curves and significance was determined by comparing the 95% CI. Error bars indicate standard error of the mean and $N=50$.


A
B
C
D
E
F
G
H
I
J
Ommatidia number
750
700
650
600
****
****
lacZ
Bl-1-RNAi 1
Bl-1-RNAi 2
Disrupted Eye Area %
50
40
30
20
10
0
***
***
lacZ
Bl-1-RNAi 1
Bl-1-RNAi 2

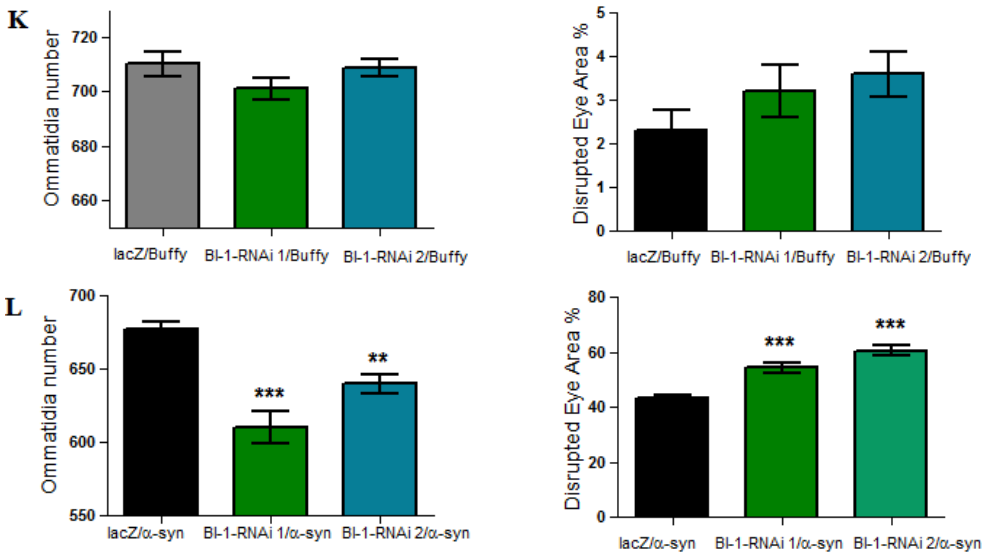


Figure 6.5 Inhibition of *BI-1* in the developing eye results in decreased ommatidia and increased degeneration of the ommatidial array

Scanning electron micrographs when *BI-1* is inhibited in the Drosophila developing eye; (A) *GMR-GAL4/ UAS-lacZ,* (B) *GMR-GAL4/ UAS-BI-1-RNAi (1)* and (C) *GMR-GAL4/ UAS-BI-1-RNAi (2)*, when inhibited along with overexpression of *Buffy*; D) *UAS-Buffy; GMR-Gal4/ UAS-lacZ,* E) *UAS-Buffy; GMR-Gal4/ UAS-BI-1-RNAi (1)* and E) *UAS-Buffy; GMR-Gal4/ UAS-BI-1-RNAi (2)* and when co-expressed with *α-synuclein*; G) *UAS-α-synuclein; GMR-Gal4/ UAS-lacZ,* H) *UAS-α-synuclein; GMR-Gal4/ UAS-BI-1-RNAi,* and I) *UAS-α-synuclein; GMR-Gal4/ UAS-BI-1-RNAi.* J) Biometric analysis when *BI-1* is inhibited in the eye indicated decreased ommatidia number and higher percentage of ommatidial disruption when compared to the control. K) The co-expression of *Buffy* with both *BI-1-RNAi* lines resulted in the suppression of the eye phenotypes, the ommatidia number and disruption of the eye were restored to control levels. L) The inhibition of *BI-1* along with *α-synuclein* expression resulted in worsened eye phenotypes, the number of ommatidia was lower and the degree of ommatidial disruption was higher than either the inhibition of both *BI-1* lines or that of *α-synuclein* when compared to controls. Comparisons were determined by one-way analysis of variance (ANOVA) with a Dunnett's multiple comparison post-test ($P<0.05$), error bars are standard error of the mean, $N=10$ and asterisks represent statistical significance (* $p<0.05$, ** $p<0.01$ and *** $p<0.001$).

and the percentage disruption to control levels as determined by a one-way analysis of variance with p = 0.2439 and 0.2342 (Figure 4D – F and 4K). Taken together, these results suggest that BI-1 may play a pro-survival role in the development of the Drosophila eye and that *Buffy* suppresses the developmental eye defects that result from the inhibition of *BI-1*. The inhibition of *BI-1* along with *α-synuclein* expression resulted in a significant decrease in the number of ommatidia or increase in percentage disruption of the eye as determined by a one-way analysis of variance with a p value less than 0.0001 in both instances (Figure 4G – I and 4L). The number of ommatidia and percentage of disruption was worse than with either *α-synuclein* expression or *BI-1* inhibition. This indicates that the inhibition of *BI-1* enhances the *α-synuclein*-induced eye defects.

Discussion

The inhibition of *BI-1* via stable inducible RNAi in the *Ddc-Gal4*-expressing neurons of Drosophila results in decreased survival and impaired climbing ability over time. Although there is no known homologue of *Bax* in Drosophila, the only pro-apoptotic *Bcl-2* homologue is *Debcl* [59-62], and has been demonstrated to possess pro-apoptotic functions. The Drosophila BI-1 is able to block *Bax*-induced cell death in yeast [7], and loss of *BI-1* function induces cell death [9]. These results suggest neuronal dysfunction may result from degeneration or death when the function of *BI-1* is reduced in the *Ddc-Gal4*-expressing neurons. The *BI-1*-induced cell death could occur through interaction with pro-survival Bcl-2 proteins at the ER membrane [9] and especially Bcl-2 and Bcl-X_L in humans and possibly Buffy in Drosophila. BI-1 seems to be involved in cellular functions that are protective to ER stress-induced apoptosis [10]. It seems to do this by

the regulation of calcium ions [13, 17] and ROS [23, 63]. BI-1 regulates ER stress by controlling ER-generated ROS accumulation and stress linked to the unfolded protein response. Therefore, the inhibition of this important ER stress regulator in the DA neurons would result in neuronal degeneration and death. The only pro-survival *Bcl-2* homologue in Drosophila is *Buffy* [36] and the overexpression of *Buffy* is known to confer survival advantages to cells under normal conditions and under conditions of stress [36, 37, 39, 40, 52, 64, 65]. The overexpression of *Buffy* along with the inhibition of *BI-1* resulted in the suppression of the *BI-1*-induced phenotypes. This Buffy action may be specific to its interaction with BI-1 or to its general pro-survival signalling pathways. The rescue of the *BI-1*-induced phenotypes in both the *Ddc-Gal4*-expressing neurons and in the developing eye may indicate a pro-survival role for *BI-1* in Drosophila, as the pro-survival action of *Buffy* can abrogate its phenotypes.

The expression of human *α-synuclein* in DA neurons of Drosophila results in impaired climbing ability [27], similar to what is observed in *BI-1* loss of function. The expression of *α-synuclein* along with the loss of *BI-1* activity significantly altered the impaired locomotor ability observed. The age-dependent loss of climbing ability could be a result of *BI-1*-induced apoptosis coupled with neurotoxicity that result from *α-synuclein* accumulation and the subsequent dysfunction of cellular mechanisms. All the same, it appears the presence of both mechanisms, vis a vis *BI-1*-induced apoptosis or *α-synuclein* aggregation neurotoxicity, confers a great disadvantage to *Ddc-Gal4*-expressing neurons. The suppression of *BI-1* in the Drosophila eye under the direction of the *GMR-Gal4* transgene results in a lower ommatidia number when compared to the control. *BI-1* is an apoptosis suppressor gene and the down-regulation of its protein product results in

programmed cell death [3]. The reduction in the ommatidia number observed is mainly due to the fusion of ommatidia and the resulting ommatidia disarray. The inhibition of *BI-1* in the Drosophila eye seems to exacerbate the *Gal4*-induced apoptosis that manifests as roughened eye phenotype [66]. The co-expression of the *Bcl-2* pro-cell survival homologue *Buffy* with *BI-1-RNAi* results in the suppression of the phenotype, with the number of ommatidia and the roughened eye restored to control levels. Buffy seems to ameliorate this phenotype and it is possibly via a general action on survival signals or an interaction with BI-1.

The expression of *α-synuclein* in the Drosophila eye results in reduced ommatidia and a highly disrupted ommatidial array [27]. This *α-synuclein*-induced developmental eye defects model is a viable system to show the effects of altered gene expression and its role in neuroprotection. The co-expression of *α-synuclein* with *BI-1-RNAi* in the Drosophila eye resulted in decreased ommatidia number and a highly disrupted ommatidial array when compared to the control that expresses *α-synuclein*. The number of ommatidia decreased further when *α-synuclein* was co-expressed with *BI-1-RNAi*. Additionally, the degree of disruption of the ommatidial array was also increased. Though it did not appear to be additive in nature, it seems that the combination of the expression of the neurotoxic *α-synuclein* and the inhibition of the activity of the anti-apoptotic BI-1 results in a worsening of the roughened eye phenotype. The accumulation of *α-synuclein* has been implicated in breakdown of cellular homeostasis that include apoptosis, ROS production, and autophagy [67]. The inhibition of *BI-1* disrupts regulation of similar mechanisms as those implicated in *α-synuclein*-induced neurotoxicity that include apoptosis, autophagy and ROS production [3]. It therefore, follows that the combined action of *α-synuclein*

expression and *BI-1* inhibition worsened the phenotypes that result from either *α-synuclein* expression or *BI-1* inhibition.

Conclusions

The inhibition of *BI-1* in the Ddc-Gal4-expressing neurons of Drosophila results in reduction in lifespan and an age-dependent loss in climbing ability, phenotypes that are strongly associated with the degeneration and loss of dopaminergic neurons. The co-expression of the pro-survival *Buffy* with *BI-1-RNAi* results in the rescue of the phenotypes observed, it is possible that Buffy and BI-1 interact to promote anti-apoptosis. Finally, *BI-1* appears to be neuroprotective as its inhibition along with *α-synuclein* expression result in enhanced phenotypes.

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Knockdown of the putative *Lifeguard* homologue *CG3814* in neurons of *Drosophila melanogaster*

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Abstract

Lifeguard is an integral transmembrane protein that modulates FasL-mediated apoptosis by interfering with the activation of caspase 8. It is evolutionarily conserved, with homologues present in plants, nematodes, zebra fish, frog, chicken, mouse, monkey and human. The *Lifeguard* homologue in *Drosophila CG3814* contains the Bax inhibitor-1 family motif that is of unknown function. The downregulation of *Lifeguard* result in disruption of cellular homeostasis and disease due to sensitizing neurons to FasL-mediated apoptosis. We identified through bioinformatic analysis CG3814, a putative homologue of Lifeguard and knocked down *CG3814/LFG* under the control of the *Dopa decarboxylase* transgene in neurons of *Drosophila melanogaster* to investigate whether it possesses neuroprotective functions that have been reported in the human homologue. The knockdown of *CG3814/LFG* in the *Ddc-Gal4*-expressing neurons results in shortened lifespan and a highly-impaired locomotor ability, phenotypes that are strongly associated with the degeneration and subsequent loss of dopaminergic neurons. Lifeguard interacts with anti-apoptotic Bcl-2 proteins and possibly with pro-apoptotic members to induce its neuroprotective function. The co-expression of *Buffy*, the sole anti-apoptotic Bcl-2 gene family member in *Drosophila*, along with *CG3814/LFG* by stable inducible RNA interference results in the suppression of the shortened lifespan and the premature age-dependent loss in climbing ability. The suppression of *CG3814/LFG* in the *Drosophila* eye under the direction of the *GMR-Gal4* transgene results in a reduction in ommatidia number and an increase in the disruption of the ommatidial array. The overexpression of *Buffy* along with the knockdown of *CG3814/LFG* counteracts the eye phenotypes. The knockdown of *CG3814/LFG* in *Ddc-Gal4*-expressing neurons in

Drosophila diminishes its neuroprotective ability and results in shortened lifespan and loss in climbing ability, phenotypes that are improved upon overexpression of the pro-survival *Buffy*.

Introduction

Lifeguard (LFG) or Fas apoptotic inhibitory molecule 2 (FAIM2), also known as Transmembrane Bcl-2 associated protein X (Bax) inhibitor motif 2 (TMBIM2), belongs to a diverse membrane-spanning protein family [1, 2] and inhibits apoptosis mediated by the Fas/CD95/Apo-1 receptor but not the closely related TNFR [3]. LFG was first identified as neuronal membrane protein 35 (NMP35) when it was found to be differentially upregulated during rat postnatal development [4] and predominantly localised to the endoplasmic reticulum (ER). A different nomenclature categorizes this protein into a family referred to as *LFG*, which is adopted from *Lifeguard* [1], and particularly classifies LFG as LFG2. This protein consists of seven transmembrane domains and is conserved being found in plants, insects, amphibians, fish and mammals [5]. LFG regulates cell death by interfering with caspase 8 activation but not its recruitment to the death-inducing signalling complex (DISC) [3], a role that is essential for the survival of neurons during development. Expression of *LFG* has been shown to be dependent on PI3K/Akt and its knockdown sensitizes neurons to FasL-induced apoptosis [6]. This appears to be through its regulation by PI3K. Another mechanism for LFG regulation of apoptosis suggests that LFG interacts with Bcl-X_L and Bcl-2 at the ER to inhibit calcium release [7]. This interaction with Bcl-X_L is contrary to previous findings where LFG was shown to interact with Bax [5]. The dysregulation of *LFG* has been implicated in disruption of cellular homeostasis that include many cancers and neuronal

diseases [8, 9]. Nevertheless, the antiapoptotic role of LFG has been demonstrated in addition to its interaction with the Bcl-2 family of proteins.

The *Bcl-2* family of genes are key regulators of cell death and survival in animals and are functionally composed of anti-apoptotic and proapoptotic members [10]. They regulate life and death decisions at the cellular level by maintaining a delicate balance of the pro-apoptotic and anti-apoptotic members. The *Bcl-2* family member homologues in *Drosophila melanogaster* are limited to the single anti-apoptotic *Buffy* and the pro-apoptotic *Debcl* [11]. In previous studies, the overexpression of *Buffy* has been shown to confer survival advantages in response to external stimuli and under conditions of stress [12-15]. A role for this gene in the mitochondrial pathway has been described during *Drosophila* oogenesis [13]. This point to an important role for this protein in aspects of cell death.

The *Drosophila* homologue was initially reported to be NMDARA1 by Schweitzer *et al*, though previous work by Pellicena-Palle and Salz reported this transcript to be NMDARA1 and a homologue of the rat glutamate binding protein (GBP) [4, 16]. It was listed as CG3798 in FlyBase and is currently Nmda1, also known as NMDA receptor associated protein [5] and the protein sequence used in Bioinformatic comparisons in this study is Nmda1 polypeptide C (http://flybase.org/reports/FBpp0088816.html). The accession number of the protein sequence used by Reimers *et al* (NP_610824.1) [5], has since been updated in both FlyBase and NCBI and currently represents CG3814 isoform A (Polypeptide Dmel\CG3814-PA) (http://flybase.org/reports/FBpp0086927.html). Interestingly, CG3814 and Nmda1 are adjacent each other on chromosome 2 (2R: 12,875,164 to 12,876,966) and (2R: 12,877,094 to 12,880,230) respectively and are

transcribed in the same direction. Recent bioinformatic studies make comparisons between *Drosophila* CG3814 and/or CG9722 with human LFG [1, 2]. The presence of homologues of this protein family implicated in the regulation of FasL-mediated apoptosis may underpin their evolutionary importance in cytoprotection. *Drosophila* is used as a model organism to study the alteration of gene expression and to model human diseases, and has produced very promising results [17]. We used *Ddc-Gal4*-expressing neurons in our studies since they are sensitive to subtle disruptions in gene expression and degenerate in an age-dependent manner which manifests as deficiency in locomotor function. Since these neurons are highly sensitive, measurements of survival and climbing ability are rapid assays to assess the organismal effects of altered gene expression and to identify genetic interaction. We investigated the outcome of the knockdown of *CG3814* alias *LFG* - which has a wider expression pattern compared to *CG9722* which is only expressed in the testes [1] – under the control of the *Dopa decarboxylase* transgene in neurons of *Drosophila*, and further we determined whether there is interaction with Bcl-2 proteins by the overexpression of the pro-survival *Bcl-2* homologue *Buffy*.

Materials and Methods

Bioinformatic analysis

The protein sequences for *Drosophila melanogaster*: NP_610824.1, *Homo sapiens*: NP_036438.2, *Xenopus tropicalis*: NP_001072357.1, and *Mus musculus*: NP_082500.2 were sourced from National Center for Biotechnology Information (NCBI; http://www.ncbi.nlm.nih.gov/protein/). The functional domains were identified using the NCBI Conserved Domain Database (CDD; http://www.ncbi.nlm.nih.gov/cdd; [18] and the Eukaryotic Linear Motif (ELM) resource (http://elm.eu.org/) [19] which focuses on

annotation and detection of eukaryotic linear motifs (ELMs), also known as short linear motifs (SLiMs). A Clustal Omega multiple sequence alignment (http://www.ebi.ac.uk/Tools/msa/clustalo/) [20, 21] was used to show conservation of the Bax inhibitor-1 domain. Additional analysis with Phyre2 (http://www.sbg.bio.ic.ac.uk/phyre2/html/page.cgi?id=index) [22], a web portal for protein modelling, prediction and analysis was performed.

Drosophila media, culture and stocks

Stocks and crosses were maintained on standard cornmeal/molasses/yeast/agar media treated with propionic acid and methylparaben. Stocks were kept at room temperature while crosses and experiments were carried out at 25°C and 29°C. The *CG3814/ P{KK104535}VIE-260B* hereby referred to as *UAS-LFG-RNAi (1)* (http://stockcenter.vdrc.at/control/product/~VIEW_INDEX=0/~VIEW_SIZE=100/~product_id=100645) was obtained from Vienna Drosophila Resource Center (Vienna, Austria), and y^1 sc^* v^1; P{y[+t7.7] v[+t1.8]=TRiP.HMS00225} attP2 hereby referred to as *UAS-LFG-RNAi (2)* was developed at Harvard Medical School Transgenic RNAi project (http://www.flyrnai.org/up-torr/GetSummaryByGene?organism=Fly) and was obtained from Bloomington Drosophila Stock Center at Indiana University, IN, USA. Information about these constructs is available online. The *GMR-Gal4* [23] and *UAS-lacZ* flies were obtained from the Bloomington Drosophila Stock Center. The *UAS-Buffy* flies [11] were a gift from Dr. L. Quinn (University of Melbourne), and *Ddc-Gal4* flies [24] by Dr. J. Hirsch (University of Virginia). The *UAS-Buffy/CyO; Ddc-Gal4* and *UAS-Buffy/CyO; GMR-Gal4* complex lines were used to overexpress *Buffy* in neurons and the developing

eye and were produced employing standard homologous recombination and marker selection methods as previously described [25, 26].

Survival assay

Several crosses of each genotype were performed and a cohort of male flies collected upon eclosion [15, 27]. For each genotype at least 200 flies were aged and scored every 2 days for the presence of deceased adults [28]. Longevity data were analysed using GraphPad Prism version 5.04 (GraphPad Software Inc., La Jolla, CA, USA), and survival curves were compared by the Log-rank (Mantel-Cox) test. Significance was determined at a 95% confidence interval and family-wise $P \leq 0.05$ with Bonferroni correction.

Climbing assay

The critical class males flies were assayed for their ability to climb using a method previously described [29]. Climbing analysis was performed and climbing indices analysed by GraphPad Prism version 5.04. The climbing curves were fitted using non-linear regression and data compared using 95% confidence intervals with a P-value of 0.05 or less being significant.

Scanning electron microscopy of the Drosophila eye

A cohort of the critical class male flies was collected upon eclosion and prepared for imaging using a standard protocol previously described [15]. 10 different scanning electron micrographs of each genotype were analysed using the National Institutes of Health (NIH) ImageJ software [30] and biometric analysis performed using GraphPad Prism version 5.04. The area of disruption of the ommatidial array was determined as detailed previously [31]. Statistical comparisons comprised one-way analyses of variance

(ANOVA) and Dunnett's multiple comparison tests. P-values less than 0.05 were considered significant.

Results

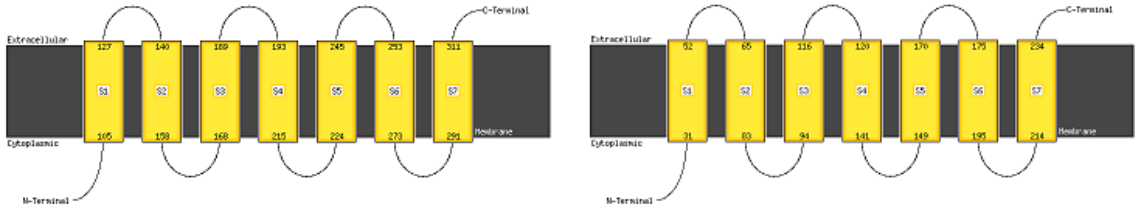
Human *LFG* is closely related to Drosophila *CG3814* and has seven TMD

To characterize the *Drosophila melanogaster* homologue of LFG, we performed bioinformatic analysis on CG3814 isoform A that is composed of 239 amino acids and has 45% identity and 65% similarity to the 316 amino acids human LFG. The *Drosophila* homologue has 7 transmembrane (TM) domains (Figure 6) and a tumour necrosis factor receptor-associated factors (TRAF2) binding consensus motif at amino acids 55 to 58 and 208 to 211 that may be involved in TNFR signalling as determined by NCBI Conserved Domain Database (CDD) [18], Eukaryotic linear motif (ELM) resource [19] and Phyre2 [22]. The alignment of protein sequences using Clustal Omega multiple sequence alignment [20, 21] shows high conservation of the Bax inhibitor-1-like domain. The 3D modelling of these proteins using Phyre2 [22] shows a close similarity in the structure and the orientation of the transmembrane domains.

Knock down of *CG3814/LFG* decreases lifespan and prematurely retards climbing ability

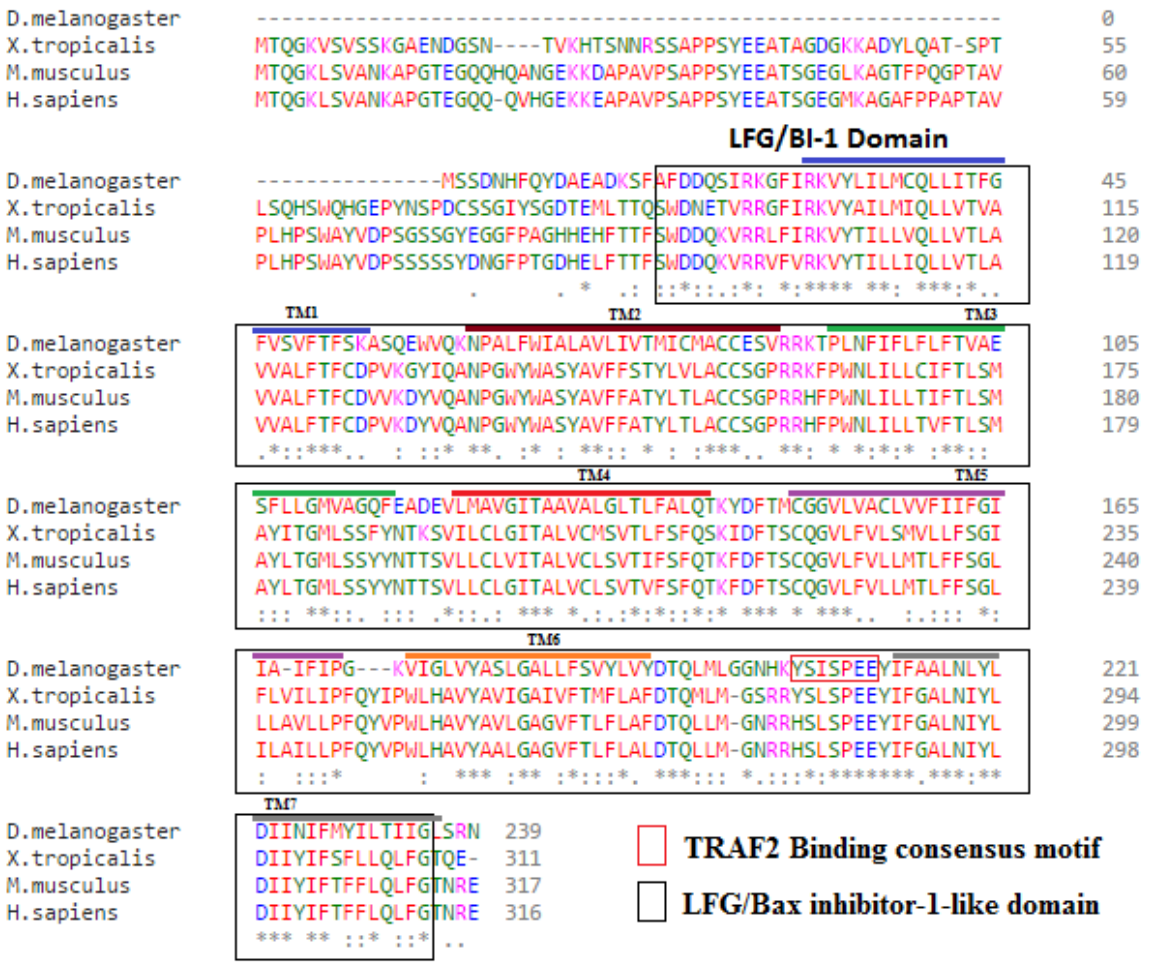
We investigated the neuroprotective role of *CG3814/LFG* in Drosophila, and the suppression of *CG3814/LFG* in the *Ddc-Gal4*-expressing neurons results in a decrease in lifespan and impairment of locomotor ability. We employed two different RNAi lines to determine the specificity of the effects of knockdown of this gene and compared them to a control line. The median lifespan of *LFG-RNAi* flies was 42 days and 46 days compared to 70 days for the *lacZ* controls as compared by Log-rank (Mantel-Cox) test with a

A

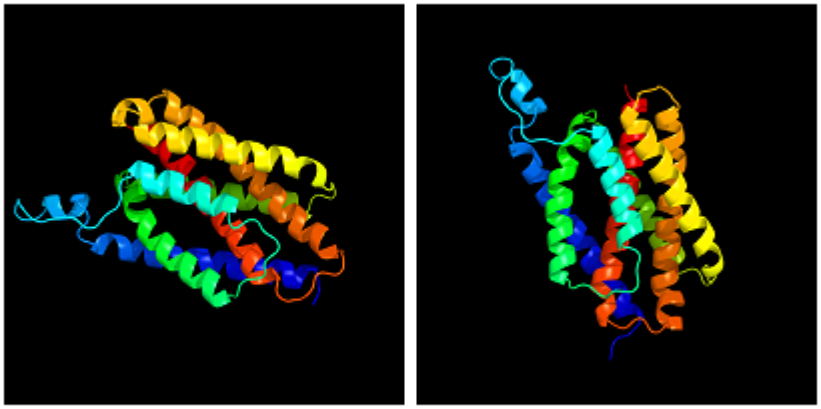


Human Lifeguard **Drosophila CG3814**

B



C



Human Lifeguard Drosophila CG3814

Figure 6.6 Drosophila CG3814 has seven TM domains that are evolutionarily conserved

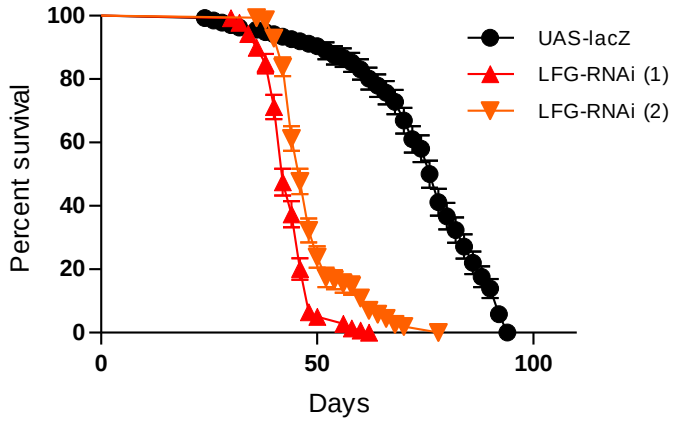
A) The seven membrane-spanning regions of *Drosophila* CG3814 and the human homologue LFG were generated by Phyre2 [22] and show the similarity between the transmembrane domain orientation. B) The *Drosophila melanogaster CG3814* isoform A is composed of 239 amino acids and is 45% identical to human LFG. Domains were identified using the NCBI Conserved Domain Database (CDD) [18] and the Eukaryotic Linear Motif [19]. Clustal Omega multiple sequence alignment [20, 21] of *Drosophila* CG3814 protein (D.melanogaster = *Drosophila melanogaster* NP_610824.1) with the human (H.sapiens = *Homo sapiens* NP_036438.2), frog (X.tropicalis = *Xenopus tropicalis* NP_001072357.1), and mouse (M.musculus = *Mus musculus* NP_082500.2) homologues shows conservation of the Bax inhibitor-1 domain. The domains were identified using the NCBI CDD. "*" indicate the residues that are identical, ":" indicate the conserved substitutions, "." indicate the semi-conserved substitutions. Colours show the chemical nature of amino acids. Red is small hydrophobic (including aromatic), Blue is acidic, Magenta is basic, and Green is basic with hydroxyl or amine groups. C) The 3D protein modelling of human LFG and the *Drosophila* CG3814 show remarkable similarity in the structure and the orientation of the transmembrane domains with the image coloured by rainbow from the N → C terminus (Image cartoons were obtained from Phyre2).

$p<0.0001$ (Figure 7A). The knockdown of *CG3814* in these neurons produces flies with significantly impaired climbing ability as determined by the nonlinear fitting of the climbing curves and comparing the CI at 95% (Figure 7B). The *LFG-RNAi* flies had CI of 0.069 to 0.091 and 0.050 to 0.071 when compared to 0.030 to 0.048 for the controls that express the benign *lacZ* transgene. These results taken together suggest a neuroprotective role for *CG3814/LFG* in *Drosophila*.

Suppression of *CG3814/LFG*-induced phenotypes are counteracted by overexpression of the pro-survival *Buffy*

We determined the pro-survival role of *Buffy* and *CG3814/LFG* in Drosophila neurons. The co-expression of the pro-survival *Bcl-2* homologue *Buffy* along with the knockdown of *CG3814/LFG* results in significant increase in lifespan and improved climbing ability. When *Buffy* is co-expressed with *LFG-RNAi,* the results indicate a median lifespan of 70 days for both RNAi constructs when compared to *Buffy* control flies with a median lifespan of 74 days as determined by Log-rank test (Figure 8A). This is an increase in survival when compared to the flies in Figure 2A that had the expression of *CG3814/LFG* reduced by RNAi. The climbing ability of the *LFG-RNAi* flies were improved as determined by comparison of the climbing curves at 95% CI (Figure 8B). The *LFG-RNAi* constructs had a CI of 0.049 to 0.068 and 0.043 to 0.057 compared to the controls with 0.035 to 0.050, that indicate the climbing curves were not significantly different. These results suggest a pro-survival role for *CG3814/LFG* in *Drosophila* neurons as phenotypes that result from its insufficient levels can be abrogated by the overexpression of the pro-survival *Buffy*.

A.



B.

