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Bax-inhibitor-1 loss of function phenotypes are suppressed by *Buffy* in *Drosophila*

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Background. Bax Inhibitor-1 (BI-1), an integral transmembrane protein, acts as a suppressor of *Bax*-induced apoptosis through regulation of endoplasmic reticulum (ER) stress-induced cell death. The gene is highly conserved being found in a diverse range of 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. We inhibited the cytoprotective *BI-1* in the *dopa decarboxylase* (*Ddc*) expressing neurons and in the developing eye of *Drosophila melanogaster* to investigate its neuroprotective functions. **Methods.** We assessed the longevity and locomotor ability of flies in response to altered *BI-1* expression in the *Ddc-Gal4*-expressing neurons where we exploited two RNAi transgenic fly lines. A control that expressed the benign *lacZ* responding transgene was used to compare against the RNAi transgenic flies. In addition, we compared the effect of the loss of *BI-1* expression in the developing eye, through biometric analysis of the number of ommatidia and extent of disruption of the ommatidial arrays. Finally, *Buffy* and *α-synuclein* were co-expressed to evaluate the potential for interactions. **Results.** The inhibition of *BI-1* in these neurons resulted in a shortened lifespan and precocious loss of locomotor ability. The co-expression of *Buffy,* the sole anti-apoptotic *Bcl-2* homologue in Drosophila, with *BI-1-RNAi* resulted in suppression of the reduced lifespan and impaired climbing ability. Expression of human *α-synuclein* in Drosophila dopaminergic neurons results in 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 decreased survival coupled with an impaired 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* results in reduction of ommatidia number and increased disruption of the ommatidial array. **Conclusions.** 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. These are suppressed upon overexpression of *Buffy* and worsened by co-expression with *α-synuclein.* This suggests that *BI-1* acts in neuroprotection and that its inhibition can be counteracted by the overexpression of the pro-survival *Bcl-2* homologue, *Buffy*.

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Abstract

Background. Bax Inhibitor-1 (BI-1), an integral transmembrane protein, acts as a suppressor of *Bax*-induced apoptosis through regulation of endoplasmic reticulum (ER) stress-induced cell death. The gene is highly conserved being found in a diverse range of 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. We inhibited the cytoprotective *BI-1* in the *dopa decarboxylase* (*Ddc*) expressing neurons and in the developing eye of *Drosophila melanogaster* to investigate its neuroprotective functions.

Methods. We assessed the longevity and locomotor ability of flies in response to altered *BI-1* expression in the *Ddc-Gal4*-expressing neurons where we exploited two RNAi transgenic fly lines. A control that expressed the benign *lacZ* responding transgene was used to compare against the RNAi transgenic flies. In addition, we compared the effect of the loss of *BI-1* expression in the developing eye, through biometric analysis of the number of ommatidia and extent of disruption of the ommatidial arrays. Finally, *Buffy* and *α-synuclein* were co-expressed to evaluate the potential for interactions.

Results. The inhibition of *BI-1* in these neurons resulted in a shortened lifespan and precocious loss of locomotor ability. The co-expression of *Buffy,* the sole anti-apoptotic *Bcl-2* homologue in Drosophila, with *BI-1-RNAi* resulted in suppression of the reduced lifespan and impaired climbing ability. Expression of human *α-synuclein* in Drosophila dopaminergic neurons results in 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 decreased survival coupled with an impaired 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* results in reduction of ommatidia number and increased disruption of the ommatidial array.

Conclusions. 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. These are suppressed upon overexpression of *Buffy* and worsened by co-expression with *α-synuclein.* This suggests that *BI-1* acts in neuroprotection and that its inhibition can be counteracted by the overexpression of the pro-survival *Bcl-2* homologue, *Buffy*.

Introduction

The Bax Inhibitor-1 (BI-1) belongs to a diverse group of proteins, known as the Transmembrane Bax Inhibitor-1 Motif-containing (TMBIM) family (Henke et al. 2011; Li et al. 2014; Reimers et al. 2008; Rojas-Rivera & Hetz 2015) that has been determined to be regulators of cell death. A different nomenclature categorizes these proteins as the *LFG* family, a designation adopted from the family member *Lifeguard (Lfg)* (Hu, Smith & Goldberger 2009), which consists of six or more highly conserved members present in a wide range of organisms (Chae et al. 2003; Henke et al. 2011; Huckelhoven 2004). These regulators of cell death, accomplish this role through the regulation of the death receptor and result in the modulation of the endoplasmic reticulum (ER) calcium homeostasis, ER stress signalling pathways, autophagy, reactive oxygen species (ROS) production, cytosolic acidification and other cellular activities (Li et al. 2014; Rojas-Rivera & Hetz 2015). The founding member of this group is *BI-1* (or *TMBIM6*), also known as *testis*

enhanced gene transcript (*TEGT*), has been demonstrated to inhibit the effect of *Bax*-induced cell death (Walter et al. 1995; Xu & Reed 1998). Members of this protein family possess a BI-1-like domain with six to seven transmembrane-spanning regions and are strongly associated with the ER membranes (Carrara et al. 2012; Chae et al. 2004; Xu & Reed 1998). *BI-1* is highly conserved across diverse species with homologues of *BI-1* able to block *Bax*-induced cell death when expressed in yeast (Chae et al. 2003), thus implying it regulates an evolutionarily conserved cytoprotective pathway.

Although not structurally related to the B cell lymphoma 2 family of proteins, this protein has been shown to form a complex with the pro-survival members Bcl-2 and Bcl-X_L but not with Bax or Bak (Lisbona et al. 2009; Xu & Reed 1998). Therefore, it is likely the anti-apoptotic activities of *BI-1/TMBIM6* are mediated through interactions with pro-survival members of the *Bcl-2* family and acts downstream of Bcl-X_L (Xu et al. 2008). *BI-1* deficient cells, including neurons, are more sensitive to apoptosis induced by ER stress and the modulation of ER calcium homeostasis has been linked to this process (Chae et al. 2004; Dohm et al. 2006). This implicates BI-1 in a variety of human diseases that may include numerous cancers, obesity, liver diseases, autoimmune response, and diabetes (Kiviluoto et al. 2012; Li et al. 2014; Lisak et al. 2015; Robinson et al. 2011; Rojas-Rivera & Hetz 2015). Neuroprotective roles include protection from oxygen-glucose deprivation, promotion of neuronal proliferation and differentiation, and stress-induced protection (Dohm et al. 2006; Hunsberger et al. 2011; Jeon et al. 2012; Krajewska et al. 2011). It regulates ROS production by modulation of unfolded protein response (UPR) induction in the ER (Lee et al. 2007), suppression of mitochondria