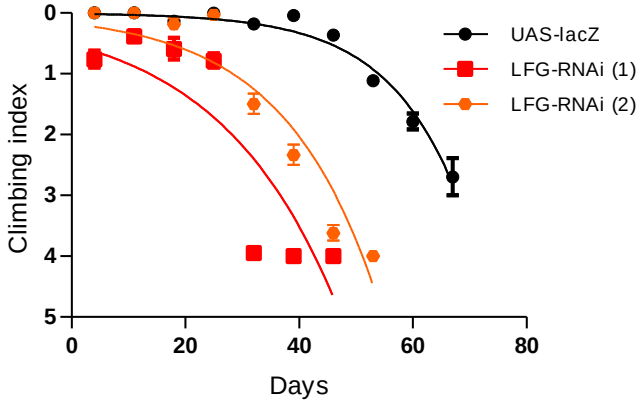
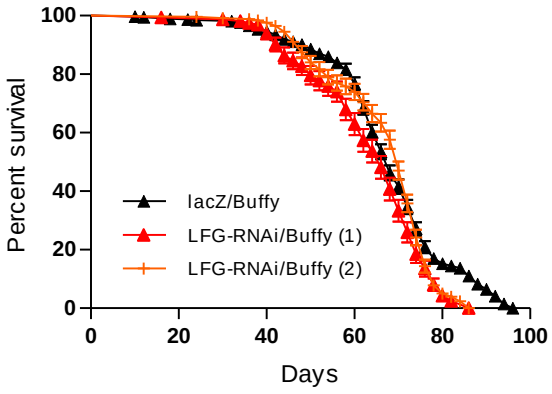


Figure 6.7 Loss of *CG3814/LFG* activity decreases survival and impairs climbing ability

A) The knockdown of *CG3814/\LFG* in the neurons under the control of the *Ddc-Gal4* transgene results in reduced lifespan, with a median survival of 42 days and 46 days when compared to 70 days for control flies that expresses the benign *lacZ* transgene. The genotypes are *Ddc-Gal4/ UAS-lacZ, Ddc-Gal4/ UAS-LFG-RNAi (1)* and *Ddc-Gal4/ UAS-LFG-RNAi (2)*. Longevity is shown as percent survival ($P < 0.05$, determined by the log-rank (Mantel-Cox) test and $N \geq 200$). B) The directed suppression of *CG3814/LFG* in the *Ddc-Gal4*-expressing neurons resulted in premature loss in climbing ability as determined by nonlinear fitting of the climbing curves and comparing 95% CI. The genotypes are *Ddc-Gal4/ UAS-lacZ, Ddc-Gal4/ UAS-LFG-RNAi (1)* and *Ddc-Gal4/ UAS-LFG-RNAi (2).* Error bars indicate standard error of the mean and $N=50$.

A.



B.

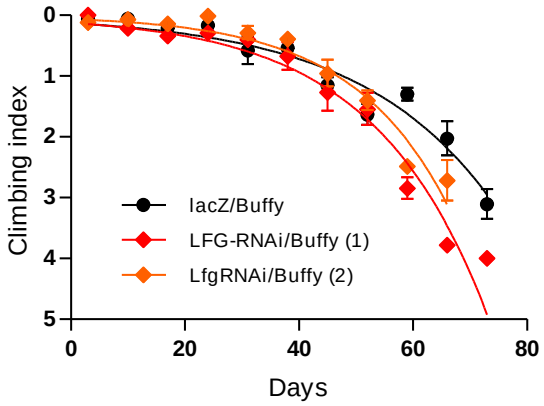


Figure 6.8 *Buffy* overexpression can counteract the *CG3814/LFG*-induced phenotypes

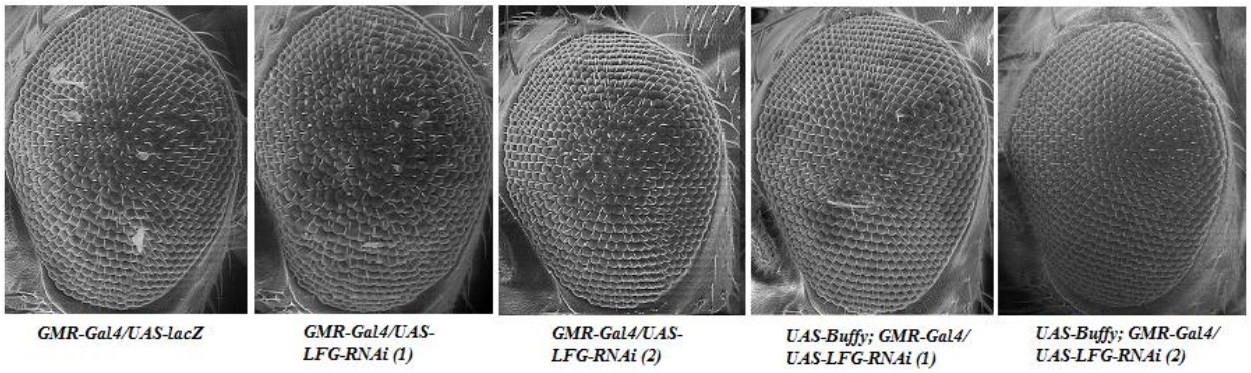
A) The overexpression of *Buffy* along with *LFG-RNAi* results in the suppression of decreased survival as indicated by a median survival of 70 days for both RNAi constructs when compared to 74 days for the control. Genotypes are *Ddc-Gal4 UAS-Buffy/ UAS-lacZ, Ddc-Gal4 UAS-Buffy/ UAS-LFG-RNAi (1)* and *Ddc-Gal4 UAS-Buffy/ UAS-LFG-RNAi (2).* Longevity is shown as percent survival ($P < 0.05$, determined by log-rank (Mantel-Cox) test with $N \geq 200$). B) The knockdown of *CG3814/LFG* along with the overexpression of *Buffy* in these neurons results in the suppression of the age-dependent loss in climbing ability. The genotypes are *Ddc-Gal4 UAS-Buffy/ UAS-lacZ, Ddc-Gal4 UAS-Buffy/ UAS-LFG-RNAi (1)* and *Ddc-Gal4 UAS-Buffy/ UAS-LFG-RNAi (2).* Analysis was done by nonlinear fitting of the climbing curves and significance was determined by comparing the 95% CI. Error bars indicate standard error of the mean and $N=50$.

Knock down of *CG3814* in the eye decreases ommatidia number and increases degeneration, phenotypes that are rescued upon *Buffy* overexpression

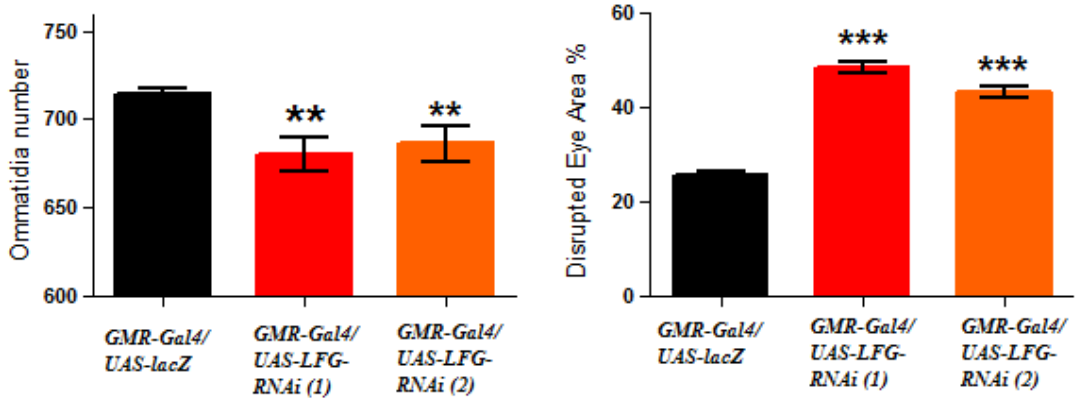
The biometric analysis of the neuron rich Drosophila compound eye can shed light on subtle differences in phenotypes that result from altered gene expression. The directed suppression of *CG3814/LFG* in the developing eye using the *GMR-Gal4* transgene results in eyes that have fewer ommatidia and a higher disruption of the ommatidial array (Figure 9A and 9B) as determined by a one-way analysis of variance (ANOVA) at a p of less than 0.050. The reduction in ommatidia number is largely from the high degree of ommatidial fusion. Co-expression of *LFG-RNAi* with *Buffy* restored the mean number of ommatidia and percentage of disruption to control levels as determined by a one-way analysis of variance (ANOVA), p>0.50 (Figure 9A and 9C). Taken together, these results suggest that *CG3814/LFG* may play a role in the development of the neuron-rich *Drosophila* eye and that *Buffy* suppresses the developmental eye defects that result from its knockdown.

Discussion

Bioinformatic analysis of protein sequences from our study shows that CG3814 is the strongest candidate for *Drosophila* LFG, with a sequence identity of 45% and similarity of 65%, but we do not exempt CG9722, the only consideration was that CG3814 was widely expressed when compared to CG9722 that is predominantly expressed in the testis [1]. Therefore, we propose that *CG3814* is the putative *Drosophila* homologue of *LFG*. The conditional knockdown of *CG3814* by stable inducible RNA interference in neurons of *Drosophila* under the direction of the *Ddc-Gal4* transgene resulted in decreased median survival and severely impaired climbing ability, phenotypes that were consistently present in both RNAi lines tested. The “healthspan” of these flies was highly A.


GMR-Gal4/UAS-lacZ
GMR-Gal4/UAS-LFG-RNAi (1)
GMR-Gal4/UAS-LFG-RNAi (2)
UAS-Buffy; GMR-Gal4/UAS-LFG-RNAi (1)
UAS-Buffy; GMR-Gal4/UAS-LFG-RNAi (2)

B.


Ommatidia number
750
700
650
600
**
**
GMR-Gal4/UAS-lacZ
GMR-Gal4/UAS-LFG-RNAi (1)
GMR-Gal4/UAS-LFG-RNAi (2)
Disrupted Eye Area %
60
40
20
0
***
***
GMR-Gal4/UAS-lacZ
GMR-Gal4/UAS-LFG-RNAi (1)
GMR-Gal4/UAS-LFG-RNAi (2)

C.

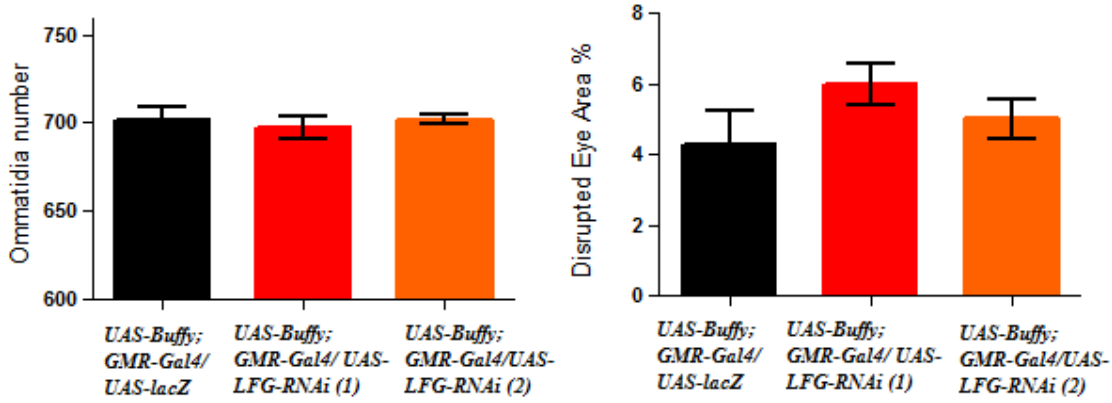

Ommatidia number
750
700
650
600
UAS-Buffy; GMR-Gal4/UAS-lacZ
UAS-Buffy; GMR-Gal4/UAS-LFG-RNAi (1)
UAS-Buffy; GMR-Gal4/UAS-LFG-RNAi (2)
Disrupted Eye Area %
8
6
4
2
0
UAS-Buffy; GMR-Gal4/UAS-lacZ
UAS-Buffy; GMR-Gal4/UAS-LFG-RNAi (1)
UAS-Buffy; GMR-Gal4/UAS-LFG-RNAi (2)

Figure 6.9 Knockdown of *CG3814/LFG* in the developing eye results in phenotypes that are counteracted by *Buffy* overexpression

A) Scanning electron micrographs when *CG3814/LFG* is suppressed in the eye and co-expressed along with *Buffy*. The genotypes are *GMR-Gal4/ UAS-lacZ*; *GMR-Gal4/ UAS-LFG-RNAi (1)*; *GMR-Gal4/ UAS-LFG-RNAi (2)*; *UAS-Buffy; GMR-Gal4/ UAS-LFG-RNAi (1)* and *UAS-Buffy; GMR-Gal4/ UAS-LFG-RNAi (2)*. B) Biometric analysis when *CG3814/LFG* is suppressed in the eye indicated decreased ommatidia number and higher percentage of ommatidial disruption when compared to the control. C) Co-expression of *Buffy* with *LFG-RNAi* resulted in the suppression of the eye phenotypes, ommatidia number and disruption of the eye were restored to control levels as determined by biometric analysis. Comparisons were determined by a one-way analysis of the variance (ANOVA), $p<0.05$, error bars are standard error of the mean, asterisks (*) represent statistical significance and $N=10$.

compromised as determined by their shortened lifespan and precocious loss in locomotor function. LFG is able to block FasL-induced cell death and has been demonstrated to be neuroprotective [4, 9, 32, 33], being highly expressed in the neurons, and its loss-of-function induces cell death. The profound loss in climbing ability that results from knockdown of *CG3814/LFG* in *Drosophila* neurons appears to be as a result of neuronal loss, since when compared to control flies around the same age and time point, there is a significant difference in the climbing abilities. In our findings, we did not perform any cell death assays and as such our conclusions are mostly based on behavioural phenotypes that are compared to controls. This does not negate the strong evidence we obtained on the effects of knocking down *CG3814/LFG*. In addition, the use of RNAi does not exclude off targets in regions that share homology with other BI-1 containing motifs, though data from Vienna Drosophila Resource Centre shows there are no off targets for these RNAi lines but in fact target the various isoforms present. Taken together, these results suggest a strong neuroprotective role for CG3814 in *Drosophila Ddc-Gal4*-expressing neurons.

The observed *CG3814*-induced phenotypes may be through a different mechanism by its interaction with pro-survival Bcl-2 proteins at the ER membrane [7], to regulate the release of calcium from the ER. Therefore, the knockdown of *CG3814/LFG* in the neurons appears to result in neuronal degeneration and death. The only known pro-survival *Bcl-2* homologue in *Drosophila* is *Buffy* [11]. The overexpression of *Buffy* is known to confer survival advantages to cells under normal conditions and under conditions of stress [11, 12, 14, 15, 26, 34, 35], the overexpression of *Buffy* along with the knockdown of *CG3814* resulted in the suppression of the *CG3814*-induced phenotypes

marked by significantly improved survival and locomotor function. This Buffy action to improve “healthspan” may be specific to an interaction with CG3814 or may be attributed to a general pro-survival signalling pathway that is initiated by Buffy in response to stress mediated by the loss in *CG3814* function. This additionally indicates a strong pro-survival role for *CG3814/LFG* since the phenotypes that result from its knockdown are rescued by the pro-survival *Buffy*.

In supportive experiments, the directed knockdown of *CG3814* in the neuron-rich developing *Drosophila* eye under the direction of the *GMR* response elements resulted in a depressed ommatidia number. The reduction in the ommatidia number was attributed to the high degree of fusion of the ommatidium and consequently resulted in ommatidia disarray. The knockdown of *CG3814* in the *Drosophila* eye seems to exacerbate the *Gal4*-induced apoptosis that manifests as roughened eye phenotype [36]. The overexpression of *Buffy* along with the knockdown of *CG3814* results in the suppression of the phenotype, with the number of ommatidia and the degree of roughened eye restored to control levels. Buffy seems to ameliorate this phenotype and it is possibly via a general action on survival signals or through a concerted function to rescue *CG3814*-induced apoptosis.

Conclusions

The knockdown of *CG3814/LFG* in the *Ddc-Gal4*-expressing neurons of *Drosophila* results in a severely shortened lifespan and marked age-dependent loss in climbing ability, phenotypes that are strongly associated with the degeneration and loss of DA neurons. The overexpression of the pro-cell survival *Buffy* along with the knockdown of

CG3814/LFG results in the rescue of the observed phenotypes that indicate a strong pro-survival and neuroprotective role for *CG3814/LFG* in *Drosophila* neurons.

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The Inhibition of *CG2076*, the *GHITM* homologue in neurons of *Drosophila melanogaster* can be rescued by *Buffy*

A version of this chapter has been accepted for publication in the Journal of RNAi and Gene Silencing (M'Angale and Staveley, 2016).

Abstract

Growth hormone-inducible transmembrane protein (GHITM) is an inner mitochondrial membrane protein that contains the Bax inhibitor-1 motif and is implicated in the regulation of mitochondrial morphology and especially cristae structure. The downregulation of GHITM results in fragmented mitochondria and the release of cytochrome c, while its upregulation delays the release of cytochrome c. We inhibited *CG2076* the Drosophila *GHITM* homologue in the neurons using RNA interference and analysed the phenotypic consequences of this mitochondrial protein. The directed expression of *GHITM-RNAi* in neurons under the control of the *Dopa decarboxylase (Ddc)* transgene results in shortened lifespan and impaired climbing ability. The co-expression of *Buffy*, the only anti-apoptotic B cell lymphoma 2 (Bcl-2) protein in Drosophila, along with *GHITM-RNAi* results in suppression of the shortened lifespan and premature age-dependent loss in climbing ability. The inhibition of *GHITM* in the Drosophila eye results in decreased ommatidia number and elevated disruption of the ommatidial array, phenotypes that are rescued upon overexpression of *Buffy*. The inhibition of the mitochondrial located *GHITM* in the *Ddc-Gal4*-expressing neurons of Drosophila results in shortened lifespan and loss in climbing ability, phenotypes that are manifest of degeneration and death of dopaminergic neurons, and are improved upon overexpression of the pro-survival *Buffy*.

Introduction

The transmembrane Bax inhibitor-1 motif containing (TMBIM) family consists of several antiapoptotic members that are evolutionarily conserved, being found in viruses, bacteria, protozoans, plants and animals [1, 2]. This group of proteins is so diversely conserved

that they are present in organisms where the Bcl-2 family of proteins have not yet been identified. Generally, 6 members or orthologues can be present in an organism, that include TMBIM1/RECS1, TMBIM2/LFG, TMBIM3/GRINA, TMBIM4/GAAP, TMBIM5/GHITM and TMBIM6/BI-1 [2]. The different members are localised to different cellular organelles, with TMBIM1/RECS1 predominantly found in the endosomal/lysosomal membranes; TMBIM2/LFG at the plasma and intracellular membranes of the Golgi and the endoplasmic reticulum (ER); TMBIM3/GRINA is primarily located at the ER and Golgi compartments; TMBIM4/GAAP to the Golgi apparatus and the ER; TMBIM5/GHITM to the mitochondrial inner membrane; and TMBIM6/BI-1 at the ER [1-4]. Growth hormone-inducible transmembrane protein (GHITM)/TMBIM5 also referred to as *Mi*tochondrial morphology and *c*ristae 1 (MICS1) is a mitochondrial inner membrane protein that is involved in mitochondria morphology and specifically the cristae and is implicated in the release of cytochrome c from the mitochondria [5]. It was named GHITM as it was found dysregulated in expression analysis of inter-capsular brown adipose tissue of mice that were expressing a growth hormone antagonist [6]. This protein consists of seven transmembrane domains with a presequence as shown by the presence of a cleavage site at the amino (N)-terminal and is ubiquitously expressed in mammals [7, 8]. GHITM/MICS1 regulates cell death by the regulation of mitochondria morphology, since the knock down of this gene results in mitochondrial fragmentation and cristae disorganization followed by the release of mitochondrial proapoptotic proteins that include the apoptogenic cytochrome c [5]. Overexpression of GHITM/MICS1 can directly block the release of cytochrome c from the inner mitochondria membrane independent of Bax-induced permeabilization though it

does not block apoptotic death. Its maintenance of mitochondrial morphology therefore, is distinct from its role in the apoptotic process.

The *Drosophila melanogaster* homologue is predicted to be CG2076 and CG1287 [2], the two putative genes have 56% and 53% protein sequence identity to the human GHITM as determined by BLAST; CG2076 is more closely related to the human homologue. Bioinformatic studies establish that the two genes are very closely related, 67% identity and 82% similarity in their protein sequences. CG2076 has two annotated transcripts on FlyBase but only one is unique, while CG1287 has only one annotated transcript [9]. Drosophila has been used as a model organism to study the phenotypic consequences of differential gene expression and to model human diseases with very promising results [10]. DA neurons are sensitive to subtle differences in gene products and degenerate in an age-dependent manner which can be quantified by scoring climbing ability of the affected flies. We investigated the outcome of the inhibition of *CG2076*, the *Drosophila melanogaster* homologue of *GHITM* in the *Ddc-GAL4*-expressing neurons, and further determined whether the Bcl-2 proteins, known to be the "guardians" of the mitochondria can rescue the *CG2076/GHITM*-induced phenotypes by the overexpression of the sole pro-survival *Bcl-2* homologue in Drosophila, *Buffy*.

Materials and Methods

Bioinformatic analysis

The protein sequences for *Drosophila melanogaster*: NP_610824.1, *Homo sapiens*: NP_036438.2, *Xenopus tropicalis*: NP_001072357.1, and *Mus musculus*: NP_082500.2 were sourced from National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/protein/). The functional domains were identified using the

NCBI Conserved Domain Database (CDD) [11] (http://www.ncbi.nlm.nih.gov/cdd) and the Eukaryotic Linear Motif [12] (http://elm.eu.org/) which focuses on annotation and detection of eukaryotic linear motifs (ELMs). A Clustal Omega multiple sequence alignment (http://www.ebi.ac.uk/Tools/msa/clustalo/) [13, 14] was used to show conservation of the Bax inhibitor-1 domain. Transmembrane domains were confirmed using TMpred [15], a program based on statistical analysis of TMbase that identifies membrane-spanning regions (http://www.ch.embnet.org/software/TMPRED_form.html). Protein modelling, prediction and analysis was performed with Phyre2 [16], (http://www.sbg.bio.ic.ac.uk/phyre2/html/page.cgi?id=index). The sub-cellular and mitochondrial targeting signal was identified using TargetP [17] (http://www.cbs.dtu.dk/services/TargetP/) and MultiLoc2 [18] (https://abi.inf.uni-tuebingen.de/Services/MultiLoc2).

Drosophila media and stocks

Stocks and crosses were maintained on standard cornmeal/molasses/yeast/agar media treated with propionic acid and methylparaben. Aliquots of media were poured into plastic vials, allowed to solidify, and refrigerated at between 4° C and 6° C. Stocks were raised at room temperature while crosses and experiments for analysis of ageing and climbing ability were carried out at 25° C while those for the eye analysis were performed at 29° C. The CG2076 stock, w^{1118}*; P{GD3308}v5537* hereby referred to as *UAS-GHITM-RNAi* was obtained from the Vienna Drosophila Resource Center. *UAS-Buffy* [19] was kindly provided by Dr. L. Quinn of University of Melbourne and *Ddc-Gal4* flies

[20] by Dr. J. Hirsch of University of Virginia. *GMR-Gal4* [21] and *UAS-lacZ* flies were obtained from the Bloomington Drosophila Stock Center.

Drosophila derivative lines

The *UAS-Buffy/CyO; Ddc-Gal4* and *UAS-Buffy/CyO; GMR-Gal4* complex lines were used to overexpress *Buffy* in neurons and the developing eye and were produced employing standard homologous recombination and marker selection methods as previously described [22, 23]. Gel electrophoresis was used to confirm recombination events via the presence of a PCR product.

Ageing assay

Flies were aged using a standard protocol as previously described [24, 25]. Briefly, more than two hundred flies were aged per genotype and scored every two days for presence of deceased adults [26]. Longevity data was analysed using GraphPad Prism version 5.04 and survival curves were compared using the Log-rank (Mantel-Cox) test. Significance was determined at 95%, at a P-value less than or equal to 0.05 with Bonferroni correction.

Climbing assay

The climbing assay was performed as previously described [27]. Climbing indices were computed and then analysed using GraphPad Prism version 5.04. The 5-climbing index is a model generated for graded climbing analysis using non-linear regression and 5 is the highest level the flies can climb. Confidence intervals were compared at 95% at a P-value of 0.05.

Scanning electron microscopy of the drosophila eye

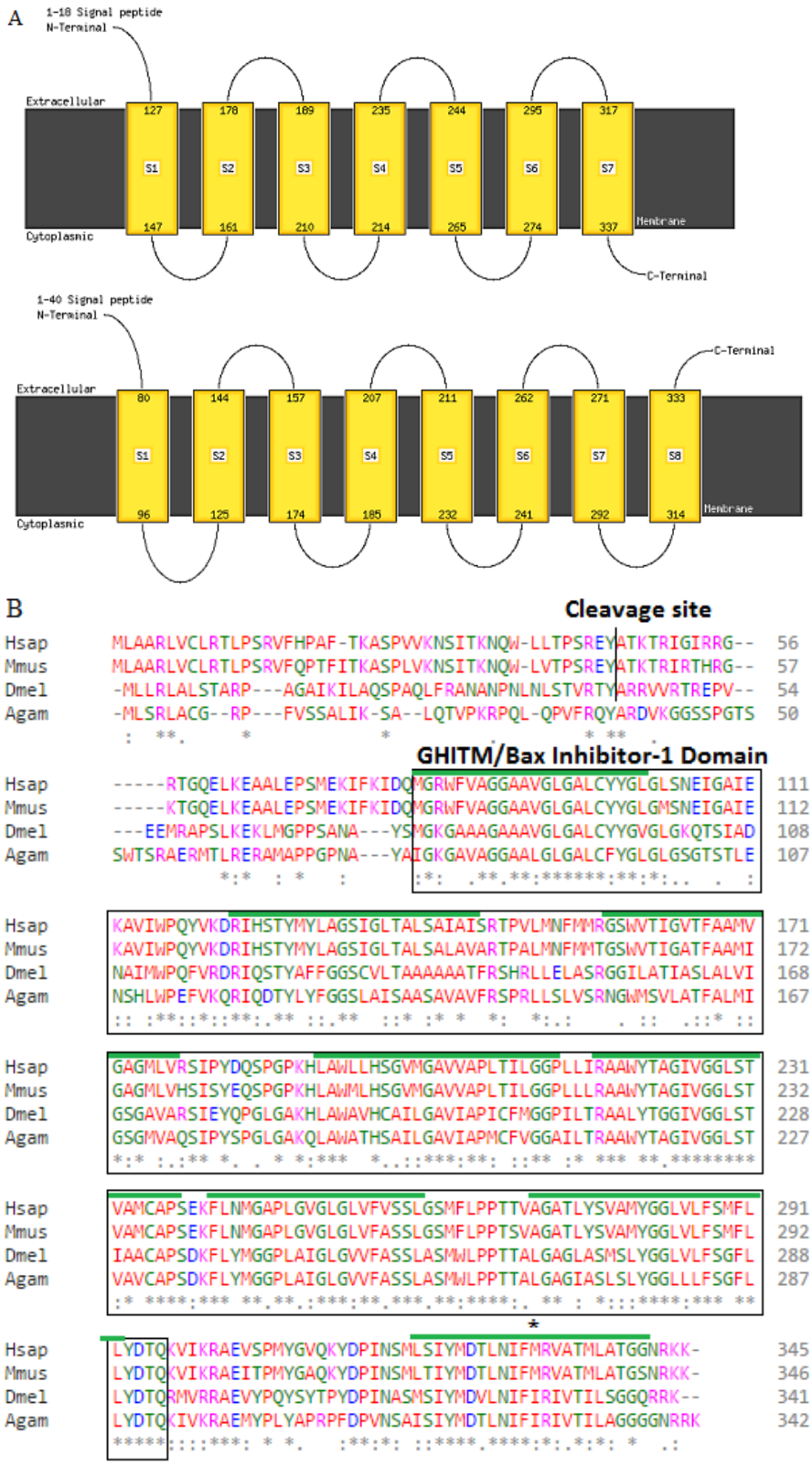
Crosses for the analysis of the Drosophila eye were made of each genotype at 29^{o} C and a batch of male flies collected and assessed using a standard protocol previously described

[25]. 10 different scanning electron micrographs of each genotype were analysed using the National Institutes of Health (NIH) ImageJ software [28] and biometric analysis performed using GraphPad Prism version 5.04. The area of disruption of the ommatidial array was determined as detailed previously [29]. Statistical comparisons were evaluated using unpaired student T-tests. P-values less than 0.05 were considered significant.

Results

Human *GHITM/MICS1* is closely related to Drosophila *CG2076*

The Drosophila *CG2076*, the human *GHITM* homologue contains the Growth hormone-inducible transmembrane hormone domain that is closely related to the Bax inhibitor-1-like superfamily as determined by NCBI Conserved Domain Database (CDD) [11]. CG2076 is composed of 341 amino acids and shows 56% identity and 73% similarity to the 345 amino acids human GHITM (Figure 10). The Drosophila homologue has eight TM domains (Figure 10A) and the human transcript has seven TM domains as determined by the Eukaryotic linear motif (ELM) resource search [12] and Phyre2 [16]. A prediction of membrane-spanning regions using TMpred [15], indicates that all the aligned sequences possesses eight TM domains, the first seven fall within the GHITM/Bax inhibitor-1-like domain and the eighth membrane-spanning region falls outside the protein family domain. A similar search using Phyre2 [16], gave similar results for CG2076 but returned seven TM domains for the human version. A multiple sequence alignment of protein sequences using Clustal Omega [13, 14] shows high conservation of the Bax inhibitor-1-like domain (Figure 10B). CG2076/GHITM is localised to the mitochondria and has a mitochondrial targeting peptide with a


A
1-18 Signal peptide
N-Terminal
Extracellular
Cytoplasmic
Membrane
C-Terminal
1-40 Signal peptide
N-Terminal
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Mmus
Dmel
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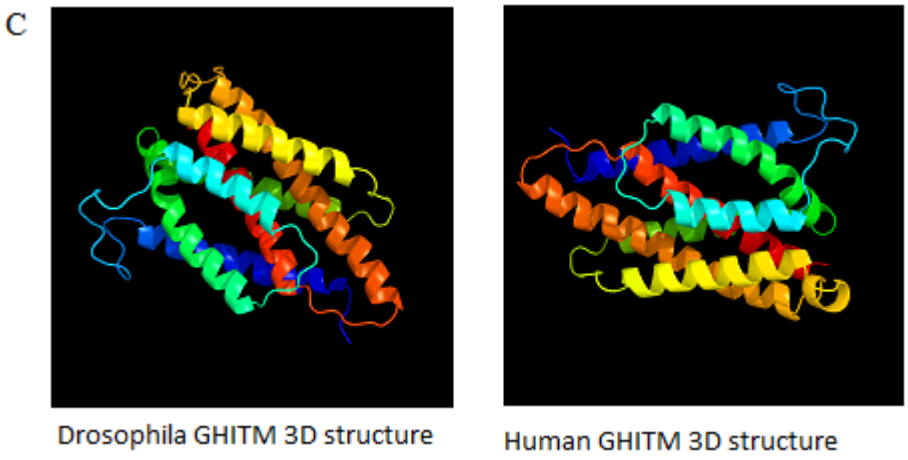


Figure 6.10 Drosophila CG2076/GHITM has eight TM domains that are evolutionarily conserved

A) The membrane-spanning regions and the location of the N-terminus signal peptide present in human GHITM and Drosophila CG2076 as generated by Phyre2 [16]. B) The GHITM/Bax inhibitor-1-like domain is highly conserved and was identified using the NCBI Conserved Domain Database (CDD) [11] and the Eukaryotic Linear Motif [12], additional membrane-spanning domains were identified using TMpred [15] and Phyre2 [16]. Clustal Omega multiple sequence alignment [13, 14] of Drosophila CG2076/GHITM protein (Dmel is *Drosophila melanogaster* NP_572681.1) with the human (Hsap is *Homo sapiens* NP_055209.2), mouse (Mmus is *Mus musculus* NP_510963.1) and mosquito (Agam is *Anopheles gambiae* XP_553771.3) homologues shows conservation of the Bax inhibitor-1 domain. "*" indicate the residues that are identical, ":" indicate the conserved substitutions, "." indicate the semi-conserved substitutions. Colours show the chemical nature of amino acids. Red is small hydrophobic (including aromatic), Blue is acidic, Magenta is basic, and Green is basic with hydroxyl or amine groups. C) A 3D modeling of both proteins using Phyre2 [16].

presequence cleavage site at amino acid 43 as predicted using TargetP [17] and MultiLoc [18]. A 3D modelling of both proteins using Phyre2 [16] is shown (Figure 10C).

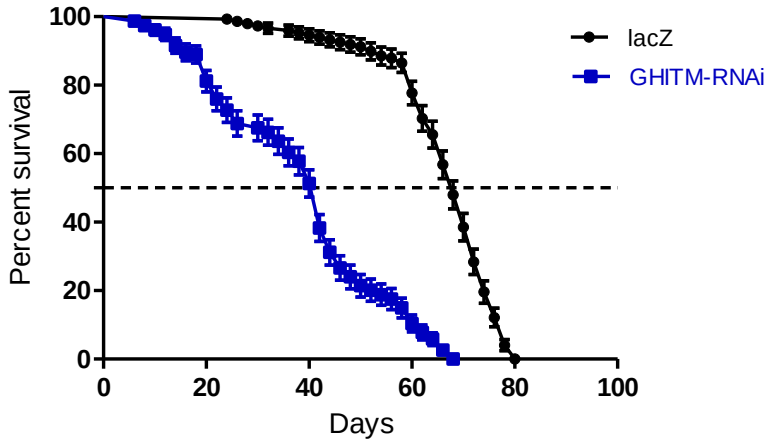
Inhibition of *CG2076/GHITM* in the *Ddc-GAL4*-expressing neurons shortens lifespan and impairs climbing ability

The suppression of *CG2076/GHITM* in the DA neurons results in severely shortened lifespan and highly impaired climbing ability. The median survival of *GHITM-RNAi* flies was 42 days compared to 68 days for the controls that express the benign *lacZ* as determined by Log-rank (Mantel-Cox) test (Figure 11A). The directed inhibition of *CG2076/GHITM* in the *Ddc-GAL4*-expressing neurons produces flies with significantly impaired climbing ability as determined by a nonlinear fit of the climbing curves (Figure 11B). The comparison of the confidence intervals (CI) at 95% indicate a significant difference between the *GHITM-RNAi* flies with 0.036 to 0.051 compared with 0.084 to 0.112 for the controls. These results suggest that *CG2076/GHITM* is required for the normal function of these neurons in Drosophila.

***Buffy* suppresses the loss of *CG2076/GHITM*-induced phenotypes**

The overexpression of the pro-survival *Bcl-2* homologue *Buffy* along with the suppression of *CG2076/GHITM* in the *Ddc-GAL4*-expressing neurons results in a significant increase in lifespan and improved climbing ability. The co-expression of *Buffy* with *GHITM-RNAi* results in increased median survival of 68 days when compared to *Buffy* control flies with a median survival of 72 days as determined by Log-rank test (Figure 12A). The climbing ability of the *GHITM-RNAi* flies was improved as determined by comparison of the climbing curves at 95% CI with 0.039 to 0.052 compared with 0.039 to 0.051 which was not significant (Figure 12B). These results suggest a pro-survival role for *Buffy;* it

A.



B.

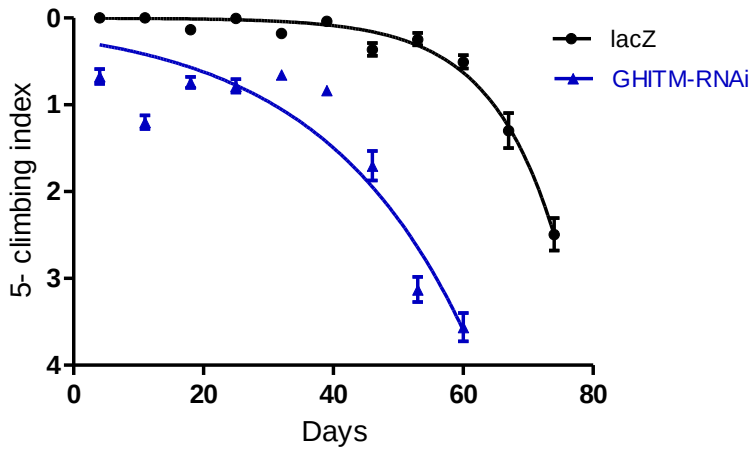
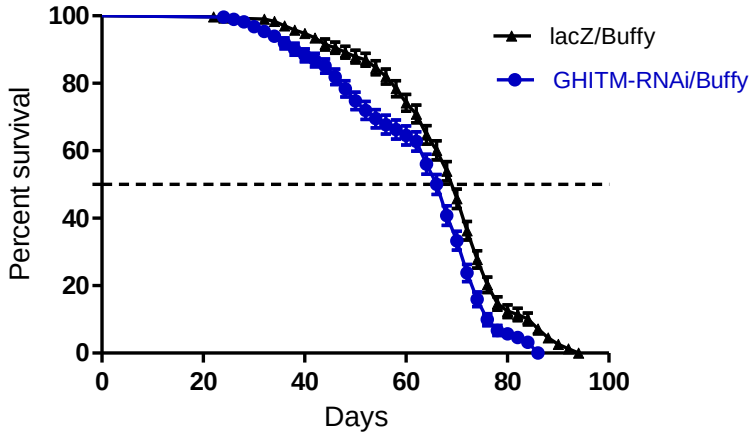


Figure 6.11 Inhibition of *CG2076/GHITM* shortens lifespan and severely impairs climbing ability

A) The inhibition of *CG2076/GHITM* in the *Ddc-GAL4*-expressing neurons results in shortened lifespan when compared to control flies expressing *UAS-lacZ*. The genotypes are *Ddc-Gal4/ UAS-lacZ* and *Ddc-Gal4/ UAS-GHITM-RNAi.* Longevity is shown as percent survival ($P < 0.0001$, determined by the log-rank (Mantel-Cox) test and n=200). B) The directed inhibition of *CG2076/GHITM* in these neurons resulted in premature loss in climbing ability as determined by nonlinear fitting of the climbing curves and comparing 95% CI (0.036 to 0.051 compared with 0.084 to 0.112). The genotypes are *Ddc-Gal4/ UAS-lacZ* and *Ddc-Gal4/ UAS-GHITM-RNAi.* Error bars indicate standard error of the mean (SEM) and n=50.

A.



B.

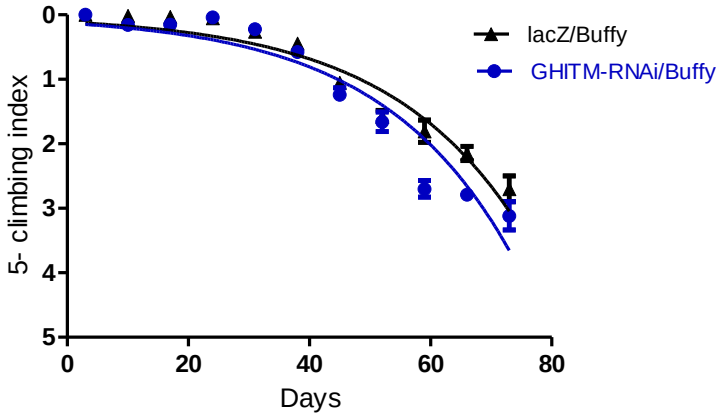


Figure 6.12 Overexpression of the pro-survival *Buffy* suppresses the *CG2076/GHITM*-induced phenotypes

A) The overexpression of *Buffy* along with *GHITM-RNAi* in the *Ddc-GAL4*-expressing neurons resulted in improved survival when compared to the control. Genotypes are *Ddc-Gal4 UAS-Buffy/ UAS-lacZ* and *Ddc-Gal4 UAS-Buffy/ UAS-GHITM-RNAi.* Longevity is shown as percent survival (P = 0.4006, determined by log-rank (Mantel-Cox) test with *n*=200). B) The inhibition of *CG2076/GHITM* along with the overexpression of *Buffy* in the DA neurons resulted in the suppression of the age-dependent loss in climbing ability. The genotypes are *Ddc-Gal4 UAS-Buffy/ UAS-lacZ* and *Ddc-Gal4 UAS-Buffy/ UAS-GHITM-RNAi.* Analysis was done by nonlinear fitting of the climbing curves and significance was determined by comparing the 95% CI (0.039 to 0.052 compared with 0.039 to 0.051). Error bars indicate SEM and *n*=50.

increases the general "healthspan" of *GHITM-RNAi* flies as it improves survival and locomotor function when *CG2076/ GHITM* is inhibited in these neurons.

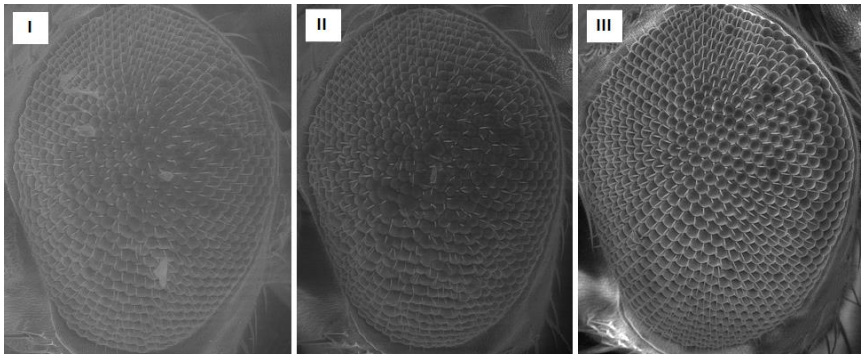
Inhibition of *CG2076/GHITM* in the eye decreases ommatidia number and increases disruption, phenotypes that are rescued upon *Buffy* overexpression

The inhibition of *CG2076/GHITM* in the eye under the direction of the *GMR-Gal4* transgene decreases ommatidia number and results in significant disruption of the ommatidial array (Figure 13A, II and 13B) as determined by an unpaired T-test $p<0.0001$. The overexpression of *Buffy* along with the inhibition of *CG2076/GHITM* restored the number of ommatidia and the percentage disruption to control levels as determined by an unpaired T-test, $p>0.50$ (Figure 13A, III and 13C). Taken together, these results suggest that CG2076/GHITM may play a developmental role in the Drosophila eye and that *Buffy* suppresses the developmental eye defects that result from its inhibition.

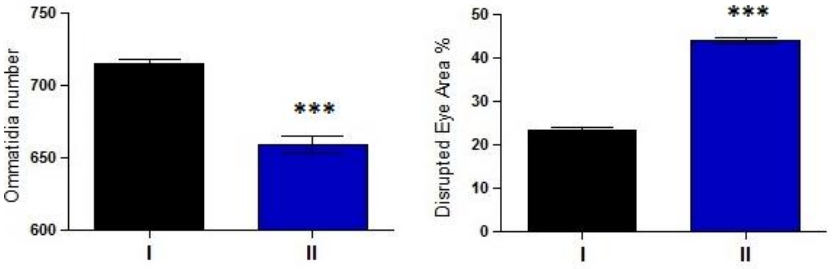
Discussion

The precise function of the BI-1 consensus motif (UPF0005) is not known, but it encodes six to seven transmembrane spanning domains that are highly conserved in many species and signifies an important biological function [8]. Bioinformatic analysis of protein sequences from previous work [2] and our own study showed CG2076 and CG1287 to be the strongest candidates for Drosophila GHITM; CG2076 appears to be the closest with a sequence identity of 56% and 73% similarity, though this does not exempt CG1287, our main consideration was the high degree of similarity to human GHITM as determined by BLAST. Therefore, we propose that CG2076 is the Drosophila homologue of GHITM/TMBIM5.

A.



B.



C.

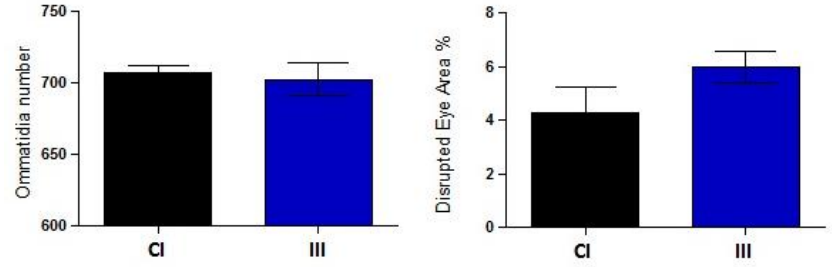


Figure 6.13 Inhibition of *CG2076/GHITM* in the eye results in decreased ommatidia and increased disruption of the ommatidial array

A) Scanning electron micrographs when *CG2076/GHITM* is inhibited in the eye and co-expressed along with *Buffy*. The genotypes are (I) *GMR-Gal4/ UAS-lacZ;* (II) *GMR-Gal4/ UAS-GHITM-RNAi;* and (III) *UAS-Buffy; GMR-Gal4/ UAS-GHITM-RNAi*. B) Biometric analysis when *CG2076/GHITM* is inhibited in the eye indicates decreased ommatidia number ($P<0.0001$) and higher percentage of ommatidial disruption ($P<0.0001$) when compared to the control. C) Co-expression of *Buffy* with *GHITM-RNAi* resulted in the suppression of the eye phenotypes, ommatidia number ($P = 0.7302$) and disruption of the eye ($P = 0.1439$) were restored to control levels (CI is *UAS-Buffy; GMR-Gal4/ UAS-lacZ*) as determined by biometric analysis. Comparisons were determined by unpaired two-tailed T-test ($P<0.05$), error bars are SEM, $n=10$ and asterisks (*) represent statistical significance.

The inhibition of *CG2076/GHITM* using RNA interference under the direction of the *Ddc-Gal4* transgene in the dopaminergic neurons of Drosophila resulted in decreased median survival and severely impaired climbing ability. The general wellbeing of these flies was highly compromised as demonstrated by the shortened lifespan and premature retardation in climbing ability. GHITM is a mitochondria inner membrane protein that possesses an N-terminal presequence that is important for its expression [7, 8]. The presence of a mitochondria targeting sequence firmly localizes it to the mitochondria, while the loss of *GHITM* function induces cell death [5]. This cell death has been attributed to the fragmentation of the mitochondria and the subsequent release of the apoptogenic factor cytochrome c. In addition, GHITM was reported to be physically associated with cytochrome c. Dopaminergic neurons are sensitive to energy requirements and mitochondrial dysfunction is the main culprit that leads to their degeneration and death [30]. The integrity of the mitochondria is vital to the survival of these important motor neurons and any disruption in their function is implicated in disease including Parkinson disease [30-32]. The results obtained suggest a strong role for *CG2076/ GHITM* in neuroprotection in Drosophila since the loss of function in the *Ddc-Gal4*-expressing neurons results in shortened lifespan and impaired climbing ability. The observed *GHITM*-induced cell death is possibly through mitochondrial dysfunction.

The pro-survival *Bcl-2* family of proteins are known to protect the mitochondria from breach by the pro-apoptotic members and releasing a variety of apoptogenic molecules that include cytochrome c [33]. The sole pro-survival *Bcl-2* homologue in Drosophila is *Buffy* [19], and the overexpression of *Buffy* along with the inhibition of *CG2076/GHITM* resulted in the suppression of the *CG2076/GHITM*-induced phenotypes. The

overexpression of *GHITM* partially blocks the release of cytochrome c during apoptosis [5], while its downregulation induced a failure to maintain normal mitochondrial network and disorganization of the cristae. We have previously shown that *Buffy* rescues *Ddc-GAL4*-expressing neurons when co-expressed with the neurotoxic *α-synuclein* [25], or the pro-apoptotic *Debcl* [22], or the Parkinson disease related *H*igh *t*emperature *r*equirement *A2* (*HtrA2*) [34]. This Buffy protection may be induced by pro-survival pathways though it is possible that Buffy regulates mitochondrial cell death as the phenotypes that result from the inhibition of *CG2076/GHITM* are rescued by *Buffy*. This in addition highlights the protective role of *CG2076/GHITM* in the neurons as its phenotypes can be rescued by the overexpression of the pro-survival *Buffy*.

The inhibition of *CG2076/GHITM* in the Drosophila eye under the direction of the *GMR-Gal4* transgene results in a depressed number of ommatidia, this was mostly due to the fusion of the ommatidia and the extensive ommatidial disarray. The inhibition of *CG2076/GHITM* in the Drosophila eye seems to exacerbate the *Gal4*-induced apoptosis that manifests as roughened eye phenotype [35]. The overexpression of *Buffy* along with the inhibition of *CG2076/GHITM* results in the suppression of the *Gal4* and the *Gal4* in addition to the *GHITM-RNAi* phenotypes, with the number of ommatidia and the degree of roughened eye restored to control levels. Buffy seems to ameliorate this phenotype possibly via a general action on survival signals through the mitochondria or through a concerted function to rescue *GHITM*-induced apoptosis at the mitochondria.

Conclusions

CG2076 appears to be the *GHITM/TMBIM5* homologue in Drosophila based on sequence homology, the presence of a mitochondria targeting signal, and a 43 amino acids

presequence, features that it strongly shares with the human *GHITM* transcript. The inhibition of *CG2076/GHITM* in the *Ddc-GAL4*-expressing neurons of Drosophila results in a severely shortened lifespan and an age-dependent loss in climbing ability, phenotypes that are strongly associated with the degeneration and loss of DA neurons, and may as well point to a novel model of Parkinson disease. The overexpression of the pro-cell survival *Buffy* along with the inhibition of *CG2076/ GHITM* results in the rescue of the observed phenotypes.

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Chapter 7 - A loss of *Pdxk* model of Parkinson Disease in Drosophila can be suppressed by *Buffy*

A version of this chapter has been submitted to BMC Research Notes (M'Angale and Staveley, 2016).

Abstract

The identification of a DNA variant in *pyridoxal kinase (Pdxk)* associated with increased risk to Parkinson disease (PD) gene led us to study the inhibition of this gene in the dopaminergic (DA) neurons of the well-studied model organism *Drosophila melanogaster*. The multitude of biological functions attributable to the vitamers catalysed by this kinase reveal an overabundance of possible links to PD, that include dopamine synthesis, antioxidant activity and mitochondrial function. Drosophila possesses a single homologue of *Pdxk* and we used RNA interference to inhibit the activity of this kinase in the DA neurons. The directed inhibition of *Pdxk* resulted in reduced lifespan and impaired locomotor ability. We investigated any association between this enhanced disease risk gene with the established PD model induced by expression of *α-synuclein* in the DA neurons. The results showed the observed decreased lifespan and impaired climbing ability were not supplemented by the inhibition of *Pdxk*. Interestingly, when we co-expressed *Pdxk-RNAi* with the anti-apoptotic *Bcl-2* homologue *Buffy*, we saw a suppression of the phenotypes. To drive the expression of *Pdxk* RNA interference in DA neurons of Drosophila we used *Ddc-Gal4* which resulted in decreased longevity and compromised climbing ability, phenotypes that are strongly associated with Drosophila models of PD. The inhibition of *Pdxk* in the *α-synuclein*-induced Drosophila model of PD did not alter longevity and climbing ability of these flies. It has been previously shown that deficiency in vitamers lead to mitochondrial dysfunction and neuronal decay, therefore, co-expression of *Pdxk-RNAi* with the sole pro-survival *Bcl-2* homologue *Buffy* in the DA neurons, resulted in increased survival and a restored climbing ability. In a similar manner, when we inhibited *Pdxk* in the developing eye using *GMR-Gal4*, we

found that there was a decrease in the number of ommatidia and the disruption of the ommatidial array was more pronounced. When *Pdxk* was inhibited with the *α-synuclein*-induced developmental eye defects, the eye phenotypes were unaltered. Interestingly co-expression with *Buffy* restored ommatidia number and decreased the severity of disruption of the ommatidial array. Though *Pdxk* is not a confirmed Parkinson disease gene, the inhibition of this kinase recapitulated the PD-like symptoms of decreased lifespan and loss of locomotor function, possibly producing a new model of PD.

Background

Parkinson disease (PD), the second most common human neurodegenerative disorder, after Alzheimer disease, afflicts about 1 % of the population over the age of 50 years of age [1]. The clinical disorders associated with PD are a variable combination of bradykinesia, postural instability, tremor, and rigidity, with a typical positive response to Levodopa [2]. Non-motor symptoms that include autonomic dysfunction, cognitive decline and psychiatric problems can also present [3]. These end-points are mainly attributed to the loss of dopaminergic (DA) neurons of the *substantia nigra* with the degeneration of the nigrostriatal dopaminergic system. However, the neuropathology of PD is known to be more widespread, with many non-dopaminergic nuclei affected, including the locus coeruleus, the brain stem, raphe nucleus, dorsal motor nucleus of the vagus, basal nucleus of the Meynert, amygdala, and hippocampus [4]. PD is characterized by the presence of neuronal inclusions composed of abnormal α-synuclein and generally referred to as Lewy-related pathology [2, 5]. This atypical protein accumulation is believed to lead to cellular toxicity and, eventually, the PD pathogenesis. A majority of PD cases are idiopathic but the emergence of familial cases led to the identification and

study of genes that are highly associated with PD [6, 7]. The understanding and exploitation of the genetic basis of PD has revealed over 20 genes that are implicated in PD pathogenesis[8], and highlighted the complexity of this neurodegenerative disease. The link between vitamin B_6 and PD incidence has been explored for years, with some studies associating dietary vitamin B_6 with reduced effectiveness of Levodopa [9]. Other studies have shown the advantages of a higher dietary vitamin B_6 and the reduced risk of PD [10] or reported low dietary intake of vitamin B_6 with increased risk to PD [11], either via its antioxidant abilities or through dopamine biosynthesis. Vitamin B_6 is comprised of three pyridine derivatives or vitamers – which are chemical compounds that have a similar molecular structure and possess similar vitamin activity - known as pyridoxine (PN), pyridoxamine (PM), pyridoxal (PL) and their phosphorylated products pyridoxine-5'-phosphate (PNP), pyridoxamine-5'-phosphate (PMP) and pyridoxal-5'-phosphate (PLP) (See Appendix 6) [12, 13]. PLP is the most metabolically active form and responsible for more than 100 enzymatic reactions [12], predominantly in amino acid metabolism, and is implicated in nervous system function (neurotransmitter synthesis), red blood cell formation (heme biosynthesis), vitamin formation, one-carbon metabolism (nucleic acid synthesis) and as a potent antioxidant [14]. In neuronal function, PLP plays a key role in the metabolism of neurotransmitters, including dopamine, serotonin, glycine, GABA, glutamate, D-serine and histamine [12]. The deficiency of vitamin B_6 has been implicated in increased risk of cancer, neural decay and accelerated ageing. Mitochondrial oxidative decay is a major contributor to ageing [15, 16]. Mitochondrial function is more dependent on PLP than any other organelle as PLP function as a coenzyme for transaminases that are involved in the catabolism of all amino acids by the

urea cycle of the mitochondria [16]. PLP is involved in diverse biochemically important roles in the mitochondria including maintaining energy pathways, homocysteine and glutathione (an antioxidant) biosynthesis. The heme biosynthesis occurs predominantly in the mitochondria and depends on PLP as a coenzyme. The inadequate synthesis of heme can cause mitochondrial decay and oxidative DNA damage [15], whereas its inhibition can cause oxidant leakage, that increases cellular endogenous ROS formation. Vitamin B_6 has a direct antioxidant activity by preventing superoxide radical formation, glycated haemoglobin formation and erythrocyte lipid peroxidation [17]. The inter-conversion of the pyridines to the biologically active phosphate derivative PLP require the action of pyridoxal kinase, thus, the activation of vitamin B_6 to its active form, PLP, requires pyridoxal kinase.

Pyridoxal kinase (Pdxk) belongs to the phosphotransferase family of proteins that are involved in the phosphorylation of vitamin B_6 to pyridoxal-5-phosphate an important co-factor in intermediary metabolism [18, 19]. They contain a ribokinase/pyridoxal domain and are highly conserved, being found in yeast, plants and animals. The association of the gene coding for *Pdxk* with Parkinson disease was through whole-genome expression profiling of human dopaminergic (DA) neurons, combined with association analysis in differentially regulated genes [20]. A DNA variant, single nucleotide polymorphism, in the *Pdxk* gene has been associated with an increased risk to PD [20], though other studies ruled out the association of the variant *rs2010795* with PD in a cohort of patients [21]. The study did not rule out the existence of other Pdxk variants that may increase the risk for PD. The development of model systems to study potential therapies is important, and as such *Drosophila melanogaster* is a good model organism to study the pathophysiology

of movement disorders [22]. The first Drosophila model of PD utilized a human α-synuclein transgene to induce the PD-like symptoms [23]. The success of this model is its ability to recapitulate features of human PD such as 1) age-dependent loss in locomotor function 2) LB-like inclusions and 3) age-dependent loss of DA neurons; and therefore has found wide use for studying the molecular basis of α-synuclein-induced neurodegeneration [22-29]. The utilization of the UAS/GAL4 spatio-temporal expression system [30], and the availability of a plethora of promoters or enhancers of which *TH-Gal4*, *elav-Gal4* and *Ddc-Gal4* are employed in modelling PD in flies, makes Drosophila a powerful model organism [22-29]. We have previously shown the pro-survival advantages of *Buffy*, the sole anti-apoptotic *Bcl-2* homologue in Drosophila by its rescue of the *α-synuclein*-induced phenotypes [31], we extended this study to investigate whether *Buffy* would rescue the loss of *Pdxk*-induced phenotypes in Drosophila. The development of PD models in Drosophila has therefore, many advantages that include extensive genetic resources including a bipartite expression system, and a genome with over 70% conservation of human disease genes.

Materials and Methods

Drosophila media and culture

Stocks and crosses were maintained on a standard medium prepared from cornmeal, molasses, yeast, agar, water and treated with propionic acid and methylparaben. Aliquots of media were poured into plastic vials, allowed to solidify, and refrigerated at 4° C until used. Stocks are kept at room temperature while crosses and experiments were carried out at 25° C and 29° C.

Drosophila stocks and derivative lines

UAS-Buffy [36] was generously supplied by Dr. Leonie Quinn (University of Melbourne), *UAS-α-synuclein* [23] by Dr. Mel Feany of Harvard Medical School, and *Ddc-Gal4* [38] by Dr. Jay Hirsch (University of Virginia). *UAS-Pdxk-RNAi (P{KK108240}VIE-260B)* was obtained from Vienna Drosophila Resource Center while *GMR-Gal4* [39] and *UAS-lacZ* flies were obtained from the Bloomington Drosophila Stock Center at Indiana University. The *UAS-α-synuclein/CyO; Ddc-Gal4/TM3; UAS-α-synuclein/CyO; GMR-Gal4; UAS-Buffy/CyO; Ddc-Gal4;* and *UAS-Buffy/CyO; GMR-Gal4* derivative lines were generated using standard recombination methods and used to overexpress either *α-synuclein* or *Buffy* in the DA neurons using the *Ddc-Gal4* transgene or in the developing eye using the *GMR-Gal4* transgene. PCR reactions and gel electrophoresis were used for analysis of recombination events.

Ageing assay

Several crosses of five females and three males were made and a cohort of males collected upon eclosion. At least two hundred flies were aged per genotype at a density of ≤ 20 flies per vial on fresh media, replenished every other day, to avoid crowding. Flies were observed and scored every two days for presence of deceased adults. As a rule, flies were considered dead when they did not display movement upon agitation [40]. Longevity data was analysed using the GraphPad Prism version 5.04. Survival curves were compared using the Log-rank (Mantel-Cox) test and significance was determined at 95%, at a $P \leq 0.05$ with Bonferroni correction.

Climbing assay

A cohort of male flies was collected upon eclosion and scored for their ability to climb according to a standard protocol [41, 42]. Every 7 days, 50 or fewer males of each genotype were assayed for their climbing ability. Climbing analysis was performed using the GraphPad Prism version 5.04 and climbing curves were fitted using non-linear regression and compared using 95% confidence interval with a 0.05 P-value.

Scanning electron microscopy of the drosophila eye

Crosses between five females and three males were made of each genotype at 29^{o} C and a cohort of adult male flies collected upon eclosion and aged for three days before being frozen at -80^{o} C. Whole flies were mounted on scanning electron microscope stubs, desiccated overnight and photographed with a FEI Mineral Liberation Analyzer 650F scanning electron microscope. For each cross ten eye images were analysed using the National Institutes of Health (NIH) ImageJ software [43] and biometric analysis performed using GraphPad Prism version 5.04. The percentage disruption of the eye was calculated as previously described [44].

Results

Pdxk is evolutionarily conserved across diverse species

The bioinformatic analysis of protein sequences encoded by the human, mouse, mosquito and fruit fly *Pdxk* gene revealed a highly conserved ribokinase/pyridoxal kinase domain (Figure 1). Comparison of the human and the Drosophila homologues reveal a 46% identity and a 64% similarity. The predicted Drosophila protein seems to possess active sites and motifs, including the ATP binding, a weak nuclear export signal (NES), nuclear localization signal (NLS), and transmembrane (TM) domains as determined by NCBI

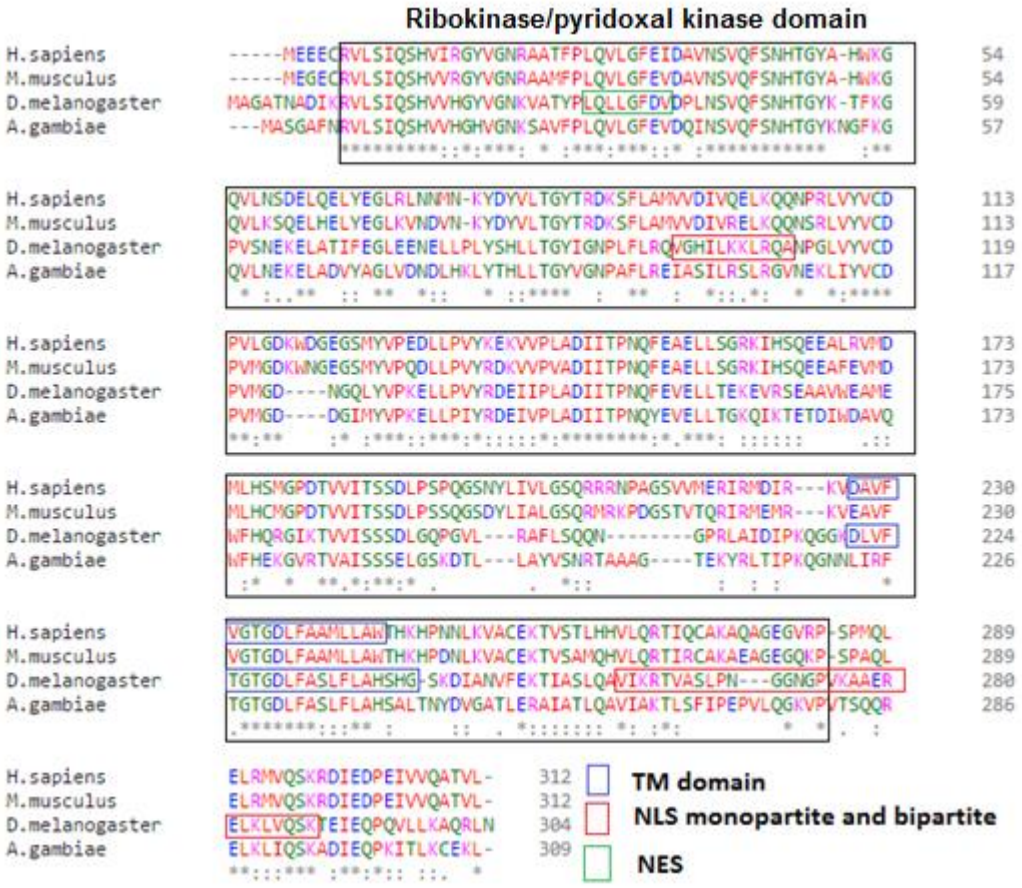


Figure 7.1 The ribokinase/pyridoxal domain is evolutionarily conserved

A Clustal Omega multiple sequence alignment [34, 35] of *Drosophila melanogaster* Pdxk with that of mammalian and insect homologues shows an evolutionarily conserved kinase domain (H.sapiens is *Homo sapiens* NP_003672.1, M.musculus is *Mus musculus* NP_742146.1, A.gambiae is *Anopheles gambiae* XP_315959.4, and D.melonogaster is *Drosophila melanogaster* NP_996031.1). The Drosophila transcript shows presence of motifs for NLS, a weak NES, MTS, and TM domains. Domains were identified using the NCBI Conserved Domain Database Search (CDD) [32] and the Eukaryotic Linear Motif resource search [33]. "*" indicate the residues that are identical, ":" indicate the conserved substitutions, "." indicate the semi-conserved substitutions. Colours show the chemical nature of amino acids. Red is small hydrophobic (including aromatic), Blue is acidic, Magenta is basic, and Green is basic with hydroxyl or amine groups.

Conserved Domain Database Search (CDD) [32] and Eukaryotic linear motif (ELM) resource search [33]. The alignment of protein sequences using Clustal Omega multiple sequence alignment [34, 35] demonstrates the high level of conservation of the kinase domain.

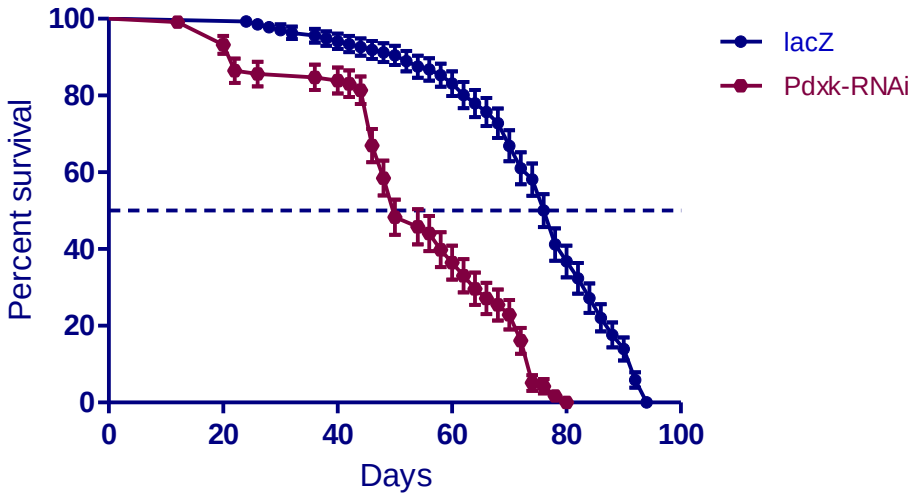
Loss of *Pdxk* decreases lifespan and locomotor ability

The inhibition of *Pdxk* in the DA neurons by RNA interference results in flies with a decreased lifespan and impaired locomotor function. The median lifespan for *Pdxk-RNAi* flies was determined to be 50 days compared to 71 days for the control flies (Figure 2A). When *Pdxk* was suppressed in the DA neurons the flies had impaired locomotor ability as determined by the nonlinear fitting of the climbing curves (Figure 2B). Taken together, these results indicate an important role for this kinase in the DA neurons of Drosophila as interference with its activity phenocopies some of the well-established phenotypes observed in other Drosophila models of PD.

Inhibiting *Pdxk* does not suppress *α-synuclein*-induced phenotypes

The co-expression of *Pdxk-RNAi* with the expression of *α-synuclein* does not alter the diminished longevity nor does it change the observed loss of climbing ability over time. The median lifespan was 58 days for *Pdxk* flies compared to 60 days for control flies (Figure 3A). A comparison of the climbing curves at 95% CI revealed they were not significantly different from each other (Figure 3B). This implies that the inhibition of *Pdxk* in the DA neurons does not influence the neurotoxic effects of α-synuclein.

A.



B.

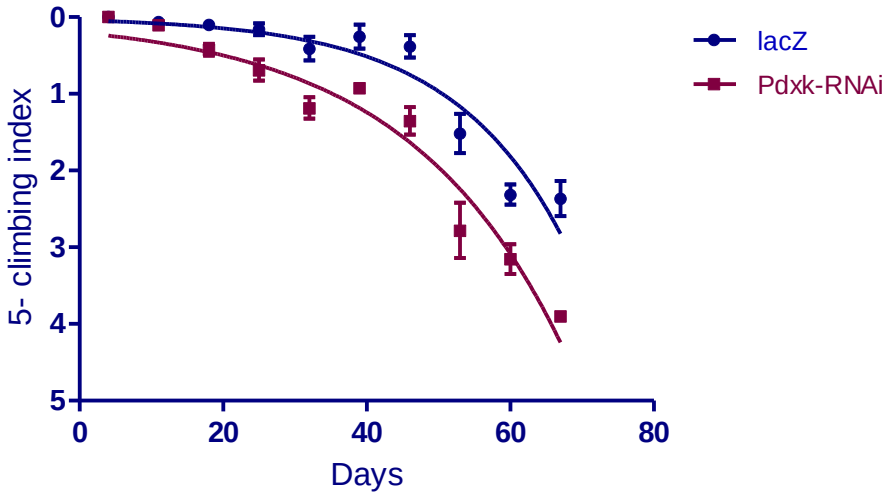
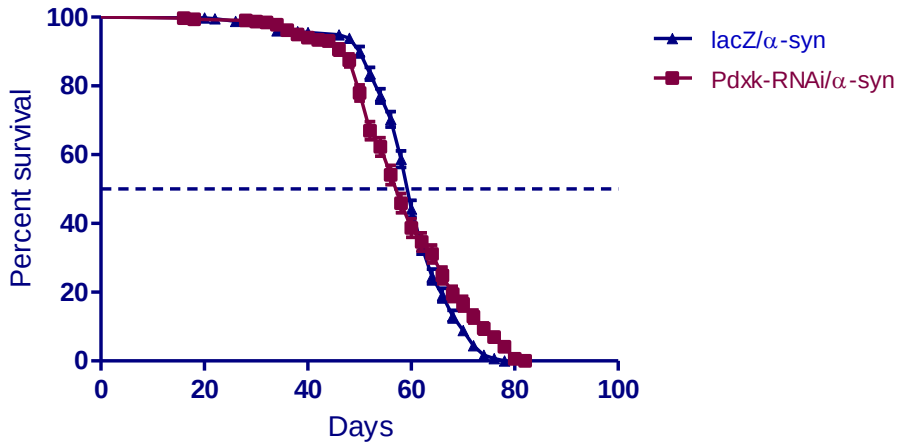


Figure 7.2 Inhibiting *Pdxk* activity in DA neurons decreases lifespan and impairs locomotor function

A) The directed expression of *Pdxk-RNAi* in DA neurons using the *Ddc-Gal4* transgene results in a decrease in median lifespan when compared to control flies expressing *UAS-lacZ*. The genotypes are *UAS-lacZ/ Ddc-Gal4* and *Pdxk-RNAi/ Ddc-Gal4.* Longevity is shown as percent survival ($P < 0.05$, determined by the log-rank (Mantel-Cox) test and $n \geq 200$). B) The inhibition of *Pdxk* in the DA neurons resulted in a significant decrease in climbing ability as determined by nonlinear fitting of the climbing curves and comparing 95% CI. The genotypes are *UAS-lacZ/ Ddc-Gal4* and *Pdxk-RNAi/ Ddc-Gal4.* Error bars indicate SEM and $n=50$.

A.



B.

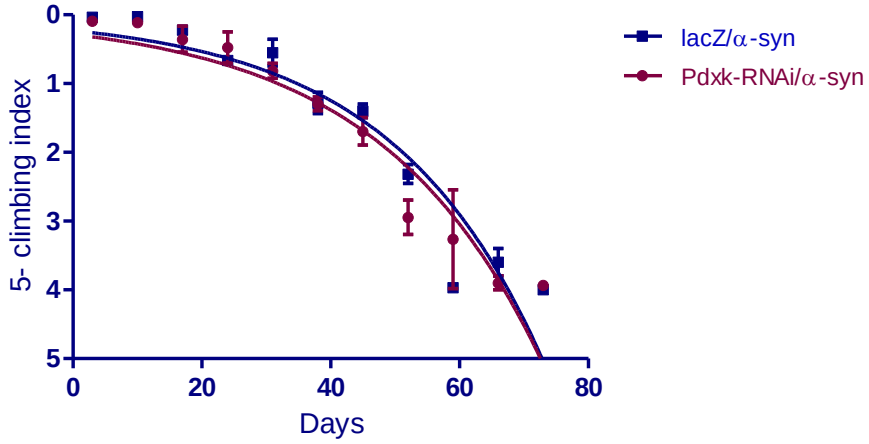


Figure 7.3 Inhibition of *Pdxk* in the *α–synuclein*-induced PD model does not alter phenotypes

A) The inhibition of Pdxk with *α-synuclein* in the DA neurons showed no significant change in lifespan when compared to the control. Genotypes are *UAS-α-synuclein; Ddc-Gal4/ UAS-lacZ* and *UAS-α-synuclein; Ddc-Gal4/ Pdxk-RNAi.* Longevity is shown as percent survival ($P < 0.05$, determined by log-rank (Mantel-Cox) test with $n \leq 200$). B) The co-expression of *Pdxk-RNAi* in the *α-synuclein* model of PD did not result in any significant age-dependent loss in climbing ability compared to the control. The genotypes are *UAS-α-synuclein; Ddc-Gal4/ UAS-lacZ* and *UAS-α-synuclein; Ddc-Gal4/ Pdxk-RNAi.* Analysis was done by nonlinear fitting of the climbing curves and significance was determined by comparing the 95% confidence intervals. Error bars indicate SEM and $n=50$.

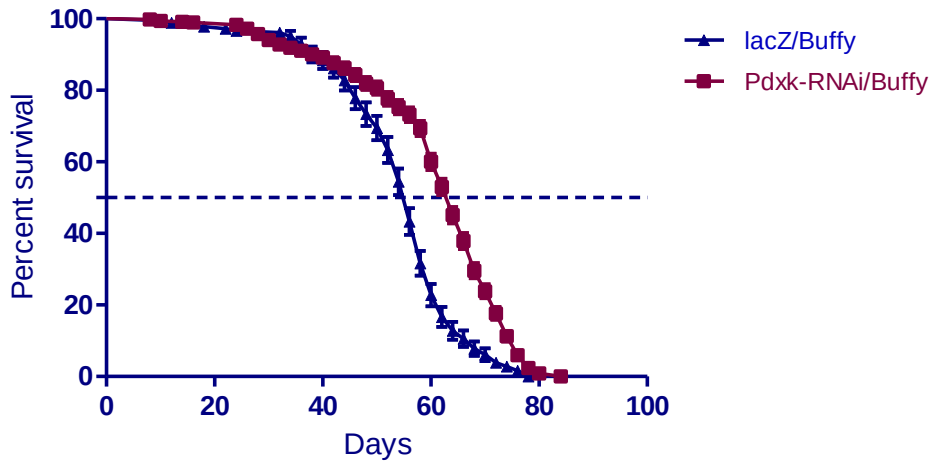
The pro-survival *Buffy* suppresses the loss of *Pdxk* phenotypes

The co-expression of the pro-survival *Bcl-2* homologue *Buffy* with *Pdxk-RNAi* in DA neurons results in a slightly increased lifespan and an improved climbing ability. The median survival of *Pdxk-RNAi* flies with co-expression of *Buffy* was 64 days when compared to 60 days for the controls, as determined by Log-rank at a $p<0.0001$ (Figure 4A). The climbing ability was slightly improved as determined by a nonlinear fitting of the climbing curves and compared at 95% CI (Figure 4B). These results suggest a pro-survival role for *Buffy*, slightly increasing survival and significantly improving locomotor function when *Pdxk* is inhibited in the DA neurons.

Inhibition of *Pdxk* in the developing eye results in disruption of the ommatidial array that can be suppressed by *Buffy*

The inhibition of *Pdxk* in the developing eye directed by the *GMR-Gal4* transgene results in eyes with a lower mean number of ommatidia and a greater disruption of the ommatidial array (Figure 5A) as determined by an unpaired T-test. The inhibition of *Pdxk* with *α-synuclein* expression did not result in marked differences in either the number of ommatidia or the disruption of the eye (Figure 5B). It seems that the inhibition of *Pdxk* in the developing eye along with *α-synuclein* expression does not result in a more roughened eye phenotype. When we co-expressed *Pdxk-RNAi* with *Buffy*, the number of ommatidia and the disruption of the array was restored to normal mean values as indicated by an unpaired T-test (Figure 5C). Taken together, these results suggest that *Buffy* suppresses the developmental eye defects resulting from the inhibition of *Pdxk* in the developing eye.

A.



B.

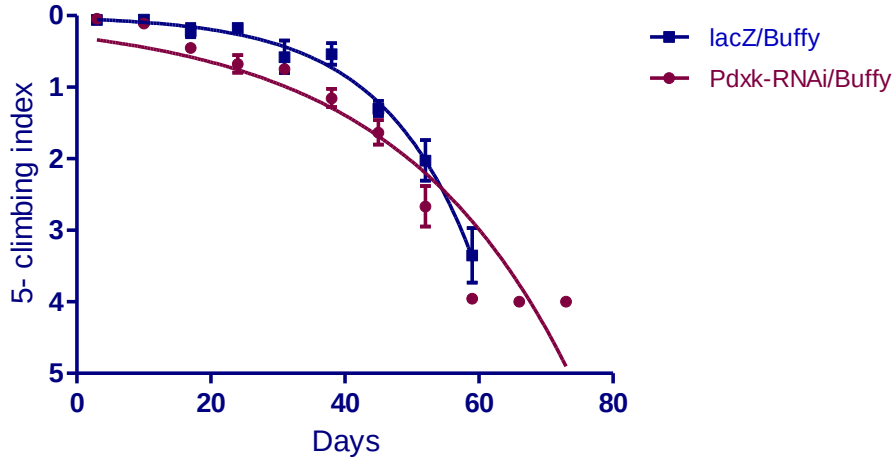


Figure 7.4 The co-expression of *Pdxk-RNAi* with *Buffy* suppresses the *Pdxk*-induced phenotypes

A) The co-expression of *Buffy* with *Pdxk-RNAi* result in the suppression of the observed phenotype of decreased survival. Genotypes are *Ddc-Gal4 UAS-Buffy/ UAS-lacZ* and *Ddc-Gal4 UAS-Buffy/ Pdxk-RNAi.* Longevity is shown as percent survival ($P < 0.05$, determined by log-rank (Mantel-Cox) test with $n \leq 200$). B) The co-expression of *Pdxk-RNAi* with *Buffy* in the DA neurons results in the suppression of the age-dependent loss in climbing ability. The genotypes are *Ddc-Gal4 UAS-Buffy/ UAS-lacZ* and *Ddc-Gal4 UAS-Buffy/ Pdxk-RNAi.* Analysis was done by nonlinear fitting of the climbing curves and significance was determined by comparing the 95% confidence interval. Error bars indicate SEM and $n=50$.

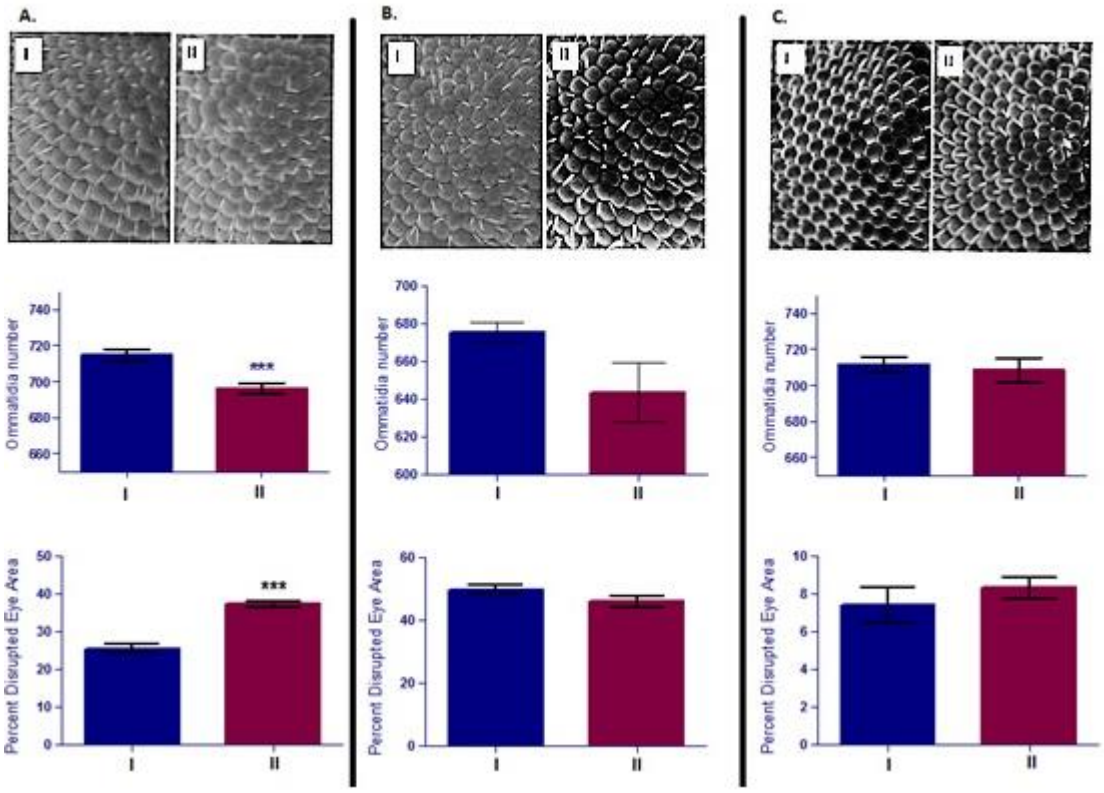


Figure 7.5 The conditional expression of *Pdxk* in the Drosophila eye results in reduced ommatidia number and increased disruption of the eyes and is suppressed upon co-expression with *Buffy*

A) Scanning electron micrographs when *Pdxk* is inhibited in the eye (I) *GMR-Gal4/ UAS-lacZ* and (II) *GMR-Gal4/ Pdxk-RNAi*. The biometric analysis of the eyes indicated a slight decrease in mean ommatidia number and a higher percent disruption of the eye when compared to the control. B) The co-expression of *Pdxk-RNAi* with *α-synuclein*-expression I) *UAS-α-synuclein; GMR-Gal4/ UAS-lacZ* and II) *UAS-α-synuclein; GMR-Gal4/ Pdxk-RNAi.* Biometric analysis of *α-synuclein*-expression and *Pdxk* inhibition in the developing eye revealed no significance in the number of ommatidia and the degree of ommatidial disruption. C) Co-expression of *Buffy* with *Pdxk-RNAi* I) *UAS-Buffy; GMR-Gal4/ UAS-lacZ* and II) *UAS-Buffy; GMR-Gal4/ Pdxk-RNAi.* Biometric analysis showed restoration of the mean number of ommatidia and ommatidial disruption to control levels. Comparisons were determined by unpaired two-tailed T-test ($P<0.05$), error bars are SEM, $n=10$ and asterisks (*) represent statistical significance.

Discussion

The directed inhibition of *Pdxk* in the DA neurons of *Drosophila melanogaster* result in decreased lifespan and an age-dependent loss in climbing ability, phenotypes strongly associated with models of PD. Pyridoxal kinase is involved in the conversion of pyridoxal into pyridoxal-5'-phosphate (PLP), an important enzyme cofactor in intermediary metabolism [14]. These B_6 vitamers seem to be involved in all vital cellular functions, from glucose metabolism to nucleic acid synthesis to being potent antioxidants. Our inhibition of this protein kinase in the DA neurons resulted in shortened lifespan and impaired climbing ability, while in supportive experiments, its inhibition in the developing eye resulted in reduced ommatidia number and a high degree of ommatidial disarray. The involvement of PLP in the conversion of dopa to dopamine is an important step in its synthesis, it seems therefore, that decreased neuronal PLP can mimic the effects of decreased dopamine, a key step in PD development, and possibly of major impact in dopamine-containing cells. It is not surprising therefore, that decrease in the availability of such an important coenzyme by inhibition of the kinase function involved in its conversion results in defective neuronal function that may lead to neurodegeneration.

The overexpression of *α-synuclein* in DA neurons can result in PD-like characteristics in Drosophila [23]. We investigated the possible existence of a link between *α-synuclein*-induced phenotypes and the inhibition of *Pdxk* in the DA neurons of Drosophila. Our results indicate that loss of *Pdxk* function in DA neurons expressing *α-synuclein* did not enhance the *α-synuclein*-induced phenotypes of decreased lifespan and impaired climbing ability and *vice versa*. Similarly, when we conducted similar comparisons in the neuron rich Drosophila eye, we observed that inhibition of *Pdxk* did not alter the consequences of

α-synuclein expression in the developing eye. Therefore, the toxic effects of α-synuclein are sufficient to generate phenotypes but inhibition of *Pdxk* activity does not confer any additional disadvantage. Alternatively, loss-of-*Pdxk*-induced toxicity may precede the effects of α-synuclein accumulation and, as such, additional phenotypes may not be observable. Additionally, the multitude of biological functions that are dependent on PLP from a dysfunctional antioxidant activity to mitochondrial dysfunction may induce neurotoxicity in these sensitive neurons.

The directed expression of the pro-survival *Bcl-2* homologue *Buffy* with *Pdxk* RNA interference resulted in the suppression of the loss of *Pdxk*-induced phenotypes of decreased survival and impaired locomotor ability. Buffy like many other pro-survival Bcl-2 proteins, is thought to be a "guardian" of the mitochondria and confers survival advantages by restricting death-promoting molecules [36, 37]. *Buffy* suppressed the *Pdxk*-induced phenotypes of reduced lifespan and locomotor ability and further restored ommatidia number and decreased the disruption of the ommatidial array. Not only do PLP deficiencies accelerate mitochondrial decay, but they increase ROS radicals [15, 16], a property that make neurons more vulnerable. It is possible that Buffy restores this balance by yet unknown pathways and cellular interactions via pro-survival signals.

Conclusions

Although *Pdxk* has not been confirmed to be a PD-causative gene, it has been associated to increased risk of the disease. The inhibition of this gene activity by the directed expression of an RNAi transgene in the DA neurons phenocopies PD-like symptoms in Drosophila, and therefore may represent a novel model of PD. This appear to corroborate other studies that show several mechanisms involved in the aetiology of PD. More studies

are required to chart out a pathway for *Pdxk* activity in Drosophila, and importantly to show at a molecular level the changes associated with the loss-of-function of this kinase in the development and function of dopaminergic neurons.

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Chapter 8 - The Loss of *Porin/VDAC* function in dopaminergic neurons of Drosophila is suppressed by *Buffy*

A version of this chapter has been published in BioMed Central Journal of Biomedical Sciences 23: 84 DOI: 10.1186/s12929-016-0300-1 (M'Angale and Staveley, 2016).

Abstract

Mitochondrial porin, also known as the voltage-dependent anion channel (VDAC), is a multi-functional channel protein that shuttles metabolites between the mitochondria and the cytosol and implicated in cellular life and death decisions. The inhibition of *porin* under the control of neuronal *Ddc-Gal4* result in short lifespan and in an age-dependent loss in locomotor function, phenotypes that are strongly associated with Drosophila models of Parkinson disease. Loss of *porin* function was achieved through exploitation of RNA interference while derivative lines were generated by homologous recombination and tested by PCR. The *UAS/Gal4* expression system was exploited with directed expression in neurons achieved with the use of the *Dopa decarboxylase* and in the developing eye with the *Glass multiple reporter* transgenes. Statistical analyses for ageing assay employed Log rank (Mantel-Cox) test, climbing indices were fitted with a non-linear curve and confidence intervals compared at 95%. Biometric analysis of the eye phenotypes was obtained by a one-way analysis of variance (ANOVA) followed by a Dunnett's post-test. The expression of *α-synuclein* in neuronal populations that include dopamine producing neurons under the control of *Ddc-Gal4* produces a robust Parkinson disease model, and results in severely reduced lifespan and locomotor dysfunction. In addition, the *porin*-induced phenotypes are greatly suppressed when the pro-survival *Bcl-2* homologue *Buffy* is overexpressed in these neurons and in the developing eye adding to the cellular advantages of altered expression of this anti-apoptotic gene. When we co-expressed *α-synuclein* along with *porin*, it results in a decrease in lifespan and impaired climbing ability. This enhancement of the *α-synuclein*-induced phenotypes observed in neurons was demonstrated in the neuron rich eye, where the simultaneous co-expression

of *porin-RNAi* and *α-synuclein* resulted in an enhanced eye phenotype, marked by reduced number of ommatidia and increased disarray of the ommatidia. The inhibition of *porin* in dopaminergic neurons among others result in reduced lifespan and age-dependent loss in climbing ability, phenotypes that are suppressed by the overexpression of the sole pro-survival *Bcl-2* homologue *Buffy*. The inhibition of *porin* phenocopies Parkinson disease phenotypes in Drosophila, while the overexpression of *Buffy* can counteract these phenotypes to improve the overall "healthspan" of the organism.

Background

The voltage-dependent anion channel (VDAC), also known as mitochondrial porin, consists of small pore-forming proteins present in the outer mitochondrial membrane that act to shuttle nucleotides, metabolites and ions between the mitochondria and the cytoplasm [1, 2]. Porin is a multi-functional protein and is involved in the regulation of metabolism and energetic functions of the mitochondria and a constituent of the mitochondrial permeability transition pore (PTP) [3]. Porin is involved in apoptosis, metabolite transport, calcium transport and signalling, ATP transport, reactive oxygen species transport and endoplasmic reticulum – mitochondrial crosstalk [3-5]. As thus porin appears to be a convergence point for cell death and survival signals, mediated by its association with a variety of ligands and proteins. Porin is implicated in mitochondria-mediated apoptosis and in regulation of apoptosis through interaction with pro-survival proteins [3]. It interacts with the pro-survival hexokinase to mediate its anti-apoptotic activity [3, 6], and the Bcl-2 family of proteins to regulate mitochondria-mediated apoptosis [7, 8]. This association can induce cell survival or death.

The *porin* gene is associated with several neurodegenerative disorders including Alzheimer disease [9], Down syndrome [10], and dopamine-induced apoptosis [11]. The association of porin with Parkinson disease-associated gene products has been established, where it recruits parkin to defective mitochondria to promote mitophagy [12], and shows high affinity interaction with α-synuclein to regulate mitochondrial-induced toxicity [13]. This study suggests that α-synuclein translocate to the mitochondria via porin to target complexes of the mitochondrial respiratory chain. The accumulation and aggregation of abnormal α-synuclein was shown to down-regulate porin [14] and possibly regulate mitochondrial permeability [15]. The association between the PD gene *α-synuclein* and the mitochondrial channel *porin* appears to be important in the progression of PD. The first PD model in Drosophila utilized the expression of a human *α-synuclein* transgene to induce the PD-like phenotypes [16]. The success of this model anchors on its ability to recapitulate features of human PD such as the age-dependent loss in locomotor function and therefore, has found application in the study of *α-synuclein*-induced degeneration [16-23]. The utilization of the *UAS/GAL4* spatio-temporal expression system [24], and the availability of a plethora of promoters or enhancers of which *TH-Gal4*, *elav-Gal4* and *Ddc-Gal4* are employed in modelling PD in flies [16-23], makes Drosophila a useful and albeit a powerful model organism.

The loss of function of Drosophila *porin/VDAC* has been shown to result in mitochondrial morphological defects [25, 26]. These mitochondrial defects were accompanied by locomotor dysfunction and male sterility. In addition, *porin* mutants displayed neurological and muscular defects, mitochondrial respiratory defects, and abnormalities in synaptic transmission and mitochondrial distribution in motor neurons. Here we

suppressed *porin* by RNA interference in Drosophila neurons under the control of the *dopa decarboxylase* transgene and analysed longevity and locomotor ability. Further we co-expressed *porin-RNAi* with *α-synuclein* to investigate its effects in the well-studied Drosophila PD model. The association of porin with Bcl-2 members is well documented, we have demonstrated the benefits of overexpression of the sole anti-apoptotic *Bcl-2* member *Buffy* in conditions of stress [27], as thus, we overexpressed *Buffy* along with *porin-RNAi*. In addition, we altered the expression of *porin* in the Drosophila developing eye and co-expressed with *α-synuclein* and *Buffy*.

Methods

Bioinformatic analysis

The protein sequences were sourced from National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/protein/) and the domains were identified using the NCBI Conserved Domain Database (CDD) [28] (http://www.ncbi.nlm.nih.gov/cdd) and the Eukaryotic Linear Motif [29] (http://elm.eu.org/) which focuses on annotation and detection of eukaryotic linear motifs (ELMs) or short linear motifs (SLiMs). A Clustal Omega multiple sequence alignment (http://www.ebi.ac.uk/Tools/msa/clustalo/) [30, 31] was used to show conservation of the porin3_VDAC domain in the selected organisms. The nuclear export signal (NES) was predicted using the NetNES 1.1 server (http://www.cbs.dtu.dk/services/NetNES/) [32] and TMpred, a program that predicts membrane-spanning regions and their orientation. The algorithm is based on the statistical analysis of TMbase, a database of naturally occurring transmembrane proteins (http://www.ch.embnet.org/software/TMPRED_form.html).

Drosophila media and culture

Stocks and crosses were maintained on standard cornmeal/molasses/yeast/agar media treated with propionic acid and methylparaben to inhibit fungal growth. Stocks were maintained on solid media for two to three weeks before transfer onto new media to re-culture. Stocks were kept at room temperature (22°C ± 2°C) while crosses and experiments were carried out at 25°C and 29°C.

Drosophila stocks

The *P{KK107645}VIE-260B* hereby referred to as *UAS-porin-RNAi (1)* was obtained from Vienna Drosophila Resource Center, *y[1] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.JF03251}attP2/TM3, Sb[1]* hereby known as *UAS-porin-RNAi (2), GMR-Gal4* [33] and *UAS-lacZ* flies were obtained from the Bloomington Drosophila Stock Center at Indiana University. *UAS-α-synuclein* [16] was generously provided by Dr. M. Feany of Harvard Medical School, *Ddc-Gal4* [34] by Dr. J. Hirsch of University of Virginia and *UAS-Buffy* [35] by Dr. Leonie Quinn of University of Melbourne.

Drosophila derivative lines

The *UAS-α-synuclein/CyO; Ddc-Gal4/TM3, UAS-α-synuclein/CyO; GMR-Gal4, UAS-Buffy/CyO; Ddc-Gal4* and *UAS-Buffy/CyO; GMR-Gal4* derivative lines were generated using standard homologous recombination methods as described elsewhere [36, 37] and were used for overexpression of either *α-synuclein* or *Buffy* in DA and other neurons using the *Ddc-Gal4* transgene or in the developing eye using the *GMR-Gal4* transgene.

Ageing assay

Crosses of each genotype were made and a cohort of critical class male flies were collected upon eclosion. At least two hundred flies were aged per genotype at a density of

20 or fewer flies per vial to avoid crowding on fresh media which was replenished every other day. Flies were observed and scored every two days for the presence of deceased adults. Flies were considered dead when they did not display movement upon agitation [38]. Longevity data was analysed using the GraphPad Prism version 5.04 and survival curves were compared using the log-rank (Mantel-Cox) test. Significance was determined at 95%, at a P-value less than or equal to 0.05 with Bonferroni correction.

Climbing assay

A group of critical class male flies was collected upon eclosion and scored for their ability to climb over their lifetime [39]. 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 in 10 repetitions. Analysis was performed using the GraphPad Prism version 5.04 and climbing curves were fitted using non-linear regression and compared using 95% confidence interval with a 0.05 P-value.

Scanning electron microscopy of the Drosophila eye

Several single vial matings were made at 29^{o} C and adult male flies collected upon eclosion and aged for three days before being frozen at -80^{o} C. Whole flies were mounted on scanning electron microscope stubs, desiccated overnight and photographed with a FEI Mineral Liberation Analyzer 650F scanning electron microscope. For each cross at least 10 eye images were analysed using the National Institutes of Health (NIH) ImageJ software [40] and biometric analysis performed using GraphPad Prism version 5.04. The percentage disruption of the eye was calculated as previously described [41]. Statistical comparisons comprised one-way analyses of variance (ANOVA) and Dunnett's multiple comparison tests. P-values less than 0.05 were considered significant.

Results

The human and Drosophila porin domain is conserved

There is 62% identity and 77% similarity between the human porin (VDAC) and the *Drosophila melanogaster* porin protein sequences, with very high conservation within the Porin3_VDAC domain (Figure 1). The putative dimerization interface and putative determinants of voltage-gated binding sites are well conserved as determined by an NCBI conserved domain search [28]. A Eukaryotic linear motif (ELM) resource search for functional sites [42] in the Drosophila transcript indicates the presence of an inhibitor of apoptosis binding motif (IBM) that function in the abrogation of caspase inhibition by inhibitors of apoptosis (IAPs) at amino acids 1 to 5, an Atg8 binding motif at amino acids 5 to 9, a nuclear export signal (NES) at amino acids 91 to 98, a PDZ domain at amino acids 277 to 282 and a transmembrane domain predicted by TMpred.

Inhibition of *porin* in neurons decreases lifespan and severely impairs locomotor function, phenotypes that are suppressed by *Buffy* overexpression

The expression of *porin-RNAi* in *Ddc-Gal4*-expressing neurons results in a slightly decreased lifespan and severely impaired locomotor function as shown by the two RNAi lines that we tested. The median lifespan for these flies was 48 days and 52 days when compared to 68 days for the controls as determined by Log-rank (Mantel-Cox) test with a $p<0.0001$ (Figure 2A). When *porin* is suppressed in these neurons, the flies have impaired locomotor ability as determined by comparison of CI after the nonlinear fit of the climbing curves (Figure 2B). These results suggest a role for *porin* in the normal function of neurons in Drosophila since its reduced activity shortens lifespan and prematurely retards climbing ability.

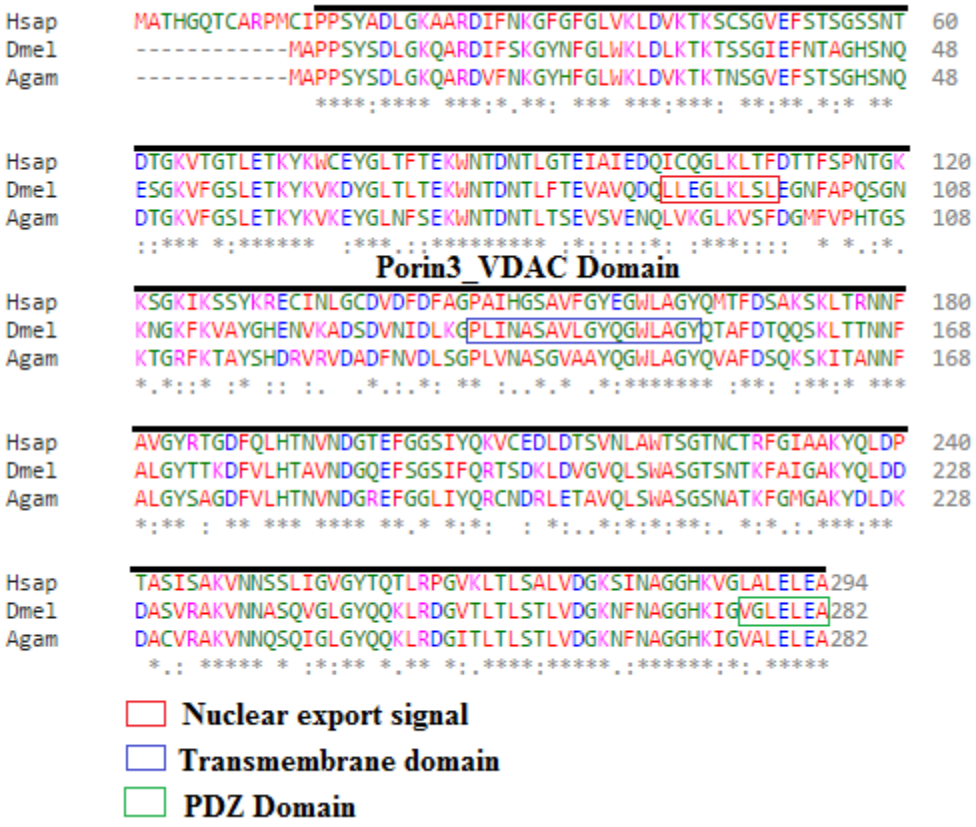
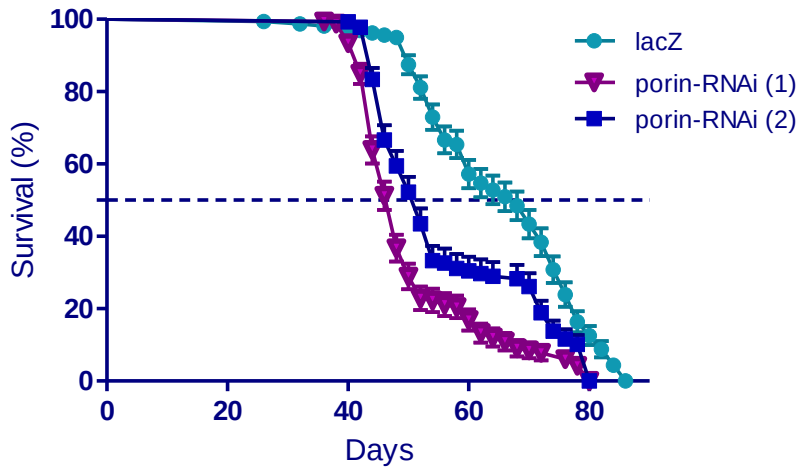


Figure 8.1 Drosophila porin has a conserved Porin3_VDAC domain

The *Drosophila melanogaster porin* gene encodes a 282 amino acids protein and the Porin domain is highly conserved when compared to the human homologue. It shows presence of a nuclear export signal (NES), a transmembrane domain, and a PDZ domain. Domains were identified using the NCBI Conserved Domain Database Search (CDD) [28] and the Eukaryotic Linear Motif resource search [29]. A Clustal Omega multiple sequence alignment [30, 31] show conservation of the porin3_VDAC domain (Hsap is *Homo sapiens* NP_003366.2, Dmel is *Drosophila melanogaster* NP_001033899.1 and Agam is *Anopheles gambiae* XP_318947.2). "*" indicate the residues that are identical, ":" indicate the conserved substitutions, "." indicate the semi-conserved substitutions. Colours show the chemical nature of amino acids. Red is small hydrophobic (including aromatic), Blue is acidic, Magenta is basic, and Green is basic with hydroxyl or amine groups.

A.



B.

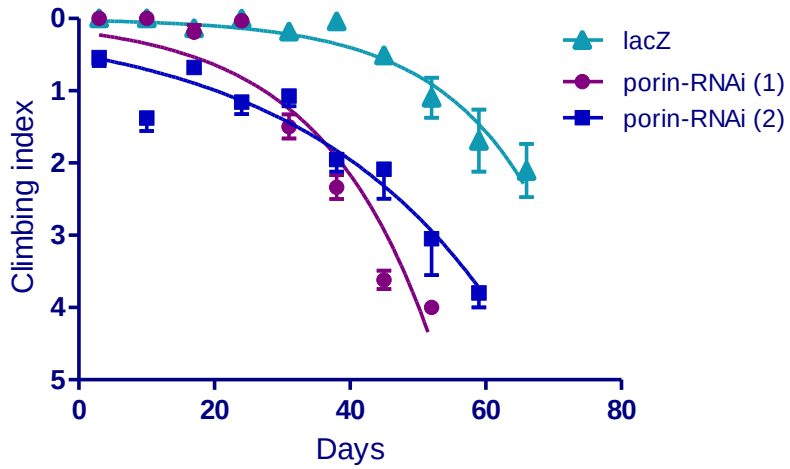


Figure 8.2 Loss of *porin* activity decreases survival and impairs climbing ability

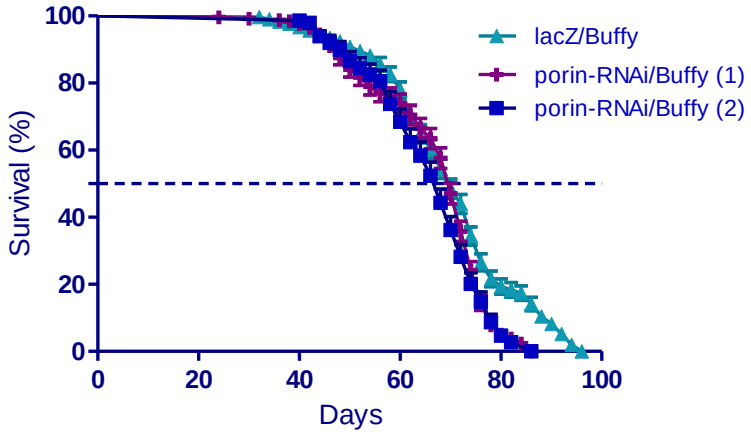
A) The inhibition of *porin* in neurons using the *Ddc-Gal4* transgene results in decreased median lifespan of 48 and 52 days when compared to 68 days for control flies that expresses *UAS-lacZ*. The genotypes are *Ddc-Gal4/ UAS-lacZ*, *Ddc-Gal4/ UAS-porin-RNAi (1)* and *Ddc-Gal4/ UAS-porin-RNAi (2).* Longevity is shown as percent survival ($P < 0.05$, determined by the log-rank (Mantel-Cox) test and $n \geq 200$). B) The inhibition of *porin* in the *Ddc-Gal4*-expressing neurons resulted in a significant decrease in climbing ability as determined by nonlinear fitting of the climbing curves and comparing 95% CI. The genotypes are *Ddc-Gal4/ UAS-lacZ*, *Ddc-Gal4/ UAS-porin-RNAi (1)* and *Ddc-Gal4/ UAS-porin-RNAi (2).* Error bars indicate SEM and $n=50$.

The directed overexpression of the pro-survival *Bcl-2* homologue *Buffy* in these neurons resulted in increased lifespan and improved climbing ability. When *Buffy* is co-expressed with the *porin-RNAi* lines, the results indicate a median lifespan of 70 and 69 days when compared to 72 days for *Buffy* co-expression with *lacZ* control flies as determined by Log-rank test (Figure 3A). The climbing ability of the *porin-RNAi* flies was significantly improved as determined by comparison of climbing curves of *porin-RNAi* flies at 95% CI (Figure 2B) with the flies that express *porin-RNAi* along with *Buffy* and with the control flies that co-expressed *Buffy* along with *lacZ* (Figure 3B). Taken together these results suggest a pro-survival role for *Buffy* as observed by significant increases in the "healthspan" of *porin*-deficient flies.

Inhibition of *porin* enhances *α-synuclein*-dependent phenotypes

The expression of *α-synuclein* in *Ddc-Gal4*-expressing neurons results in impaired locomotor function that has been attributed to cellular toxicity due to the accumulation of this protein. The co-expression of the *porin-RNAi* lines along with *α-synuclein,* decreased survival and impaired climbing ability over time (Figure 4). The median lifespan was 50 and 56 days for flies that expressed *porin-RNAi* along with *α-synuclein,* compared to 60 days for control flies that co-expressed *α-synuclein* along with *lacZ*, a significant decrease in survival for both RNAi lines (Figure 4A) as determined by Log-rank (Mantel-Cox) test ($p<0.0001$). A comparison of the climbing curves by nonlinear fitting at 95% CI revealed they were significantly different (Figure 4B), with CI of 0.04691 to 0.06795 and 0.030 to 0.050 for flies that expressed *porin-RNAi* along with *α-synuclein,* compared to 0.06842 to

A.



B.

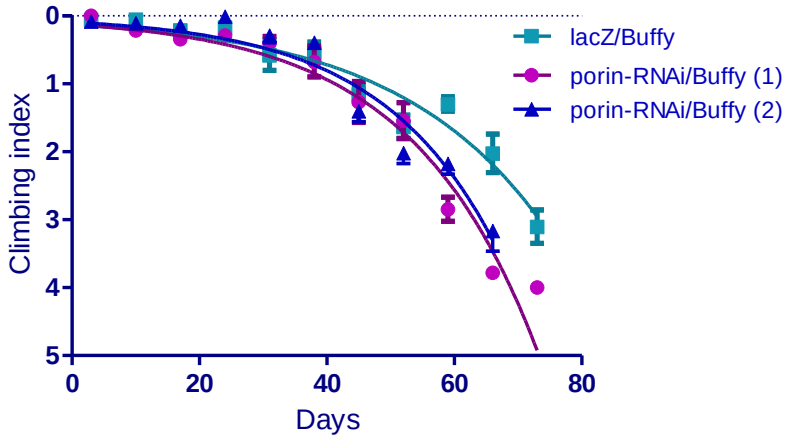
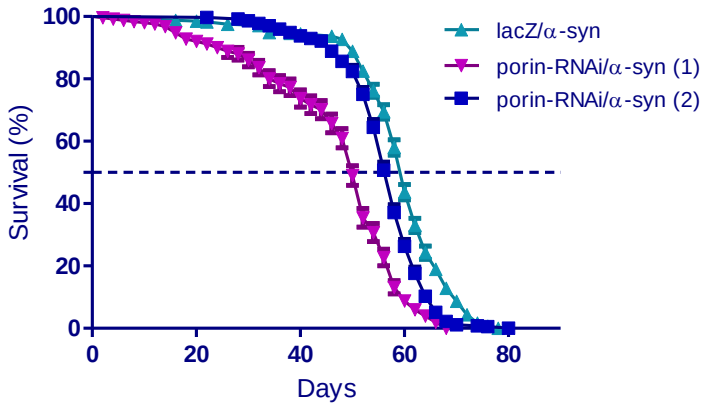


Figure 8.3 Loss of *porin* activity is suppressed by *Buffy*

A) The co-expression of *Buffy* with *porin-RNAi* result in the rescue of the observed phenotype of decreased survival, with a median survival of 70 and 69 days when compared to 72 days for controls. Genotypes are *Ddc-Gal4 UAS-Buffy/ UAS-lacZ, Ddc-Gal4 UAS-Buffy/ UAS-porin-RNAi (1)* and *Ddc-Gal4 UAS-Buffy/ UAS-porin-RNAi (2).* Longevity is shown as percent survival ($P < 0.05$, determined by log-rank (Mantel-Cox) test with $n \leq 200$). B) The inhibition of *porin* along with the overexpression of *Buffy* in the DA neurons results in the suppression of the age-dependent loss in climbing ability. The genotypes are *Ddc-Gal4 UAS-Buffy/ UAS-lacZ, Ddc-Gal4 UAS-Buffy/ UAS-porin-RNAi (1)* and *Ddc-Gal4 UAS-Buffy/ UAS-porin-RNAi (2).* Analysis was done by nonlinear fitting of the climbing curves and significance was determined by comparing the 95% CI. Error bars indicate SEM and $n=50$.

A.



B.

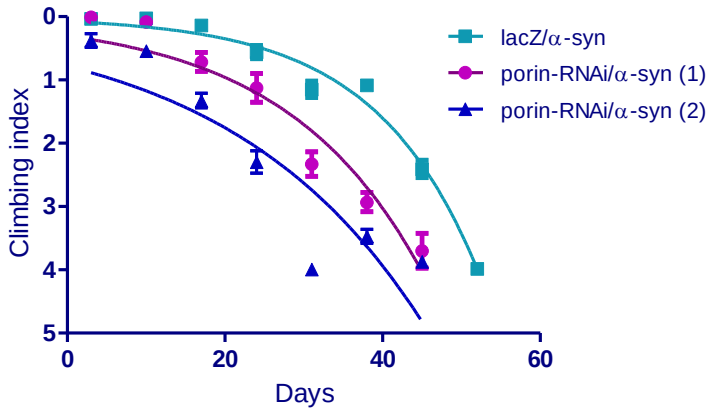


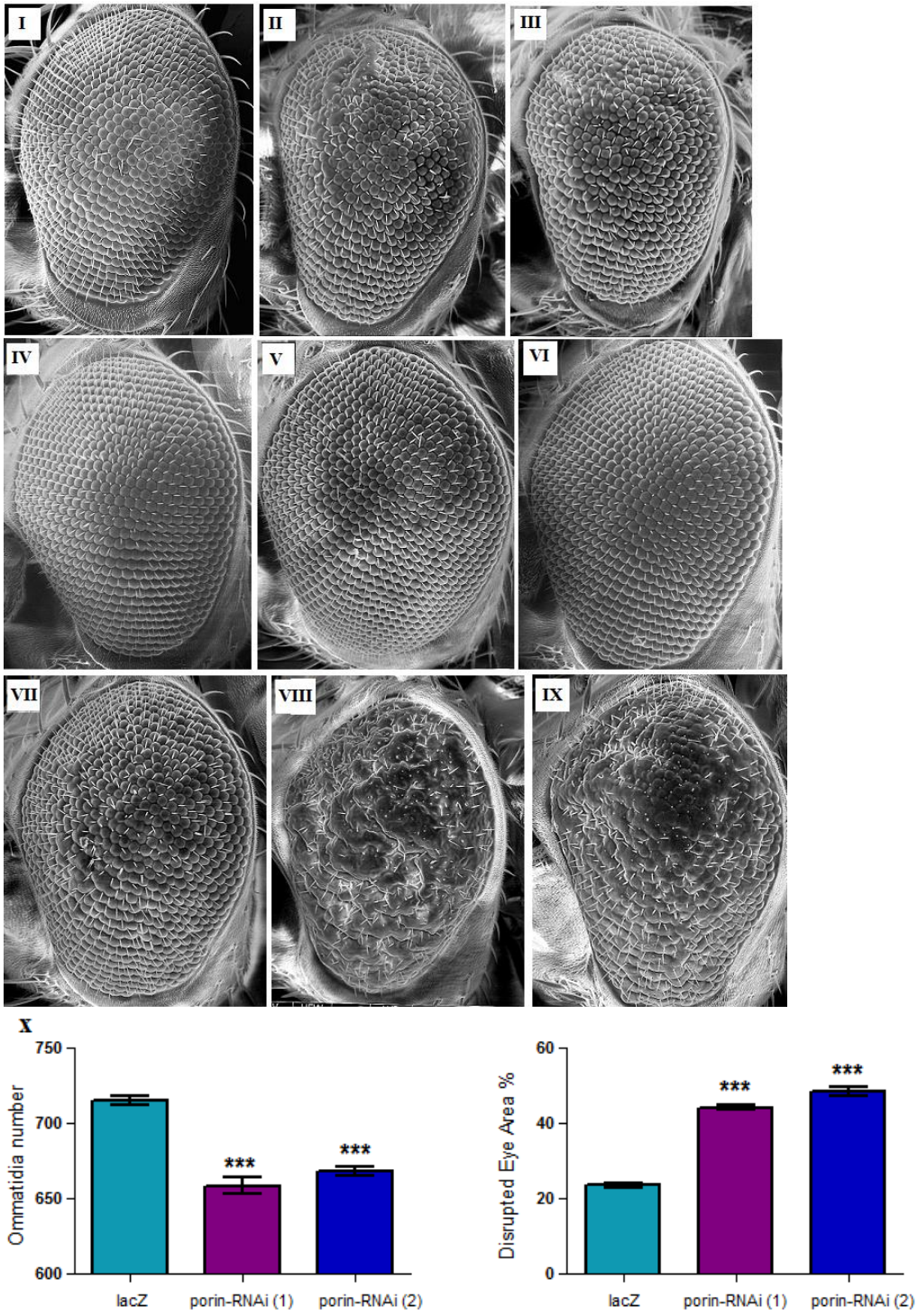
Figure 8.4 Loss of *porin* function enhances the *α-synuclein*-induced reduction in lifespan and age-dependent loss of climbing ability

A) The directed inhibition of *porin* along with *α-synuclein* expression in the neurons decreased lifespan with a median survival of 50 and 56 days when compared to 60 days for the control flies that express *α-synuclein* along with the *lacZ* transgene. Genotypes are *UAS-α-synuclein; Ddc-Gal4/ UAS-lacZ, UAS-α-synuclein; Ddc-Gal4/ UAS-porin-RNAi (1)* and *UAS-α-synuclein; Ddc-Gal4/ UAS-porin-RNAi (2).* Longevity is shown as percent survival ($P < 0.05$, determined by log-rank (Mantel-Cox) test with $n \leq 200$). B) The co-expression of *porin-RNAi* with *α-synuclein* resulted in reduction of climbing ability over time when compared to the controls. The genotypes are *UAS-α-synuclein; Ddc-Gal4/ UAS-lacZ, UAS-α-synuclein; Ddc-Gal4/ UAS-porin-RNAi (1)* and *UAS-α-synuclein; Ddc-Gal4/ UAS-porin-RNAi (2).* Analysis was done by nonlinear fitting of the climbing curves and significance was determined by comparing the 95% CI. Error bars indicate SEM and $n=50$.

0.08366 for control flies that co-expressed *α-synuclein* along with *lacZ*. This suggests that the inhibition of *porin* together with the expression of *α-synuclein* in these neurons confers a significant health disadvantage, with marked decreases in survival and premature loss of climbing ability.

Inhibition of *porin* in the eye decreases ommatidia number and increases ommatidial disarray, phenotypes that are rescued by *Buffy* overexpression

When *porin-RNAi* is directed in the developing eye using the *GMR-Gal4* transgene, it results in eyes with decreased number of ommatidia and higher disruption of the ommatidial array (Figure 5ii, iii and x) as determined by a one-way analysis of variance followed by a Dunnett's multiple comparison test $p<0.0001$. Co-expression of *porin* with *Buffy* restored the mean number of ommatidia and the percentage disruption to control levels as determined by a one-way analysis of variance followed by a Dunnett's multiple comparison test $p>0.05$ (Figure 5v, vi and xi). Taken together, these results suggest that *porin* may play a role in the development of the Drosophila eye and that *Buffy* suppresses the developmental eye defects that result from the inhibition of *porin*. The inhibition of *porin* along with *α-synuclein* overexpression resulted in a significant decrease in the number of ommatidia due to fusion of ommatidia and an increase in the percentage disruption of the eye (Figure 5viii, ix and xii) as determined by a one-way analysis of variance followed by a Dunnett's multiple comparison test $p<0.0001$. This suggests an enhancement of the neurotoxic effects of the *α-synuclein*-induced developmental eye defects in the presence of reduced *porin* activity.


I
II
III
IV
V
VI
VII
VIII
IX
X
Ommatidia number
750
700
650
600
***
***
lacZ
porin-RNAi (1)
porin-RNAi (2)
Disrupted Eye Area %
60
40
20
0
***
***
lacZ
porin-RNAi (1)
porin-RNAi (2)

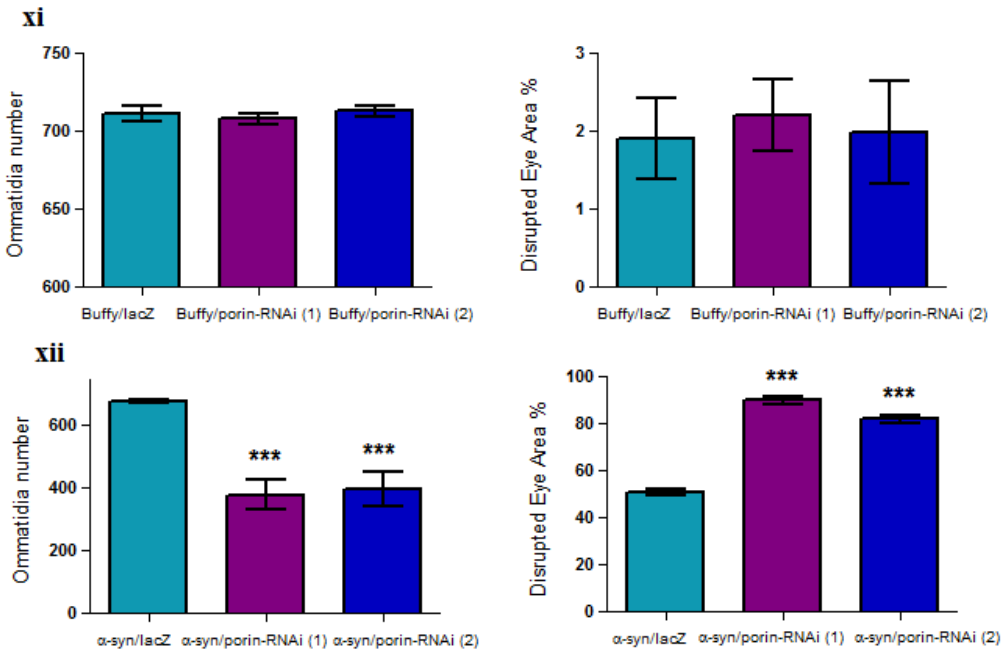


Figure 8.5 Inhibition of *porin* in the developing eye results in phenotypes that may be suppressed by *Buffy* and enhanced by *α-synuclein*

(i – ix) Scanning electron micrographs when *porin* is inhibited in the developing eye and co-expressed along with either *Buffy* or *α-synuclein.* The genotypes are (i) *GMR-Gal4/ UAS-lacZ,* (ii) *GMR-Gal4/ UAS-porin-RNAi (1),* (iii) *GMR-Gal4/ UAS-porin-RNAi (2),* (iv) *UAS-Buffy; GMR-Gal4/ UAS-lacZ,* (v) *UAS-Buffy; GMR-Gal4/ UAS-porin-RNAi (1),* (vi) *UAS-Buffy; GMR-Gal4/ UAS-porin-RNAi (2),* (vii) *UAS-α-synuclein; GMR-Gal4/ UAS-lacZ,* (viii) *UAS-α-synuclein; GMR-Gal4/ UAS-porin-RNAi (1),* and (ix) *UAS-α-synuclein; GMR-Gal4/ UAS-porin-RNAi (2).* Biometric analysis when (x) *porin* is inhibited in the eye indicated decreased ommatidia number and higher percentage of ommatidial disruption when compared to the control. (xi) The overexpression of *Buffy* with *porin-RNAi* results in restoration of the number of ommatidia and the degree of ommatidial disruption to below the control levels. (xii) The inhibition of *porin* along with *α-synuclein* expression resulted in the enhancement of the eye phenotypes when compared to controls as displayed by the low number of ommatidia coupled by the high degree of disruption of the ommatidial array. All comparisons were determined by a one-way analysis of variance followed by a Dunnett's multiple comparison test ($P<0.05$), error bars are SEM, asterisks (*) represent statistical significance and $n=10$.

Discussion

The multitude of functions attributed to mitochondrial porin or VDAC and its control of the entry and exit of mitochondrial metabolites makes it a key player in the cellular decisions that lead to either survival or death [1]. The expression of *porin-RNAi* in neurons under the direction of the *Ddc-Gal4* transgene results in shortened lifespan and a premature loss in locomotor ability, results that were consistent in both RNAi lines tested and that corroborate previous studies [25, 26]. This gene product is involved in maintaining mitochondrial morphology, and its disruption leads to a host of phenotypes among them locomotor defects. In our study, we disrupted this protein in DA and other neurons, the results obtained signifies a close connection between *porin* and the progression of the PD-like phenotypes of shortened lifespan and an age-dependent loss in locomotor function. The comparison of climbing indices of flies at 40 days when most of them are alive to the control lines indicate a significant change in the phenotypes, these appears to be a strong indication of possible neurodegeneration.

The relationship between mitochondrial porin and PD susceptibility gene products has been investigated in other organisms [12-14, 43, 44]. The inhibition of *porin* along with the expression of *α-synuclein* in *Ddc-Gal4*-expressing neurons of *Drosophila melanogaster* results in the enhancement of the loss of *α-synuclein*-induced phenotypes, with a decrease in lifespan and an impairment in climbing ability. Some studies have attributed the neurotoxicity of *α-synuclein* to its interaction with electron transport chain components among them Complex I [45]. It has been suggested that α-synuclein blocks the activity of porin and uses this channel to translocate into the inner mitochondria [13] and that it preferentially interacts with mitochondrial membranes compared to other

organelle membranes [46]. This association inhibits mitochondrial function and promotes reactive oxygen stress. Our study firstly inhibited the mitochondria *porin* and secondly expressed *α-synuclein* in the same neurons, this resulted in the enhancement of the observed phenotypes, with shortened lifespan and severe reduction in climbing ability over time. It seems therefore that the combination effect of the directed inhibition of *porin*, and expression of *α-synuclein* confers a greater disadvantage to "healthspan", albeit when altered in neurons. When altered individually, *α-synuclein*-induced PD model, a well-studied and robust disease model in Drosophila [16, 22] result in shortened lifespan and impaired climbing ability. Inhibition of *porin* in the developing eye results in extensive ommatidial disruption and fewer ommatidia number, as a result of intensive fusion of the ommatidia with no distinct ommatidia detectable in most of the eyes analysed. We suggest that though α-synuclein interacts with the mitochondria to result in disruption of mitochondria homeostasis, loss of *porin* in neurons seem to be independent of *α-synuclein*-induced phenotypes and this highlights the complexity of mechanisms involved in the pathogenesis of PD.

The association of porin with members of the Bcl-2 family is well documented [7], and has been suggested to be a point of convergence for cell survival and death signals [3]. When we overexpressed *Buffy*, the sole pro-survival *Bcl-2* homologue, in Drosophila neurons, along with inhibition of *porin* via RNAi, the phenotypes associated with the loss-of-function of *porin*, shortened lifespan and impaired climbing ability, were suppressed. The survival-induced advantages of Buffy especially under conditions of stress are well documented [27, 35], and so is the regulation of porin by Bcl-2 proteins that underscores the importance of Bcl-2 protein in life and death decisions. The

overexpression of *Buffy* along with the inhibition of *porin* in *Ddc-Gal4*-expressing neurons and in the developing eye resulted in a suppression of the phenotypes. The excess Buffy product must therefore confer cellular advantages to the target cells and counteracts the toxic effects of *porin* inhibition, and demonstrates a wider role for the Drosophila pro-survival homologue, with potential involvement in the mitochondria-mediated cell death. One study has suggested that *porin* was not involved in *debcl*-induced cell death [25] and found that apoptosis induced by *debcl* overexpression was not inhibited by *porin* loss of function. As such it seems that the rescue of *porin*-induced phenotypes by *Buffy* are consistent with its action on the mitochondria directly or through other proteins in a dedicated pro-survival signalling pathway.

Conclusions

The inhibition of *porin* in the *Ddc-Gal4*-expressing neurons and the developing eye is rescued upon the overexpression of *Buffy*, a pro-survival *Bcl-2* homologue. The co-expression of *porin-RNAi* along with *α-synuclein* results in enhanced phenotypes, this highlights the complexity of *α-synuclein*-induced mechanisms in the pathogenesis of PD, and in deed demonstrates the multi-faceted mechanisms involved in the aetiology of PD.

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Chapter 9 - Summary and Future Directions

The Drosophila Bcl-2 homologue Buffy is pro-survival

The *Bcl-2* family of genes is one of the most conserved gene families and is present in a wide range of organisms [1-3]. Earlier studies investigated the presence of *Bcl-2* homologues in Drosophila, after their identification in the nematode and in mammals, the fly was the missing link, and in 2000 several groups independently published research that showed the presence of this important gene family in Drosophila [4-8]. The pro-survival Bcl-2 homologue Buffy was identified by Quinn and colleagues [9] and in their study they showed its anti-apoptotic functions. Our studies further corroborate the pro-survival functions of *Buffy*; it rescues the *α-synuclein*-induced phenotypes [10] when overexpressed in the DA neurons and in the developing eye [11], it increased lifespan, significantly improved climbing ability, and restored the roughened eye phenotype that is generally associated with cell death [12] to control levels. The overexpression of the pro-apoptotic *Bcl-2* homologue *debcl* in DA neurons and the developing eye resulted in severely shortened lifespan, impaired locomotor function and significant ablation of the eye [13]. These phenotypes were suppressed upon the overexpression of the pro-survival *Buffy*. This highlighted the antagonistic relationship between the two *Bcl-2* family members; *debcl* overexpression promotes cell death while *Buffy* overexpression favours survival. We also updated the bioinformatic data for the two Drosophila Bcl-2 members, we showed the presence of four BH domains, TMD and presence of several motifs that may aid in their function [11, 13]. Indeed, the BH4 domain was previously associated with the pro-survival Bcl-2 members, but has recently been shown to occur in both anti- and pro-apoptotic multi-domain members [14]. This domain is known to interact with

pro-apoptotic members such as Bax, and with non-Bcl-2 proteins such as IP3R, ryanodine receptor and VDAC to prevent cell death.

In general, *Buffy* shows strong pro-survival functions when overexpressed in DA neurons, this was demonstrated with different neuronal cell death models. As previously mentioned, the overexpression of *Buffy* along with expression of *α-synuclein* in DA neurons counteracts the *α-synuclein*-induced phenotypes. The *α-synuclein*-induced PD model in Drosophila makes a robust model for the study of neuronal degeneration and death [10]. This survival enhancement was demonstrated in other PD models in Drosophila that included the mitochondrial serine protease HtrA2 model, where the loss of *HtrA2* function in DA neurons or in the developing eye resulted in shortened lifespan, impaired locomotor function and developmental eye defects [15, 16]. We show the suppression of these phenotypes upon overexpression of *Buffy* in either the DA neurons to enhance survival and locomotor ability in an age-dependent manner, or in the developing eye to restore the developmental eye defects to control levels (M'Angale and Staveley, unpublished work). We developed novel models of PD in Drosophila by the inhibition of anti-apoptotic genes such as the ancient *TMBIM* family, the loss of function of mitochondrial channel proteins such as *VDAC* and *MICU1*, and loss of function of *Pdxk* – a kinase that phosphorylates vitamers of vitamin B6 to the metabolically active PLP. The inhibition of *Bax inhibitor-1* (*BI-1/ TMBIM6*) in DA neurons resulted in shortened lifespan and a precocious loss in climbing ability, whereas its suppression in the developing eye appeared to enhance the roughened eye phenotype (M'Angale and Staveley, unpublished work). This appears to indicate an involvement of BI-1 in the normal function of DA neurons, which might be through its regulation of ER calcium

homeostasis. Evolutionarily, the TMBIM family appears to be more ancient than the well-studied and diverse Bcl-2 family, being present in species where no Bcl-2 members have been identified that include plants, yeast, some bacteria and viruses [17]. Regardless, present evidence points to interaction between these two regulators of cell death, especially with the discovery of putative BH3 domain in some of the TMBIM family members. In our experiments, we showed a rescue of the inhibition of *BI-1* by *Buffy*, when co-expressed in the DA neurons or in the developing eye (M'Angale and Staveley, unpublished work). Though we did not show it in our experiments, pro-survival Bcl-2 proteins interact with other proteins of the same family or non-Bcl-2 proteins through their BH4 or BH3 domains [14, 18]. Other than BI-1, we investigated Lifeguard (LFG/ TMBIM2), a member of the TMBIM family that is known to modulate FasL-mediated apoptosis [19]. The inhibition of *CG3814*, the Drosophila *LFG* homologue in DA neurons or in the developing eye produced flies with severely shortened lifespans, highly decreased locomotor function and increased degree of ommatidial disarray (M'Angale and Staveley, unpublished work). The overexpression of the *Bcl-2* pro-survival homologue *Buffy* suppressed the phenotypes that resulted from the inhibition of *LFG*. Additionally, we investigated the suppression of *CG2076*, the Drosophila homologue of Growth hormone-inducible transmembrane protein (GHITM/ TMBIM5), an inner mitochondrial localized TMBIM member that is involved in the regulation of mitochondrial morphology [20] in DA neurons and in the developing eye. When we inhibited the expression of *GHITM* in the DA neurons or developing eye, it resulted in shortened lifespan, impaired climbing ability and a high degree of ommatidial disruption (M'Angale and Staveley, unpublished work), phenotypes that were rescued upon

overexpression of *Buffy*. The role of Drosophila *Bcl-2* homologues in mitochondrial mediated cell death are still very unclear, though recent studies indicate a possible involvement under certain cellular conditions [21]. The loss of *GHITM/ MICS1* function causes mitochondrial fragmentation and cristae disorganization and stimulates the release of apoptogenic molecules that include cytochrome c [20]. GHITM appears to facilitate the tight association of cytochrome c with the inner membrane, and thus its up-regulation can delay the release of cytochrome c. The overexpression of *Buffy* in this background suppresses the *GHITM*-induced phenotypes, the mechanism for this protection is unknown yet, but it is possibly through the association of Buffy with the mitochondria and the up-regulation of survival mechanisms in response to changes in mitochondrial dynamics and homeostasis. Regardless of the mechanism involved, Buffy counteracts these phenotypes by an increase in survival.

Mitochondrial dysfunction is one of the main mechanisms implicated in the pathogenesis of PD [22]; the altered expression of genes with a function at the mitochondria is known to result in neurodegeneration as a result of mitochondrial dysfunction [23]. With this premise, we pursued the hypothesis that the overexpression of the pro-survival Bcl-2 homologue *Buffy* would suppress the phenotypes that result from inhibition of *porin*, a voltage-dependent anion channel (VDAC) and *mitochondrial calcium uptake 1* (MICU1). The inhibition of *porin* or *VDAC* is known to result in mitochondrial morphological defects [24]. In our study, the directed inhibition of *porin* in the DA neurons resulted in an age-dependent decrease in survival and locomotor dysfunction coupled with developmental eye defects (M'Angale and Staveley, unpublished work). These phenotypes were rescued upon the overexpression of *Buffy*. It seems that Buffy possesses

potent pro-survival signalling mechanisms, and as such this is an area of possible research to highlight the survival advantages that result from Drosophila Bcl-2 and its potential role in mitochondrial dynamics. Similar results were obtained with the inhibition of *MICU1,* and the overexpression of *Buffy* suppressed the *MICU1*-induced phenotypes of shortened lifespan and impaired climbing ability (M'Angale and Staveley, unpublished work). The role of calcium homeostasis in cell death is well known. The rescue of *MICU1* phenotypes by *Buffy* points to a wider role of Drosophila Bcl-2 in the regulation of mitochondrial dynamics and in the modulation of calcium homeostasis. As to whether Buffy interacts with any of the mitochondrial channel proteins is yet to be studied, but regardless of this, the potential benefits of Buffy are obvious.

Buffy influences the autophagy pathway

Autophagy is one of the mechanisms implicated in the pathogenesis of PD [25, 26]. The accumulation of Lewy bodies suggests the involvement of the autophagic-lysosomal pathway in their clearance. In our study, we inhibited *Atg6* and *Pi3K59F;* which are important in the formation of the autophagosome, in DA neurons and in the developing eye (M'Angale and Staveley, unpublished work). This resulted in flies with compromised survival and locomotor function and ommatidial disarray. These results suggest a strong involvement of autophagy or at least Atg6 and Pi3K59F in PD pathogenesis and in the normal function of neurons. We obtained strong evidence that showed the importance of this cellular process in disease pathogenesis by the expression of *α-synuclein* along with the inhibition of either *Atg6* or *Pi3K59F*. This co-expression did not alter either of the phenotypes obtained by the expression of *α-synuclein* or inhibition of *Atg6* or *Pi3K59F*,

that ruled out synergy and suggested that the impairment of the autophagy pathway result in PD-like phenotypes in Drosophila. This is a key finding from our study that indicate the complexity of the aetiology of PD; several failed cellular processes can in deed result in disease states.

The Bcl-2 pro-survival members are implicated in the regulation of autophagy, Atg6 or Beclin-1 is considered a BH3-only Bcl-2 protein (BOP) whereas its regulation relies heavily on other Bcl-2 members [27]. The formation of the Atg6-PI3K complex is an important regulatory step, with Atg6 sequestration being achieved by the association of its BH3 domain with Bcl-2 or Bcl-$X_{L.}$ Phosphorylation of the BH3 domain reverses this inhibition [28]. When we co-expressed *Buffy* with *Atg6-RNAi*, there was an enhancement of the phenotypes that suggested interaction between Buffy and Atg6 (M'Angale and Staveley, unpublished work). It is possible that Buffy interacts with the putative Atg6 BH3 domain we discovered through bioinformatic analysis, or Buffy survival signals are unable to alleviate the cellular disadvantages that result from the inhibition of Atg6, but regardless of the mechanism of action, it appears that *Buffy* overexpression enhances the *Atg6*-induced phenotypes. The putative BH3 domain present in Atg6 may be the interaction point between Buffy and Atg6, but this remain to be determined by further studies and will possibly highlight the role of Drosophila Bcl-2 in autophagic homeostasis.

Buffy has a complex effect on the Pink1/Parkin pathway

The Pink1/Parkin pathway is a key process in the regulation of mitochondrial dynamics and homeostasis [29, 30]. The Pink1/Parkin pathway is responsible for the turnover of

defective mitochondria via mitophagy. The Pink1 [31-34] and parkin [35] models of PD are well-studied, and in our study we utilized these models to investigate the effect of the overexpression of Buffy. In the Pink1 model, the overexpression of *Buffy* resulted in the suppression of the loss of *Pink1*-induced phenotypes, with a general increase in "healthspan" [32] M'Angale and Staveley, unpublished work). This rescue of *Pink1* LOF increases evidence for the involvement of Buffy in mitochondrial-mediated cell death. Our study shows the beneficial overexpression of *Buffy*, by increasing lifespan and restoring locomotor ability to control levels.

In contrast, *parkin* LOF was enhanced by overexpression of *Buffy* (M'Angale and Staveley, unpublished work). This finding indicates the involvement of Buffy in a pro-apoptotic signalling mechanism that is potentially triggered by the excess *Buffy* product, coupled by the diminished levels of parkin. This is an important finding that first, shows that the Pink1/Parkin pathway converge at the mitochondria and Pink1 and parkin have additional cellular roles independent of the Pink1/Parkin pathway. Second, Buffy has additional cellular roles that need to be determined to provide insight into the neuronal consequences of *parkin* LOF and *Buffy* overexpression. And finally, Buffy stimulates survival under strict cellular conditions that appear to require certain molecules among them parkin, and deficiencies in parkin tips the survival balance in favour of cell death. As to whether Buffy and parkin participate in the same survival pathway remains to be elucidated and until such experimental evidence is obtained, this attractive pathway that can serve as a therapeutic target will remain elusive.

Debcl is pro-cell death

The only identified pro-apoptotic *Bcl-2* homologue in Drosophila is *Debcl* [4-6, 8], it shares sequence similarity with the mammalian pro-apoptotic Bok. The overexpression of *debcl* in DA neurons resulted in severely shortened lifespan and a significant impairment of climbing ability (M'Angale and Staveley, unpublished work). Previously it has been shown that the overexpression of *debcl* in the developing eye causes severe eye ablation [5], which was corroborated by our studies. The overexpression of *debcl* along with the expression of the human transgene *α-synuclein* in the DA enhanced the *debcl*- and *α-synuclein*-induced phenotypes. The *debcl*-induced eye ablation was worsened with co-expression of *α-synuclein*, the combined effect appears to worsen either of the phenotypes. Anti-apoptotic and pro-apoptotic Bcl-2 proteins antagonizes each other to balance life and death signals, in our study the co-expression of *debcl* with the pro-survival *Buffy* resulted in suppression of the *debcl*-induced phenotypes, these flies had a longer lifespan, climbed better and had the eye ablation slightly restored to control levels. It is evident that Buffy confers survival advantages in conditions of stress and appears to overcome the cellular disadvantages of *debcl* overexpression.

Ddc-Gal4-expressing neurons are highly sensitive to altered gene expression

Ddc-Gal4-expressing neurons appear to be selectively sensitive to cellular stress due to their high oxygen consumption, high generation of reactive oxygen and nitrogen species from neurochemical reactions and the deposition of metal ions in the brain that comes with ageing [36]. As such alterations in gene products appear to impact these sensitive neurons greatly, and thus make an excellent model to study subtle differences in gene

products. We took advantage of these neurons to study the effects of gene inhibition, with an aim to recapitulate some of the PD-like symptoms such as shortened lifespan and impaired climbing ability. The inhibition of *Pdxk* in DA neurons is such one study we performed that resulted in shortened lifespan and an age-dependent loss in climbing ability (M'Angale and Staveley, unpublished work). The importance of this kinase is underscored by the product of its phosphorylation, pyridoxal-5'-phosphate (PLP), that is an important co-factor responsible for more than 100 enzymatic reactions [37], predominantly in amino acid metabolism, and is implicated in nervous system function (neurotransmitter synthesis), red blood cell formation (heme biosynthesis), vitamin formation, one-carbon metabolism (nucleic acid synthesis) and as a potent antioxidant [38]. The disruption of Pdxk function therefore has far ranging consequences that can result in mitochondrial dysfunction, neuronal decay and degeneration. The survival advantages of the overexpression of *Buffy* extended to phenotypes induced by the inhibition of this kinase.

Future directions

This study put into perspective the immense pro-survival advantages that result from overexpression of the Drosophila Bcl-2 homologue *Buffy*. We fell short of direct evidence for the involvement of Buffy at the mitochondria, but indirectly extended the theory that Drosophila Bcl-2 homologues have an active function at the mitochondria. Further investigation is required to unequivocally show this. Interaction studies are required between Buffy and the several proteins whose inhibition it suppresses. The relationship between Buffy and debcl is paramount to decipher the pathways that are involved in

Buffy survival signals, the presence of four BH domains in both proteins require further studies to determine which domain(s) are involved. This study should extend to Atg6, which we showed has a putative BH3 domain, the enhancement of *Atg6*-induced phenotypes points to an interaction between Buffy and Atg6, possibly within the BH3 domain of Atg6.

It appears that Buffy may have a role in ER stress response due to its suppression of *BI-1*-induced phenotypes. It would be beneficial to investigate ER UPR states when *BI-1* is inhibited and co-expressed with *Buffy*. The UPR response is pro-survival and only becomes pro-apoptotic after a sustained signal. The combined effects of ER stress and *Buffy* overexpression may likely act synergistically to promote cell survival and may prove to be an important therapeutic target. In addition, we need to investigate the mitochondrial morphological changes associated with suppression of the inner mitochondrial protein GHITM and its suppression by Buffy, this likely could shed more light on the involvement of Buffy in mitochondrial dynamics and homeostasis. Finally, further investigation into the interaction between Buffy and parkin is needed to explain the effects the overexpression of *Buffy* has on the inhibition of *parkin*.

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Appendix 1: Co-expression of *Buffy* with *Buffy-RNAi* produces an intermediate phenotype in the Drosophila eye.

A version of this chapter has been published in Drosophila Information Services 99: 14 – 16 (M'Angale and Staveley, 2017).

Introduction

The use of ribonucleic acid interference (RNAi), a post-transcriptional gene silencing mechanism, to inhibit gene function is widely applied to analyse phenotypic consequences of gene suppression [1-3]. RNAi is an evolutionary conserved cellular mechanism which is present in protozoa, fungi, nematodes, plants, flies and mammals [4]. This method is used in genome-wide screens [5], functional genomics, genetic therapeutics, crop and animal improvements among many upcoming applications [3]. In most of the organisms currently being used for studies, RNAi is systemic and cannot therefore, be restricted to a specific cell type [5]. Alternately, in *Drosophila melanogaster* RNAi is cell autonomous, and can be triggered in a spacio-temporal manner using the bipartite UAS/Gal4 expression system [5, 6]. Gene function can be analysed using an appropriate assay by examining the phenotypic effect of the directed inhibition (RNAi) or overexpression. To investigate the phenotypic effect of directed overexpression on directed RNA interference, the overexpression of *Buffy*, the sole pro-cell survival *Bcl-2* homologue [7], and an RNAi based on *Buffy* was used in the Drosophila developing eye. We investigated the possibility that intermediate developmental phenotypes can be generated from this interaction that are subject to modification by other genes.

Materials and Methods

Drosophila stock and culture: *UAS-Buffy* [7] was kindly provided by Dr. Leonie Quinn (University of Melbourne). *UAS-Buffy-RNA*$_i$ (w[*]; P{w[+mC]=UAS-Buffy.RNAi}c3), *GMR-Gal4* [8] and *UAS-lacZ* flies were obtained from the Bloomington Drosophila Stock Center at Indiana University. The *UAS-Buffy/CyO; GMR-GAL4* line was generated using standard recombination methods and was used to overexpress *Buffy* in the developing eye

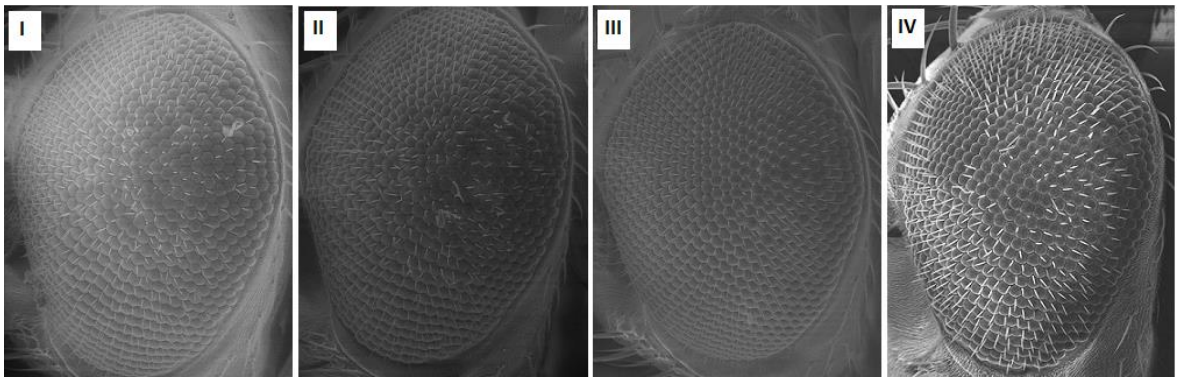
under the direction of the *GMR-Gal4* transgene. Stocks and crosses were maintained on standard medium containing cornmeal, molasses, yeast, and agar. Stocks were kept at room temperature (23°C ± 2°C) while crosses and experiments were carried out at 29°C.

Biometric analysis of the Drosophila eye: A number of single vial crosses of each genotype were made at 29°C, a cohort of the critical class male flies was collected upon eclosion and aged for three days before being frozen at -80°C. Whole flies were mounted on scanning electron microscope stubs, desiccated overnight and photographed with a FEI Mineral Liberation Analyzer 650F scanning electron microscope. For each cross at least 10 eye images were analysed using the National Institutes of Health (NIH) ImageJ software [9] and biometric analysis performed using GraphPad Prism version 5.04. The percent area of eye disruption was calculated as previously described [10].

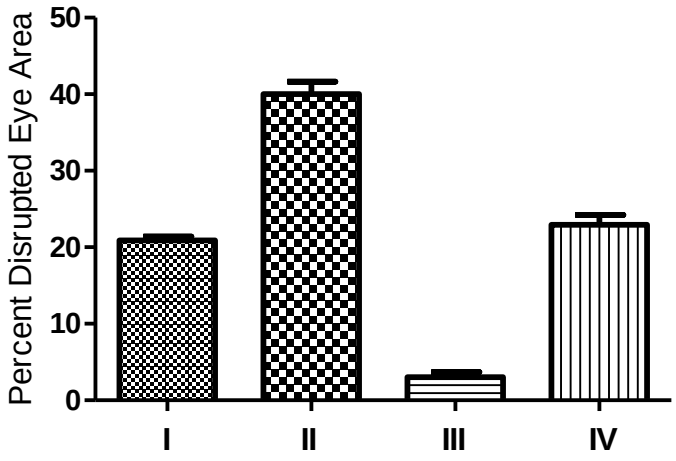
Results and Discussion

The directed expression of *Gal4* in the Drosophila eye at 29º C results in a roughened eye phenotype characterised by uneven, enlarged and fused ommatidia [11, 12]. Analysis of scanning electron micrographs shows ommatidial disarray as a result of expression of *Gal4* and the inhibition of *Buffy* in the developing eye (Figure 1A, I-II). *Gal4*-expressing flies show a disrupted ommatidia morphology, with 20% disruption of the eye (Figure 1B, I), whereas *Buffy-RNAi* flies display a much more severe phenotype of 45% disruption (Figure 1B, II). The co-expression of *Buffy* along with *Gal4* results in suppression of the roughened eye phenotype with a disruption of 3% (Figure 1B, III). The overexpression of *Buffy* along with its inhibition results in a disruption of the eye with a mean of 22% (Figure 1B, IV); intermediate between *Buffy* overexpression (3%) and its inhibition

A.



B.



A1-1 The phenotypic consequences of the directed expression and inhibition of Buffy in the Drosophila developing eye.

A) Scanning electron micrographs of the eye of the following genotypes (I) *GMR-Gal4/ UAS-lacZ;* (II) *GMR-Gal4/ UAS-Buffy-RNAi;* (III) *UAS-Buffy; GMR-Gal4/ UAS-lacZ;* and (IV) *UAS-Buffy; GMR-Gal4/ UAS-Buffy-RNAi.* B) The biometric analysis of the developing eye showing the percent area of disruption (I-IV). There is suppression of the *Gal4*-expression phenotype by *Buffy* (III) and an intermediate phenotype when *Buffy* is overexpressed along with *Buffy-RNAi* (IV) as determined by one-way ANOVA and Dunnett's multiple comparison test ($P<0.05$ and 95% CI), error bars indicate the SEM and $n=10$. (*Data for GMR-Gal4/UAS-Buffy-RNAi is adapted from M'Angale and Staveley, 2016* [13])

(45%). While similar to the control, this intermediate phenotype results from a balance of a rescue of the RNAi inhibition by the directed expression of *Buffy* and interference of the overexpression of *Buffy* by the RNAi transgene.

Previously, our research group demonstrated the expression of the yeast transcription factor *Gal4* in the Drosophila eye results in apoptosis-dependent developmental defects of the ommatidial array [11]. The overexpression of *Buffy*, a *Bcl-2* pro-cell survival homologue results in the suppression of this phenotype, similar to the suppression of these developmental defects by *Pink1* [12]. These results suggest that the alteration of *Buffy* expression in the developing eye may subtly influence neurogenesis. The overexpression of *Buffy* along with its inhibition results in disrupted area of the eye that is intermediate to the two extremes. Intermediate phenotypes are important in determining gene function, neuropathology of neurological diseases and therapeutics [14, 15]. The inhibition of gene function by RNA interference relies on the degradation of the mRNA by the introduction of a dsRNA molecule [16]. One consequence of using RNAi, for better or worse, is the generation of phenotypes that may or may not be the equivalent of null mutants.

A priori, if the inhibition of *Buffy* is extremely efficient, coupled with directed overexpression of *Buffy-RNAi* could be expected to generate a phenotype like *Buffy-RNAi* expression. Interestingly, the resulting intermediate phenotype reveals 1) that *Buffy* partially rescues the effects of *Buffy-RNAi*; 2) that *Buffy-RNAi* reduces the consequences of the directed expression of *Buffy* and 3) that both transgenes are biologically functional. Alternatively, the overexpression of the pro-cell survival *Buffy* might be acting in a general manner to counteract the downstream effects of an overloaded “RNAi system”,

with the elevated levels of *Buffy* gene product being sufficient to abrogate the *Buffy-RNAi*-induced developmental eye defects. In conclusion, *GMR-Gal4* produces a cell death-dependent rough eye phenotype that can be suppressed by the pro-survival *Buffy,* enhanced by its loss of function and the co-expression of *Buffy* along with its inhibition by RNAi results in an intermediate phenotype.

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Appendix 2: Overexpression of Buffy has differing effects on the Pink1 and parkin Drosophila models of Parkinson disease

A version of this chapter has been published in Genome (M'Angale and Staveley, 2017)

Abstract

Mutations in *parkin* and *Pink1* are responsible for autosomal recessive early onset Parkinson disease (PD). This has been attributed to the failure of the cell to clear dysfunctional mitochondria that lead to nigrostriatal neuronal loss. The Pink1/parkin pathway is a quality control checkpoint for defective mitochondria and its failure results in phenotypes marked by reduced lifespan, impaired locomotor ability, among others. Our inhibition of *parkin* or *Pink1* in the *Ddc-Gal4*-expressing neurons through a stable inducible RNAi results in compromised survival and precocious loss in climbing ability, results that corroborate other studies that supress the activity of these PD-associated genes. To evaluate the effects of the overexpression of the *Bcl-2* homologue *Buffy,* we analysed lifespan and climbing ability in both *parkin-RNAi* and *Pink1-RNAi* flies, in addition to investigating the effect of *Buffy* overexpression in *parkin*-induced developmental eye defects. Our results show that *Buffy* overexpression had differing effects on *parkin-RNAi* and *Pink1-RNAi*, the *parkin*-induced phenotypes were enhanced whereas the *Pink1*-enhanced phenotypes were suppressed. Interestingly, the overexpression of *Buffy* along with the inhibition of *parkin* in the neuron rich eye results in the suppression of the developmental eye defects.

Introduction

Parkinson disease is the most common human neurodegenerative movement disease which is mainly characterized by the selective degeneration and loss of dopaminergic neurons in the s*ubstantia nigra pars compacta* (*SNpc*) region of the midbrain. Some of its more common symptoms include muscle rigidity, resting tremors, postural instability, and

bradykinesia paired in some cases with non-motor symptoms such as autonomic, cognitive and psychiatric problems [1, 2]. The majority of PD cases are sporadic with no known causative agents, but familial forms with a genetic link have been identified and studied extensively in model organisms [3, 4]. Mutations in *parkin*, a gene that encodes a cytosolic ubiquitin E3 ligase that selectively targets misfolded proteins to the ubiquitin proteasome system (UPS) for degradation [5, 6], and *Pink1*, that encodes the Phosphatase and tensin homologue (PTEN)-induced kinase1 (Pink1) [7-9], are associated with autosomal recessive early onset PD. Pink1 and parkin are implicated in the same pathway that clears dysfunctional mitochondria via a specialized form of autophagy called mitophagy [10-12]. The Pink1/parkin pathway is responsible for aspects of mitochondrial quality control.

Studies in Drosophila and cell systems demonstrated that parkin is recruited by Pink1 to depolarized mitochondria to mediate the selective autophagic removal of damaged mitochondria [13]. Pink1 localizes to damaged mitochondria then directly phosphorylates and activates parkin [14] to ubiquitinate targets on the defective mitochondria to mark them for autophagic removal [10, 15]. Although parkin and Pink1 participate in this important cellular pathway, they have been shown to function in separate cellular pathways that supplement mitophagy such as the action of parkin on UPS, autophagy and the lysosomal system. Mutant *parkin* flies show a reduction in lifespan, locomotor defects among other phenotypes [16], similar to those observed in *Pink1* mutant flies [17-21]. It is this similarity in phenotypes that aided in the elucidation of the Pink1/parkin pathway. The sole pro-survival *Bcl-2* homologue in *Drosophila melanogaster*, *Buffy*, has been shown to contribute to cell survival [22-24]. Previous studies have shown the suppression

of loss of *Pink1*-induced phenotypes by the overexpression of Buffy [19]. Here we determined the effect of directed overexpression of Buffy along with the inhibition of *parkin* or *Pink1* via directed expression of stable RNAi transgenes in the *Ddc-Gal4*-expressing neurons of Drosophila. We also co-expressed *Buffy* along with *parkin-RNAi* in the developing eye under the direction of the *GMR-Gal4* transgene and evaluated the role of this overexpression on the *parkin*-induced developmental eye defects.

Materials and Methods

Drosophila stocks, derivative lines and media

The *UAS-parkin-RNAi* and *UAS-Pink1-RNAi* were provided by Dr. B. Lu [21, 25], *UAS-Buffy* [24] by Dr. Leonie Quinn, and *Ddc-Gal4* [26] by Dr. J. Hirsch. *GMR-Gal4* [27] and *UAS-lacZ* were obtained from the Bloomington Drosophila Stock Center. The *UAS-Buffy Ddc-Gal4/ CyO* and *UAS-Buffy GMR-Gal4/ CyO* complex lines were generated and tested by PCR as previously described (M'Angale and Staveley, in press). They were used to overexpress *Buffy* in neurons under the direction of the *Ddc-Gal4* transgene or in the developing eye using the *GMR* response elements. Stocks and crosses were maintained on standard cornmeal/molasses/yeast/agar media treated with propionic acid and methylparaben in plastic vials. Stocks were kept at room temperature while crosses and experiments were carried out at 25°C and 29°C.

Ageing assay

Males flies of the selected critical class genotypes were carefully aged following a standard protocol [22, 28]. Briefly, more than two hundred male flies were aged per genotype and scored every two days for evidence of dead adults [29]. Male flies were

selected for ageing experiments to avoid the reproductive stresses associated with female fertility. Longevity data was analysed with GraphPad Prism version 5.04 and survival curves were compared using the Log-rank (Mantel-Cox) test. Significance was determined at 95%, at a P-value less than or equal to 0.05 with Bonferroni correction.

Climbing assay

The climbing assays were performed as described in previous studies [30] and climbing indices were generated and analysed using GraphPad Prism version 5.04. As before, the climbing curves were fitted using non-linear regression and compared using 95% confidence interval with a 0.05 P-value.

Scanning electron microscopy of the drosophila eye

The eyes for scanning electron microscopy were prepared using our research group's standard protocol [22]. For each cross at least 10 different eye images per critical class genotype were analysed using the National Institutes of Health (NIH) ImageJ software [31]. The ratio of disruption of the eye was calculated as previously described [32] and biometric analysis performed using GraphPad Prism version 5.04. Comparisons were determined by an unpaired two-tailed T-test ($P<0.05$).

Results

Inhibition of *parkin* or *Pink1* in *Ddc-Gal4*-expressing neurons decreases lifespan and severely impairs locomotor function

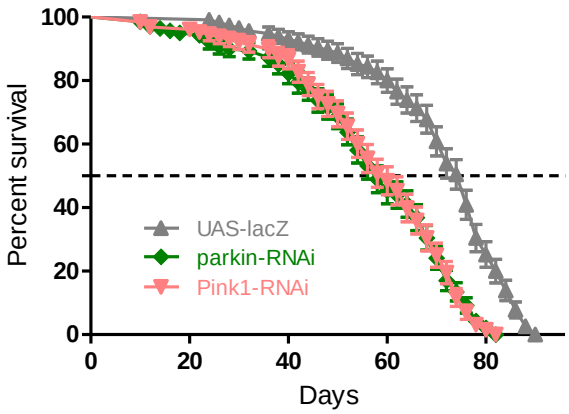
The directed expression of *parkin-RNAi* in the *Ddc-Gal4*-expressing neurons results in decreased lifespan and severely impaired locomotor function. The median lifespan for these flies was 58 days compared to 70 days for the controls that express the benign *lacZ* transgene as compared by Log-rank (Mantel-Cox) test (Figure 1A). Similarly, the

inhibition of *Pink1* in these neurons results in a decreased lifespan with a median survival of 60 days when compared to the controls. When either *parkin* or *Pink1* is suppressed in these neurons, the flies display a marked impairment of locomotor ability as determined by the nonlinear fitting of the climbing curves (Figure 1B). These results corroborate previous studies that show a role for parkin and Pink1 in the protection of neurons in Drosophila as its reduced activity severely shortens lifespan and prematurely retards climbing ability.

Overexpression of *Buffy* enhances the loss of *parkin*-induced phenotypes

The directed overexpression of the pro-survival *Bcl-2* homologue *Buffy* in *Ddc-Gal4*-expressing neurons results in increased lifespan and improved climbing ability [22]. When *Buffy* is co-expressed with *parkin-RNAi*, the results show a significant decrease in lifespan with a median lifespan of 36 days when compared to 74 days for *Buffy* co-expression with the benign *lacZ* control flies as determined by Log-rank test (Figure 2A). The climbing ability of the *parkin-RNAi* flies was slightly depressed as determined by comparison of the climbing curves (Figure 2B) with the control curve of *Buffy* co-expression with the benign *lacZ* at 95% CI. Taken together these results suggest the pro-cell survival role for *Buffy* is not active when *parkin* is inhibited and, it seems, may be anti-survival when the function of *parkin* is inhibited.

A.



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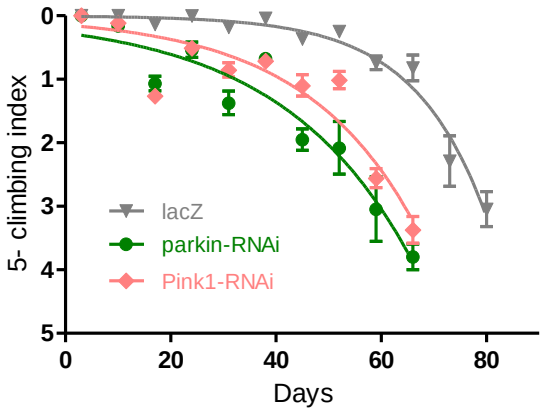
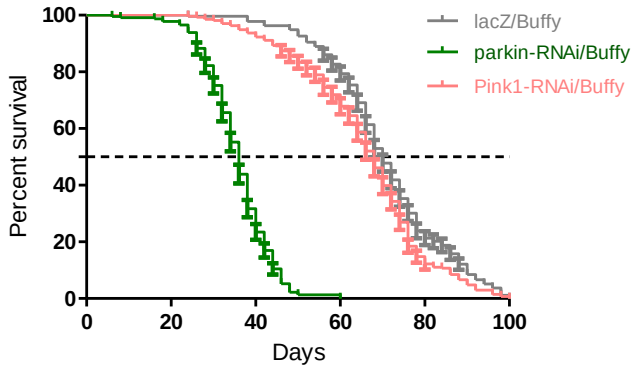


Figure A2.1 Loss of parkin or Pink1 activity in neurons decreases survival and impairs climbing ability

A) The inhibition of *parkin* in neurons under the direction of the *Ddc-Gal4* transgene results in decreased lifespan with a median survival of 58 days compared to 70 days for the control flies that express the benign *UAS-lacZ* transgene. Similarly, the co-expression of *Pink1-RNAi* in the *Ddc-Gal4*-expressing neurons reduces lifespan, with a median survival of 60 days when compared to the controls. The genotypes are *Ddc-Gal4/ UAS-lacZ, Ddc-Gal4/ UAS-parkin-RNAi*, and *Ddc-Gal4/ UAS-Pink1-RNAi.* Longevity is shown as percent survival ($P < 0.05$, determined by the log-rank (Mantel-Cox) test and $n \geq 200$). B) The inhibition of *parkin* or *Pink1* in these neurons results in a significant decrease in climbing ability as determined by nonlinear fitting of the climbing curves and comparing the 95% CI. The genotypes are *Ddc-Gal4/ UAS-lacZ, Ddc-Gal4/ UAS-parkin-RNAi* and *Ddc-Gal4/ UAS-Pink1-RNAi.* Error bars indicate SEM and $n=50$.

A.



B.

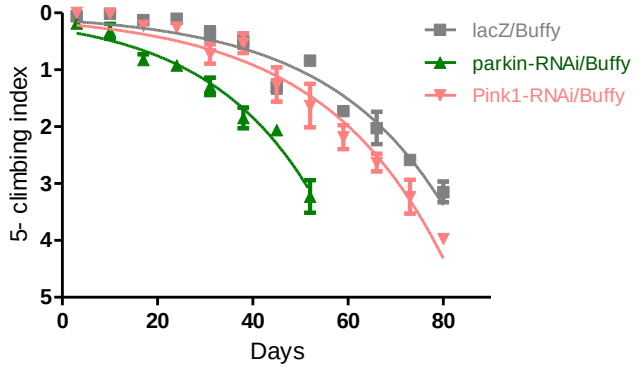


Figure A2.2 Pro-survival Buffy enhances the parkin-induced phenotypes and suppress the Pink1-induced phenotypes

A) The co-expression of *Buffy* with *parkin-RNAi* in Ddc-Gal4-expressing neurons result in enhancement of decreased lifespan, the median survival of these flies was 36 days compared to 74 days for the controls that overexpress *Buffy* along with the benign *lacZ* transgene. In contrast, the overexpression of *Buffy* along with the inhibition of *Pink1* results in increased lifespan, with a median survival of 68 days compared to 74 days for the controls. Genotypes are *Ddc-Gal4 UAS-Buffy/ UAS-lacZ, Ddc-Gal4 UAS-Buffy/ UAS-parkin-RNAi* and *Ddc-Gal4 UAS-Buffy/ UAS-Pink1-RNAi.* Longevity is shown as percent survival ($P < 0.05$, determined by log-rank (Mantel-Cox) test with $n \leq 200$). B) The inhibition of *parkin* or *Pink1* along with the overexpression of *Buffy* in these neurons results in an enhanced *parkin*-induced phenotype and suppression of the *Pink1*-induced age-dependent loss in climbing ability. The genotypes are *Ddc-Gal4 UAS-Buffy/ UAS-lacZ, Ddc-Gal4 UAS-Buffy/ UAS-parkin-RNAi* and *Ddc-Gal4 UAS-Buffy/ UAS-Pink1-RNAi.* Analysis was done by nonlinear fitting of the climbing curves and significance was determined by comparing the 95% CI. Error bars indicate SEM and $n=50$.

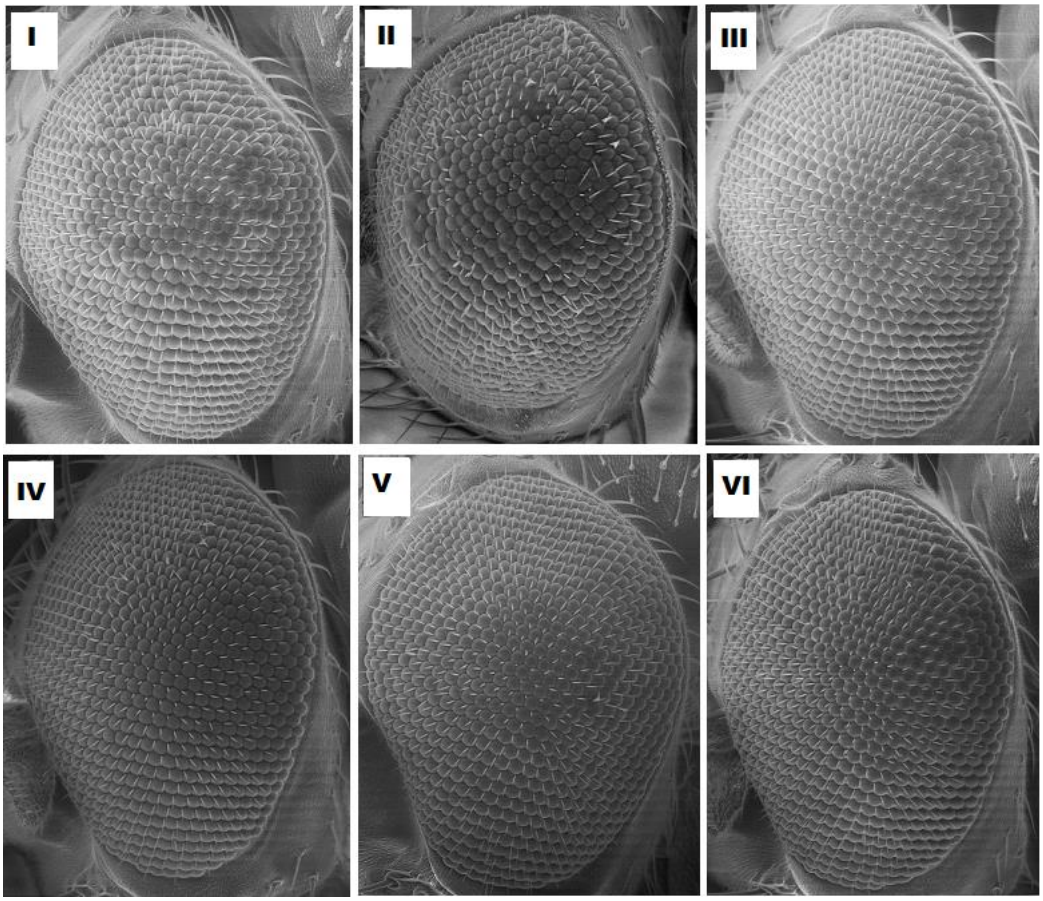
Co-overexpression of *Buffy* with *Pink1-RNAi* suppresses phenotypes

The directed overexpression of *Buffy* along with the inhibition of *Pink1* in the *Ddc-Gal4*-expressing neurons results in the suppression of the reduced survival phenotype observed with *Pink1-RNAi* expression (a median survival of 60 days, see Figure 1B) with a median lifespan of 68 days compared to 74 days for *Buffy* co-expression with the benign *lacZ* control flies. This was a significant increase (Figure 2A). A comparison of the climbing curves by nonlinear fitting at 95% CI revealed they were not significantly different (Figure 2B). This implies that the overexpression of the pro-survival *Buffy* confers survival advantage when *Pink1* is inhibited in the *Ddc-Gal4*-expressing neurons.

Loss of *parkin*-induced developmental eye defects are suppressed by *Buffy,* while inhibition of *Pink1* decreases disruption of the eye

The ectopic expression of *parkin-RNAi* in the developing eye under the direction of the *GMR-Gal4* transgene results in decreased number of ommatidia and a higher disruption of the ommatidial array (Figure 3A, II and 3B). The inhibition of *Pink1* in the developing eye results in normal ommatidia numbers and significantly lower levels of disruption of the ommatidial array (Figure 3A, III and 3B) as determined by a one-way analysis of variance followed by a Dunnett's multiple comparison test $p<0.05$. Co-expression of *parkin-RNAi* with *Buffy* restored the mean number of ommatidia and the percentage disruption to control levels (Figure 3A, V and 3C). The co-expression of *Pink1-RNAi* with *Buffy* in the developing eye results in control level number of ommatidia and disruption of the eye (Figure 3A, VI and 3C) as determined by a one-way analysis of variance followed by a Dunnett's multiple comparison test $p<0.05$. Taken together, these results suggest that parkin may play a role in the normal development of the Drosophila

A.


I
II
III
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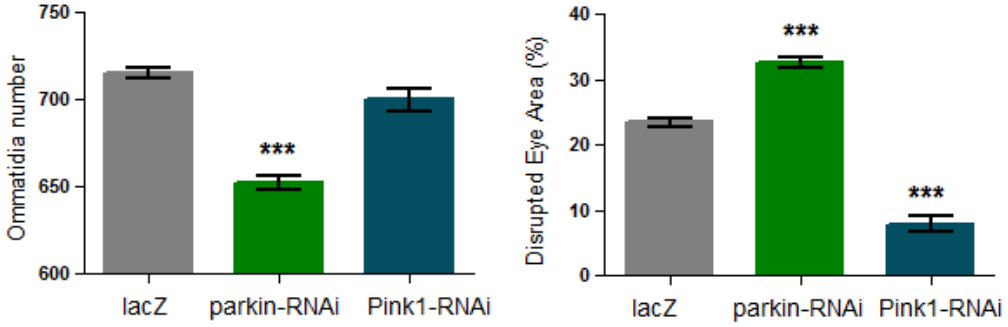
B.


Ommatidia number
750
700
650
600
***
lacZ
parkin-RNAi
Pink1-RNAi
Disrupted Eye Area (%)
40
30
20
10
0
***
***
lacZ
parkin-RNAi
Pink1-RNAi

C.

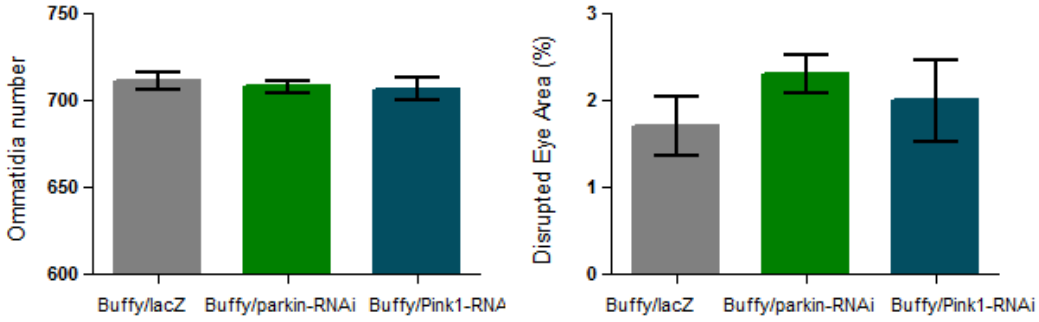


Figure A2.3 Inhibition of parkin in the developing eye is suppressed by Buffy

A) Scanning electron micrographs when *parkin* and *Pink1* are inhibited in the eye and co-expressed along with *Buffy*. The genotypes are (I) *GMR-Gal4/ UAS-lacZ;* (II) *GMR-Gal4/ UAS-parkin-RNAi;* (III) *GMR-Gal4/ UAS-Pink1-RNAi;* (IV) *UAS-Buffy; GMR-Gal4/ UAS-lacZ;* (V) *UAS-Buffy; GMR-Gal4/ UAS-parkin-RNAi* and (VI) *UAS-Buffy; GMR-Gal4/ UAS-Pink1-RNAi*. B) Biometric analysis of the inhibition of *parkin* and *Pink1* in the developing eye show a decrease in ommatidia number and increase in the percentage of ommatidial disruption for *parkin-RNAi* when compared to the control, whereas that of *Pink1-RNAi* resulted in a reduction of the disruption of the ommatidial array. C) The overexpression of *Buffy* with *parkin-RNAi* and *Pink1-RNAi* in the developing eye results in the suppression of the *parkin-RNAi*-induced phenotypes: the ommatidia number and disruption of the eye were restored to control levels. Comparisons were determined by a one-way analysis of variance followed by Dunnett's multiple comparison test ($P<0.05$), error bars are SEM, asterisks (*) represent statistical significance and $n=10$.

eye and that the activity of *Buffy* suppresses the developmental eye defects that result from the inhibition of *parkin*. In addition, the inhibition of *Pink1* seems to confer some survival advantages by counteracting the defects of *Gal4* expression in the developing eye.

Discussion

The inhibition of *parkin* or *Pink1* function in Drosophila is known to produce robust models of PD [16, 18-21]. In our study, we showed that the inhibition of *parkin* or *Pink1* in *Ddc-Gal4*-expressing neurons results in significant reduction in lifespan and an age-dependent loss in locomotor function, results that corroborate previous studies. The overexpression of *Buffy* suppressed the loss of *Pink1*-induced phenotypes in *Ddc-Gal4*-expressing neurons, of shortened lifespan and age-dependent loss in climbing ability. A previous study showed a rescue of loss of *Pink1*-induced phenotypes when overexpressed under the direction of *hs-Gal4*, but did not report the suppression of the shortened lifespan we have shown here [19]. Importantly, this study indicated that the survival benefits conferred by Buffy were from its protection of the mitochondria, as it restored mtDNA and ATP levels. All together this suggests that overexpression of *Buffy* strongly counteracts the phenotypes that result from the inhibition of *Pink1*.

Recent studies have shown an increased array of parkin functions, in addition to its role in the Pink1/Parkin pathway [10, 11]. Contrary to the finding that the overexpression of *Buffy* suppressed the loss of *Pink1*-induced phenotypes, the loss of *parkin*-induced phenotypes was severely enhanced. The overexpression of *Buffy* in a *parkin-RNAi* background significantly shortened lifespan and severely impaired climbing ability. The neuroprotective function of the ubiquitin E3 ligase parkin has been shown by its

regulation of the proapoptotic Bax through ubiquitination [33]. The parkin-dependent ubiquitination of Bax prevented its translocation under basal and stress conditions to the mitochondria [34]. This appears to be an important function for parkin as it prevents overt cell death and supports cell viability during mitochondrial injury and repair. A loss in parkin function failed to inhibit the translocation of Bax to the mitochondria. We speculate that since parkin is well conserved in Drosophila, some of the neuroprotective functions are maintained. The BH3 domain of Bcl-2 proteins has been determined to be important for interaction between parkin and the Bcl-2 proteins [34]. The Bcl-2 homologues in Drosophila *Buffy* and *Debcl* both possess a BH3 domain [24, 35]. It is possible that parkin influences neuronal apoptosis through its interaction with Debcl and Buffy. Its action on Buffy may be at several levels of regulation, first, the overexpression of Buffy may inhibit mitochondrial quality control as it antagonizes the parkin-dependent mitophagy [36]. Pro-survival Bcl-2 proteins inhibit the translocation of parkin to the mitochondria and thus antagonizes the elimination of defective mitochondria by the Pink1/Parkin pathway. Multifaceted roles for *parkin* and *Pink1* have been demonstrated when co-expressed with *Foxo* [37], with differences in the severity of the *Foxo*-induced eye phenotypes. It therefore appears that parkin and Pink1 have specific unique cellular functions, separate from their role in the Pink1/parkin pathway.

The overexpression of pro-survival *Bcl-2* homologues has been shown to inhibit the translocation of parkin to the mitochondria and further suppress the activity of the Pink1/Parkin pathway [36]. Consistent with our studies, the overexpression of the pro-survival *Buffy* enhanced the *parkin*-induced phenotypes, this could be due to the accumulation of defective and dysfunctional mitochondria, that leads to an increase in

oxidative stress, release of apoptogenic molecules from the mitochondria and depressed mitochondria turnover. As thus the zealous protection of the mitochondria by excess Buffy product countermand the pro-survival signal into a pro-apoptotic signal. A secondary theory for the mechanism and that require to be tested in future experiments is that the pro-survival actions of Buffy require ubiquitination of Buffy by parkin, that is, parkin activates Buffy by ubiquitination. It seems therefore the pro-survival function of Buffy requires normal parkin function This may be cell specific, as we only observed this in *Ddc-Gal4*-expressing neurons and not in the developing eye.

Conclusion

The overexpression of *Buffy* differentially affects the Pink1/Parkin pathway, it enhances the loss of *parkin*-induced phenotypes and counteracts the loss of *Pink1*-induced phenotypes.

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Appendix 3: Loss of MICU1 function in Drosophila neurons alters longevity and motor ability

A version of this chapter has been accepted for publication in Genetics and Molecular Research (M'Angale and Staveley, 2016)

Abstract

The mitochondrial calcium uptake 1 (MICU1) is a regulatory subunit of the mitochondrial calcium uniporter that function in calcium sensing. MICU1 contain two EF hand domains that are well conserved across diverse species from protozoa, to plants and metazoans. The loss of function in *MICU1* is attributed to several neurological disorders that include movement dysfunction. The inhibition of *MICU1* in the *Ddc-Gal4*-expressing neurons of *Drosophila melanogaster* presented with reduction in survival coupled with a precocious loss in locomotor ability. We attempted to rescue these phenotypes by overexpressing the sole *Bcl-2* homologue in *Drosophila Buffy*. The co-expression of *MICU1-RNAi* along with *Buffy* resulted in the suppression of the loss of *MICU1*-induced phenotypes. Subsequently, the inhibition of MICU1 in the *Drosophila* developing eye resulted in reduced number of ommatidia paired with highly fused ommatidial array. These developmental eye defects were rescued by the overexpression of *Buffy*.

Introduction

The mitochondrial calcium uptake 1 (*MICU1*) encodes a mitochondrial EF hand protein essential for the uptake of calcium [1]. It is the regulatory component of the calcium uniporter that is proposed to regulate signalling, energy metabolism and cell death [1-3]. The mitochondrial calcium uniporter (MCU) is the pore-forming subunit while MICU1 together with other subunits are the regulatory components [4]. MICU1 is a peripheral membrane protein that possess two EF-hand domains that are used for calcium sensing. MICU1 is evolutionarily diverse being present in some protozoa, protists, plants, and metazoans [5]. It is in most cases found strongly co-expressed with MCU and in some cases, share a potential promoter.

MICU1 is required for MCU-mediated calcium uptake [1] and plays an essential gatekeeping role to regulate cell survival [6]. The two MICU1 calcium binding EF-hands are key to its inhibitory functions. The knockdown of *MICU1* inhibits mitochondrial calcium uptake [7], additionally mutations that abrogates EF-hand functions result in *MICU1* knockdown-like phenotypes [6]. Loss of function mutations in the *MICU1* gene were found to cause a brain and muscle disorder that was linked to mitochondrial calcium signalling [8]. Individuals suffering from this disorder suffered from proximal myopathy, learning difficulties and a progressive extrapyramidal movement disorder. This study shows a genetic link between mutations in *MICU1* and a neuromuscular disease in children. Homozygous deletions in this gene have been additionally implicated with fatigue and lethargy in children [9], while single nucleotide polymorphisms analysis indicate a role for the EF-hand in bipolar disorder.

The *Bcl-2* family of genes are key regulators of cell death and survival in animals and regulate cell fate decisions following developmental or stress signals [10]. The family is classified into pro-death and pro-survival members, with the later said to be the "guardians of the mitochondria". The *Bcl-2* family member homologues in *Drosophila melanogaster* are limited to the single anti-apoptotic *Buffy* and the pro-apoptotic *Debcl* [11]. The overexpression of *Buffy* is reported to confer survival benefits in response to external stimuli and under conditions of stress [12-15]. To determine the pro-survival effect of the *Bcl-2* homologue *Buffy*, and that of *MICU1*, we attempted to suppress the *MICU1*-induced phenotypes by overexpressing *Buffy* along with *MICU1* in the *Ddc-Gal4*-expressing neurons and in the *Drosophila* developing eye.

We propose that *CG4495*, through bioinformatic analysis to be the *MICU1* homologue in *Drosophila*. Additionally, we show that the inhibition of *CG4495/MICU1* in *Drosophila* neurons under the direction of the *Dopa decarboxylase* transgene results in shortened lifespan and locomotor dysfunction. Interestingly, these phenotypes are counteracted by the overexpression of the sole pro-survival *Bcl-2* homologue *Buffy*.

Materials and Methods

Bioinformatic analysis

The protein sequences were sourced from the National Center for Biotechnology Information (NCBI; http://www.ncbi.nlm.nih.gov/protein/). The functional domains were identified using the NCBI Conserved Domain Database (http://www.ncbi.nlm.nih.gov/cdd) [16] and the Eukaryotic Linear Motif (ELM) resource (http://elm.eu.org/) [17] which focuses on annotation and detection of eukaryotic linear motifs (ELMs), also known as short linear motifs (SLiMs). The Clustal Omega multiple sequence alignment (http://www.ebi.ac.uk/Tools/msa/clustalo/) [18, 19] was performed to indicate conservation of the domains. Transmembrane domains were identified by TMpred (http://www.ch.embnet.org/software/TMPRED_form.html) [20]. Further analysis of protein domains, modelling, prediction and analysis was performed with Phyre2 (http://www.sbg.bio.ic.ac.uk/phyre2/html/page.cgi?id=index) [21]. More information on this protein is available on http://www.uniprot.org/uniprot/A2VEI2.

Drosophila media, stocks and derivative lines

Stocks and crosses were maintained on standard cornmeal/molasses/yeast/agar media treated with propionic acid and methylparaben. Stocks were raised at room temperature while crosses and experiments were performed at 25°C and 29°C. The *CG4495* RNA

interference lines, *w^{1118}; P{GD4927}v49349* and *w^{1118}; P{GD4927}v49350* hereby referred to as *UAS-MICU1-RNAi (1)* and *UAS-MICU1-RNAi (2)* respectively were obtained from the Vienna Drosophila Resource Center (Vienna, Austria). The *UAS-Buffy* [11] was kindly provided by Dr. L. Quinn (University of Melbourne) and *Ddc-Gal4* flies [22] by Dr. J. Hirsch (University of Virginia). *GMR-Gal4* [23] and *UAS-lacZ* flies were obtained from the Bloomington Drosophila Stock Center (Indiana University).

The *UAS-Buffy/CyO; Ddc-Gal4* and *UAS-Buffy/CyO; GMR-Gal4* derivative lines were generated using standard homologous recombination methods and were used for overexpression of *Buffy* in neurons using the *Ddc-Gal4* transgene or in the developing eye using the *GMR* response elements following a standard protocol that has previously been described [24, 25].

Survival assay

Crosses of each genotype were made and a cohort of male flies collected upon eclosion and survival analysis assessed using a standard protocol [15, 26, 27]. At least two hundred flies were assayed per genotype and observed every 2 days for presence of deceased adults. Longevity data were analysed using the GraphPad Prism version 5.04 (GraphPad Software, Inc., La Jolla, CA, USA). Survival curves were compared using the Log-rank (Mantel-Cox) test and significance was determined at a 95% confidence interval ($P \leq 0.05$ with Bonferroni correction.

Climbing assay

A cohort of male flies was collected upon eclosion and scored for their ability to climb every 7 days [28]. Climbing analysis was performed using the GraphPad Prism version

5.04 from climbing indices. The climbing curves were fitted using non-linear regression and compared using 95% confidence intervals with a P-value of 0.05.

Scanning electron microscopy of the *Drosophila* eye

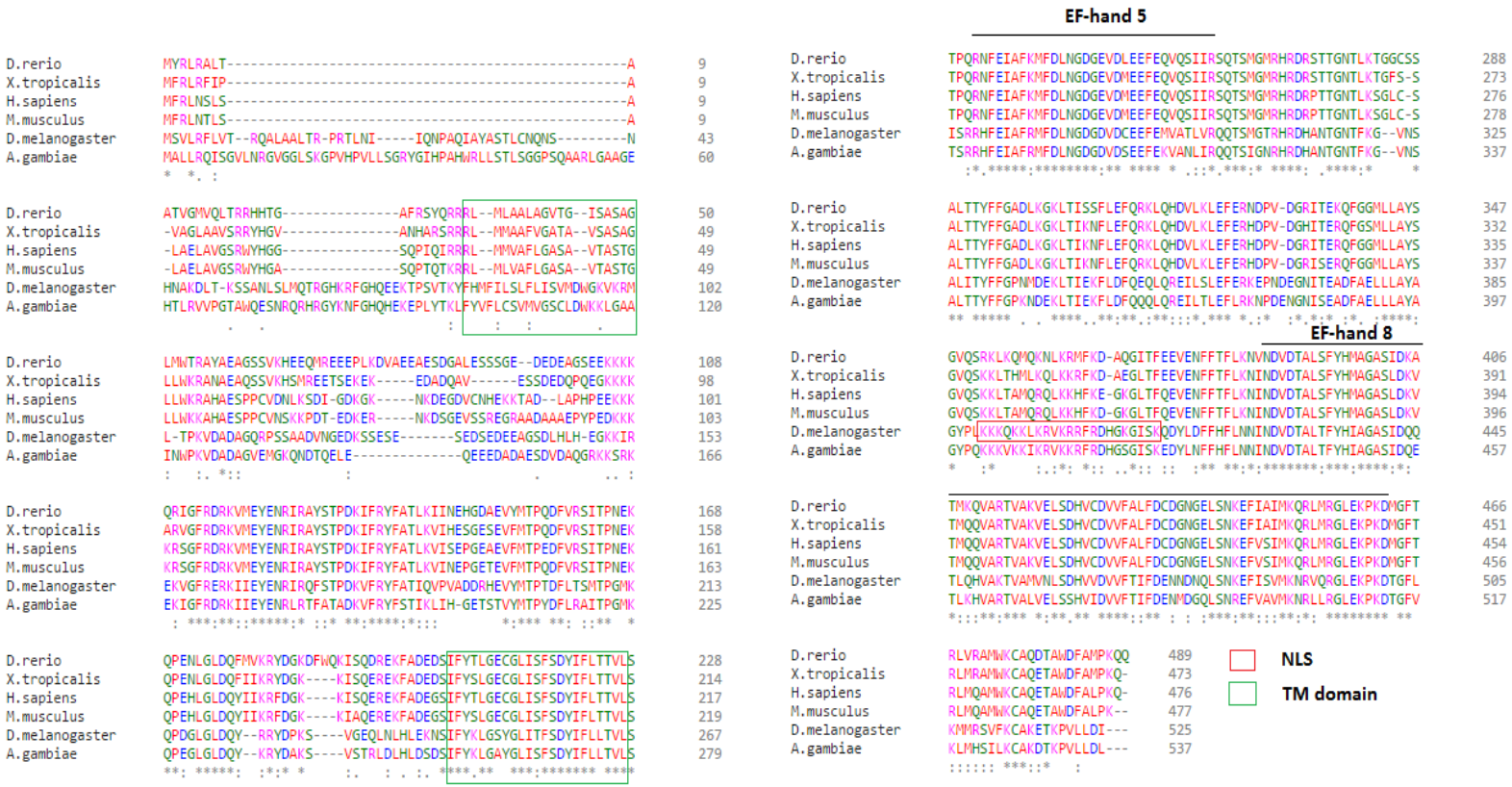
Crosses were made of each genotype at 29^{o} C and a batch of male flies collected upon eclosion and prepared for scanning electron microscopy following a standard protocol that has been previously described [15]. A minimum of 10 different eye images per genotype were analysed using ImageJ software (National Institutes of Health) [29] and biometric analysis performed using GraphPad Prism version 5.04. Disruption area of the eye was calculated as previously described [30]. Statistical comparisons comprised one-way analyses of variance and Dunnett's multiple comparison tests. P-values less than 0.05 were considered significant.

Results

Human *MICU1* and *Drosophila CG4495* EF-Hand domains are highly conserved

The *Drosophila* CG4495 and human MICU1 homologue contains 2 EF-hand domains that are closely related and evolutionarily conserved as determined by NCBI Conserved Domain Database (CDD) [16]. CG4495 is composed of 525 amino acids and shows 57% identity and 75% similarity to the 476 amino acids human MICU1 (Figure1). The *Drosophila* homologue has 2 EF-hand domains at amino acids 272 to 300 and 463 to 491, while in the human transcript they are located at amino acids 222 to 250 and 412 to 440 as determined by CDD and the Eukaryotic linear motif (ELM) [17]. Two to three transmembrane (TM) domains in the *Drosophila* and human transcripts are predicted using TMpred [20]. A nuclear localisation signal (NLS) was detected in both CG4495 (390 to 404) and human MICU1 (87 to 105) using ELM. A multiple sequence alignment

A.



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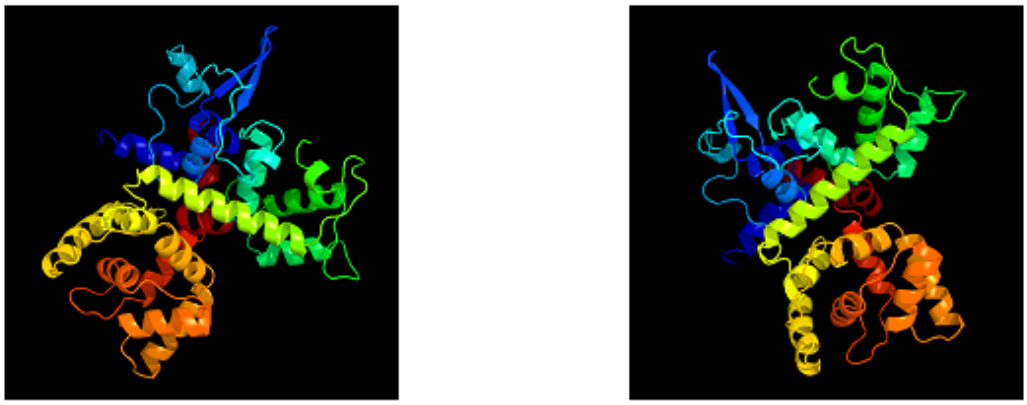


Human MICU1 Drosophila CG4495

Figure 1 - *Drosophila* CG4495/MICU1 has two EF-hand motifs that are evolutionarily conserved

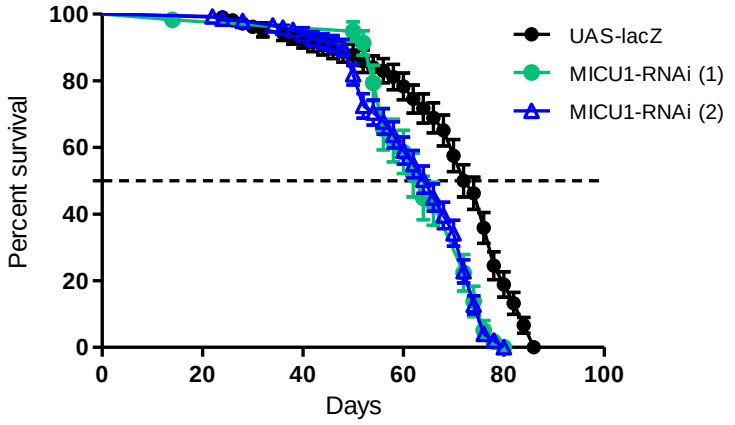
The 2 EF-hand domains are highly conserved and were identified using the NCBI Conserved Domain Database (CDD) [16] and the Eukaryotic Linear Motif (ELM) [17], in addition to the nuclear localisation signal. The membrane-spanning domains were identified using TMpred [20] and Phyre2 [21]. The mitochondrial targeting sequence in both human and Drosophila MICU1 were identified by TargetP [31]. Clustal Omega multiple sequence alignment [18, 19] of *Drosophila* CG4495 protein (D.melanogaster = *Drosophila melanogaster* NP_001097110.1) with the human (H.sapiens = *Homo sapiens* NP_001182447.1), mouse (M.musculus = *Mus musculus* NP_659071.1), zebra fish (D.rerio = *Danio rerio* NP_001077302.1), frog (X.tropicalis = *Xenopus (Silurana) tropicalis* NP_001106411.2), and mosquito (A.gambiae = *Anopheles gambiae* XP_319870.3) homologues shows conservation of the 2 EF-hand domains. "*" indicate the residues that are identical, ":" indicate the conserved substitutions, "." indicate the semi-conserved substitutions. Colours show the chemical nature of amino acids. Red is small hydrophobic (including aromatic), Blue is acidic, Magenta is basic, and Green is basic with hydroxyl or amine groups. A 3D modeling of both proteins using Phyre2 [21] is shown in the bottom panel.

of protein sequences using Clustal Omega [18, 19] shows high conservation of the EF-hand domains (Figure 1A). CG4495/MICU1 is localised to the mitochondria and has a mitochondrial targeting peptide with a presequence cleavage site at amino acid 115, human MICU1 has a presequence length of 33 amino acids as predicted using TargetP [31]. A 3D modelling of both proteins using Phyre2 [21] is shown (Figure 1B).

Inhibition of *MICU1* in the *Ddc-GAL4*-expressing neurons shortens lifespan and impairs climbing ability

To suppress *MICU1* in the DA neurons, two different RNAi lines were utilised to determine the specificity of the effects of inhibition of this gene and to rule out possible off-target effects. The inhibition of this gene results in shortened lifespan coupled with age-dependent loss in climbing ability. The median survival of *MICU1-RNAi* flies was 63 and 64 days when compared to 70 days for the controls that express the benign *lacZ* as determined by Log-rank (Mantel-Cox) test (Figure 2A). The directed inhibition of *MICU1* in the *Ddc-GAL4*-expressing neurons produces flies with significantly impaired climbing ability as determined by a nonlinear fit of the climbing curves (Figure 2B). The comparison of the confidence intervals (CI) at 95% indicate a significant difference between the *MICU1-RNAi* flies and the controls. These results suggest that *MICU1* is essential for the normal function of these neurons in *Drosophila*.

A



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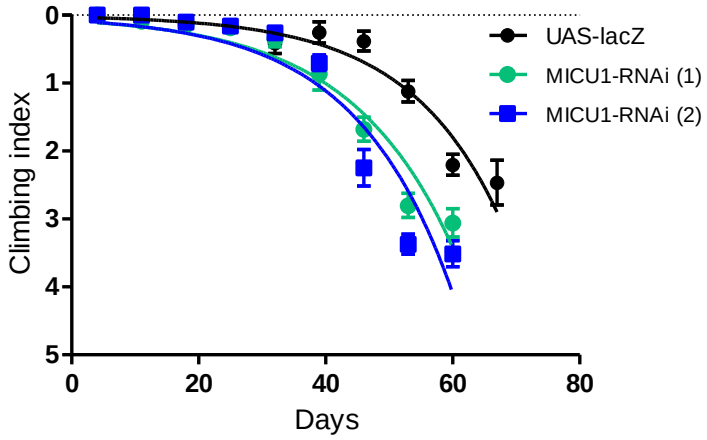


Figure 2 - Inhibition of *MICU1* shortens lifespan and severely impairs climbing ability and is counteracted by the overexpression of *Buffy*

A) The inhibition of *CG4495/MICU1* in the neurons under the control of the *Dopa decarboxylase* transgene results in reduced lifespan, with a median survival of 42 days and 46 days when compared to 70 days for control flies that expresses the benign *lacZ* transgene. The genotypes are *Ddc-Gal4/ UAS-lacZ, Ddc-Gal4/ UAS-MICU1-RNAi (1)* and *Ddc-Gal4/ UAS-MICU1-RNAi (2).* Longevity is shown as percent survival ($P < 0.05$, determined by the log-rank (Mantel-Cox) test and $N \geq 200$). B) The directed suppression of *CG4495/MICU1* in the *Ddc-Gal4*-expressing neurons resulted in premature loss in climbing ability as determined by nonlinear fitting of the climbing curves and comparing 95% CI. The genotypes are *Ddc-Gal4/ UAS-lacZ, Ddc-Gal4/ UAS-MICU1-RNAi (1)* and *Ddc-Gal4/ UAS-MICU1-RNAi (2).* Error bars indicate standard error of the mean and $N=50$.

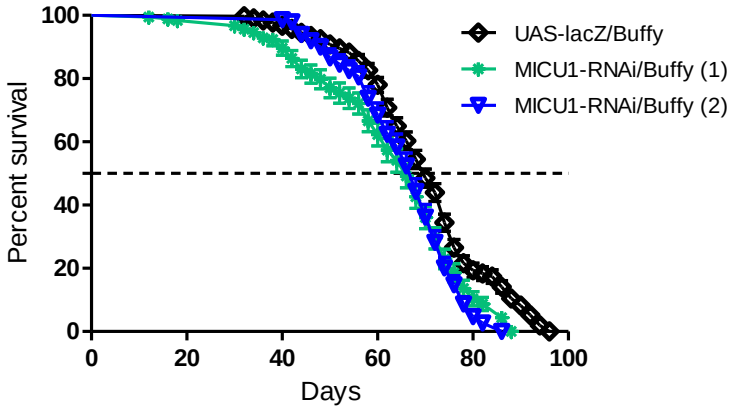
Buffy counteracts the loss of *MICU1*-induced phenotypes

The overexpression of the pro-survival *Bcl-2* homologue *Buffy* along with the suppression of *MICU1* in the *Ddc-GAL4*-expressing neurons results in a significant increase in lifespan and improved climbing ability. The co-expression of *Buffy* with *MICU1-RNAi* results in increased median survival of 70 and 71 days when compared to *Buffy* control flies with a median survival of 74 days as determined by Log-rank test (Figure 3A). The climbing ability of the *MICU1-RNAi* flies was improved as determined by comparison of the climbing curves at 95% CI with 0.039 to 0.052 compared with 0.039 to 0.051 which was not significant (Figure 3B). These results suggest a protective role for *Buffy* as it improves the lifespan and locomotor function in *MICU1* deficient neurons.

Inhibition of *MICU1* in the developing eye decreases ommatidia number and increases disruption of the ommatidial array

The inhibition of *MICU1* in the eye under the direction of the *GMR-Gal4* transgene decreases ommatidia number and results in significant disruption of the ommatidial array in the two RNAi lines tested when compared to the control flies (Figure 4A-C and 4G) as determined by a one-way analysis of variance (ANOVA) $p<0.0001$. The overexpression of *Buffy* along with the inhibition of *MICU1* restored the number of ommatidia and the percentage disruption to control levels as determined by a one-way analysis of variance, $p>0.50$ (Figure 4D-F and 4H). Taken together, these results suggest that CG4495/MICU1 may play a developmental role in the *Drosophila* eye and that *Buffy* suppresses the developmental eye defects that result from the inhibition of this developmentally important gene.

A



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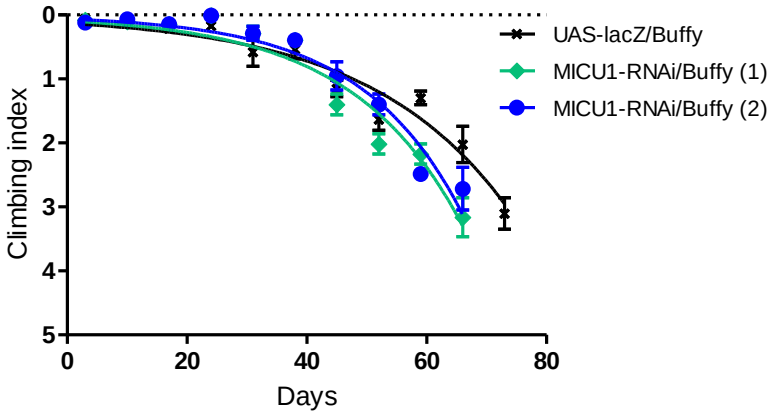
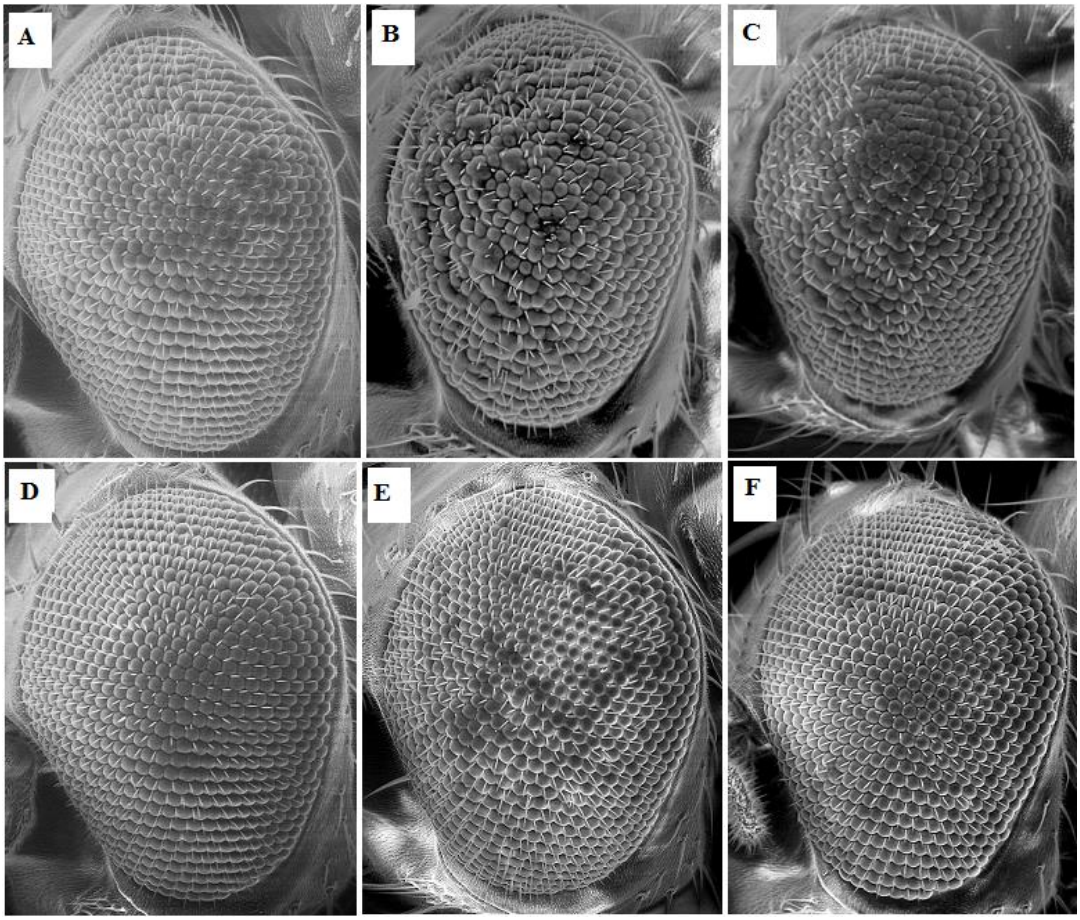


Figure 3 - Inhibition of *MICU1*-induced phenotypes can be counteracted by the overexpression of *Buffy*

A) The overexpression of *Buffy* along with *MICU1-RNAi* results in the suppression of decreased survival as indicated by a median survival of 70 days for both RNAi constructs when compared to 74 days for the control. Genotypes are *Ddc-Gal4 UAS-Buffy/ UAS-lacZ, Ddc-Gal4 UAS-Buffy/ UAS-MICU1-RNAi (1)* and *Ddc-Gal4 UAS-Buffy/ UAS-MICU1-RNAi (2).* Longevity is shown as percent survival ($P < 0.05$, determined by log-rank (Mantel-Cox) test with $N \geq 200$). B) The inhibition of *MICU1* along with the overexpression of *Buffy* in these neurons results in the suppression of the age-dependent loss in climbing ability. The genotypes are *Ddc-Gal4 UAS-Buffy/ UAS-lacZ, Ddc-Gal4 UAS-Buffy/ UAS-MICU1-RNAi (1)* and *Ddc-Gal4 UAS-Buffy/ UAS-MICU1-RNAi (2).* Analysis was done by nonlinear fitting of the climbing curves and significance was determined by comparing the 95% CI. Error bars indicate standard error of the mean and $N=50$.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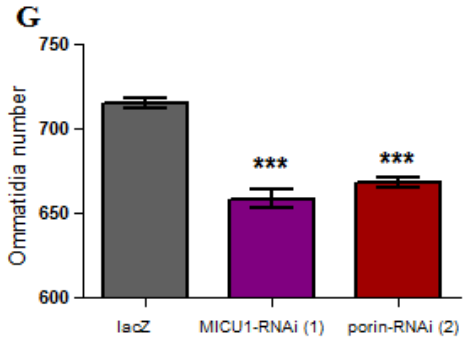
G
Ommatidia number
750
700
650
600
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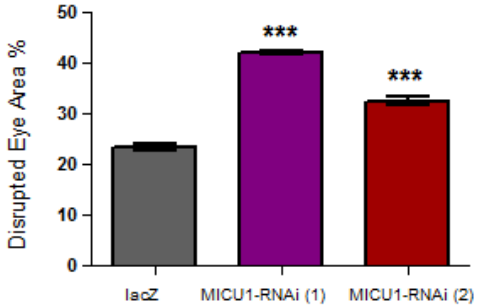
lacZ
MICU1-RNAi (1)
porin-RNAi (2)


Disrupted Eye Area %
50
40
30
20
10
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lacZ
MICU1-RNAi (1)
MICU1-RNAi (2)

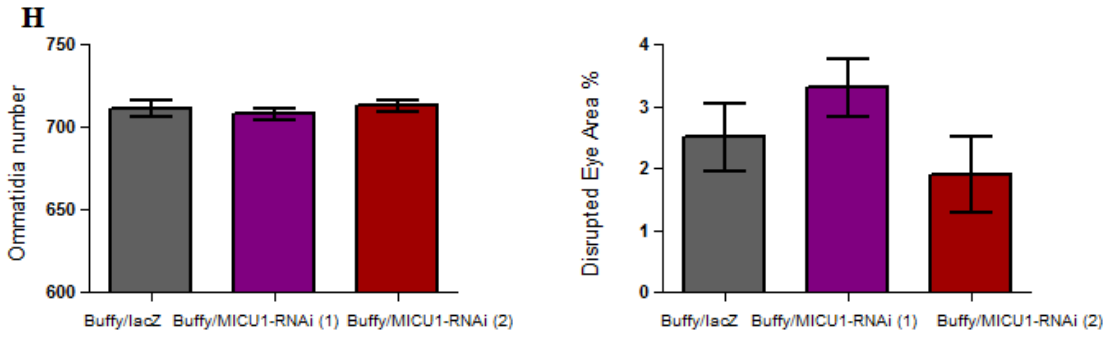

Figure 4 – Inhibition of *MICU1* in the eye results in decreased ommatidia and increased disruption of the ommatidial array

(A - F) Scanning electron micrographs of the inhibition of *CG4495/MICU1* in the *Drosophila* developing eye and its co-expression along with *Buffy*. The genotypes are (A) *GMR-Gal4/ UAS-lacZ*, (B) *GMR-Gal4/ UAS-MICU1-RNAi (1)*, (C) *GMR-Gal4/ UAS-MICU1-RNAi (2)*, (D) *UAS-Buffy; GMR-Gal4/ UAS-lacZ*, (E) *UAS-Buffy; GMR-Gal4/ UAS-MICU1-RNAi (1)*, and (F) *UAS-Buffy; GMR-Gal4/ UAS-MICU1-RNAi (2)*. G) Biometric analysis of the loss of *CG4495/MICU1* function in the developing eye indicates decreased ommatidia number and higher percentage of ommatidial disruption when compared to the control. H) Similarly, the co-expression of *Buffy* with *MICU1-RNAi* results in the suppression of the developmental eye phenotypes. Comparisons were determined by a one-way analysis of the variance (ANOVA), $p<0.05$, error bars are standard error of the mean, asterisks (*) represent statistical significance and $N=10$.

Discussion

The importance of mitochondrial calcium uptake 1 as a regulatory subunit of mitochondrial calcium uniporter (MCU) is exemplified by the disorders that result from its misexpression or in mutations that alter the EF hand function [8, 9, 32, 33]. Bioinformatic analysis reveals very conserved EF-hand domains, this may point to an evolutionarily conserved cellular pathway that is involved in calcium uptake and possibly in calcium signalling. Further analysis show presence of transmembrane domains and a nuclear localisation signal motif that contained residues that were not well conserved among the species investigated. It therefore appears that CG4495 is the closest homologue of MICU1 in *Drosophila*.

In both the RNAi fly lines we tested, there was a consistent reduction in lifespan and a profound early-onset loss in climbing ability. The loss of *MICU1* function in humans alters mitochondrial calcium signalling and result in proximal skeletal muscle weakness and brain disorder [8]. The *MICU1-RNAi* flies exhibited erratic climbing and at a very early time complete lack of climbing, they attempted to climb on the apparatus but were unsuccessful and dropped to the bottom of the climbing tube within the first level. The loss in climbing ability was not matched by decreased survival and from Figure 2A and 2B it is evident that at 40 days when most the flies in both the control and the RNAi lines were alive, the *MICU1-RNAi* flies had lost their climbing ability in an age-dependent manner, which is indicative of neuronal degeneration. We established that the suppressed activity of *MICU1* in *Drosophila* results in significant reduction in survival and a precocious loss in climbing ability. We co-expressed the pro-survival *Bcl-2* homologue

Buffy along with *MICU1-RNAi* and observed that this counteracted the induced phenotypes. This was an important finding as it includes a role for Buffy in calcium signalling and possibly a role in mitochondrial calcium uptake. The pro-survival role of Buffy in conditions of stress is well documented [15, 24, 25, 34], while this new role for Buffy deserves further investigation and may point to the conserved role for Bcl-2 proteins in regulation of calcium signalling [35]. Additionally, the rescue by Buffy highlights the pro-survival function of MICU1, being protective to the mitochondria as its loss can be compensated in yet unknown mechanisms by Buffy.

In complimentary experiments in the neuron rich *Drosophila* eyes, the inhibition of *MICU1* by stable inducible RNA interference under the direction of the *GMR-Gal4* transgene results in a reduction in the number of ommatidia and an increase in the area of the eye that had fused ommatidia. *MICU1-RNAi (1)* showed a greater penetrance of the eye phenotypes, with significantly lower numbers of ommatidia and greater disruption of the ommatidial array than *MICU1-RNAi (2)*. This indicates that mitochondrial calcium homeostasis is important in the development of the Drosophila eye. Indeed, as previously observed, the co-expression of *Buffy* along with *MICU1-RNAi* in the developing eye resulted in the restoration of the eye defects to normal levels. It appears that *MICU1* plays a significant role in *Drosophila Ddc-Gal4*-expressing neurons and in the developing eye.

Conclusions

The bioinformatic candidate for MICU1 in *Drosophila* appears to be *CG4495*, experimental evidence shows that the inhibition of this gene in neurons results in reduced survival and an age-dependent onset of locomotor dysfunction. These phenotypes are

strongly associated with neurodegeneration and are rescued upon overexpression of the pro-survival *Buffy*, highlighting the protective nature of *MICU1* in *Drosophila* neurons.

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Appendix 4: Parkinson disease genes and their function

PD locus	Gene/ Protein	Inheritance	Function
PARK1/4	SNCA α-synuclein [1]	Autosomal dominant (AD)	Unknown, presynaptic vesicles
PARK 2	Parkin [2]	Autosomal recessive (AR)	E3 ubiquitin ligase
PARK 3	Unknown		
PARK 5	Ubiquitin C-terminal hydrolase – L1 (Uchl-1) [3]	AR	Thiol protease involved in deubiquitinating
PARK 6	Phosphatase and tensin homologue (PTEN) induced kinase 1 (Pink1) [4]	AR	Protein kinase
PARK 7	DJ-1 [5]	AR	Unknown but possibly antioxidant and/or chaperone
PARK 8	Leucine rich repeat kinase 2 (LRRK2) [6, 7]	AD	Multifunctional with kinase, GTPase, and WD40 domain
PARK 9	ATP 13A2 [8]	AR	Lysosomal type 5 P-type ATPase
PARK 10	Unknown		
PARK 11	Grb10-interacting GYF protein-2 (GIGYF2) [9]	AD	Insulin signalling
PARK 12	Unknown		
PARK 13	High temperature requirement A2 (HTRA2) [10]	AR	Mitochondrial serine protease

PARK 14	Phospholipase A2 (PLA2G6) [11]	AR	Fatty acid hydrolysis
PARK 15	F-box only protein 7 (FBXO7) [12]	AR	Ubiquitination
PARK 16	Unknown		
PARK 17	Vacuolar protein sorting 35 (VPS35) [13, 14]	AD	Component of the retromer cargo-recognition complex
PARK 18	Eukaryotic translation initiation factor 4 gamma 1 (EIF4G1) [15]	AD	mRNA translation initiation
PARK 19	DnaJ homologue subfamily C member 6 (DNAJC13) [16]	AR	Clathrin–uncoating co-chaperone (clathrin mediated endocytosis)
PARK 20	Synaptojanin 1 (SYNJ1) [17, 18]	AR	Phosphoinositide phosphatase (synaptic vesicle recycling)

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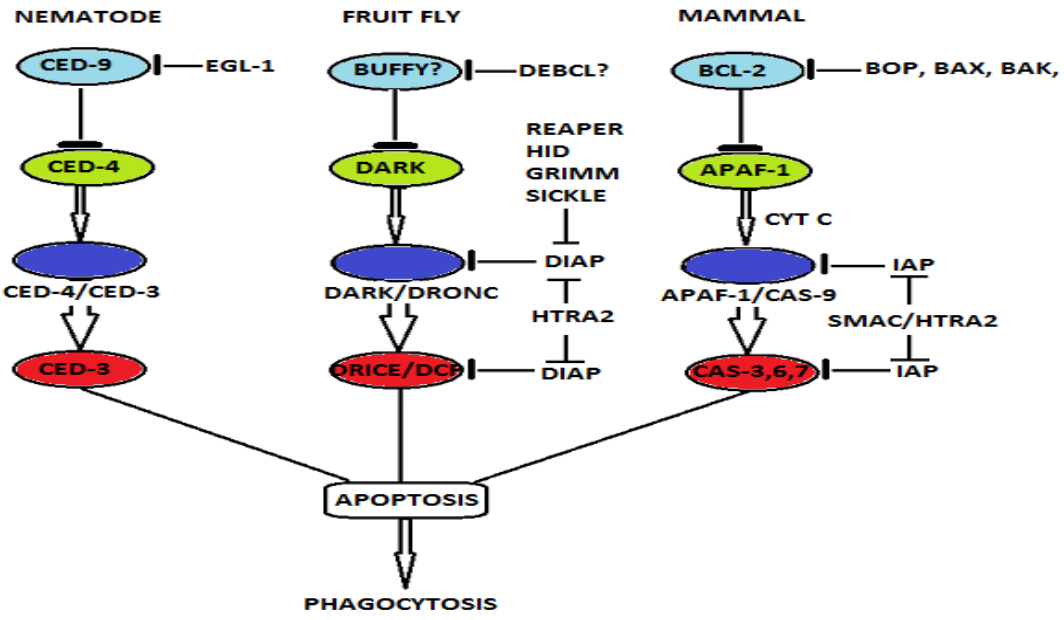
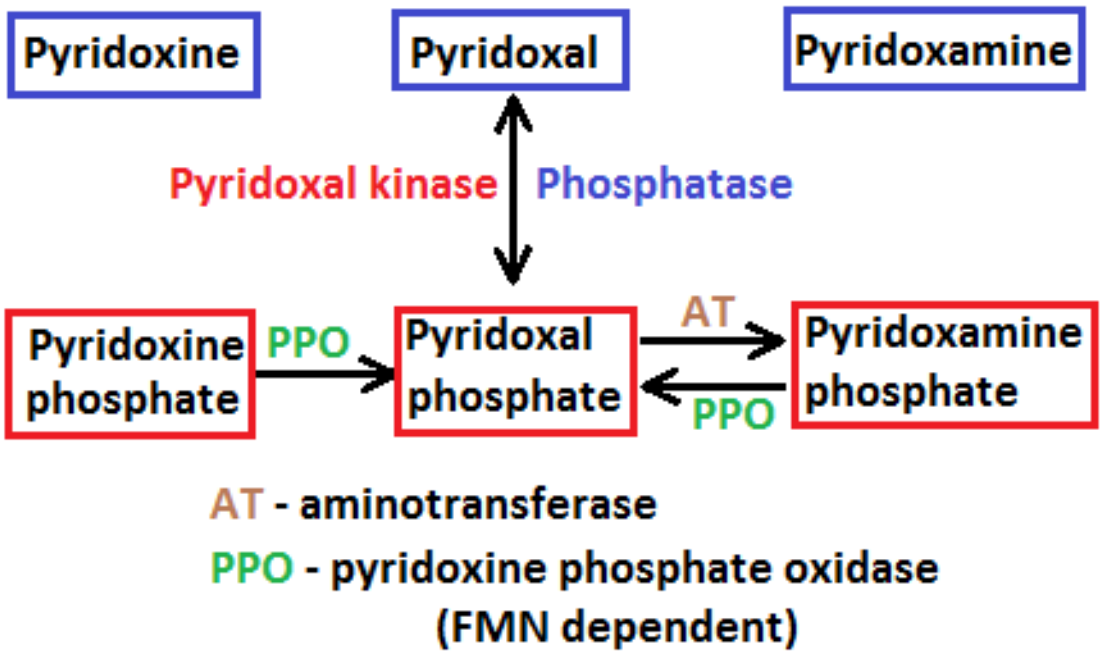
Appendix 5: The initiation of apoptosis in three model organisms: a comparison of the main apoptotic molecules



A comparison of the three main model organisms that are used to study aspects of apoptosis to highlight conservation of various molecules used in different stages of apoptosis. The different levels are indicated by different coloured molecules. Though the mechanisms for apoptosis differ in all three organisms, the molecules involved are conserved and interact with other molecules across species.

Appendix 6: Pyridoxal kinase and formation of pyridoxal phosphate



The vitamin B6 consists of three vitamers, pyridoxine (PN), pyridoxal (PL) and pyridoxamine (PM). Pyridoxal kinase phosphorylates them to PNP, PLP and PMP. PLP is the active form of vitamin B6 and requires pyridoxal kinase, PNP and PMP are further converted into PLP via the action of pyridoxine 5'-phosphate oxidases that requires Flavin mononucleotide (FMN) as a cofactor.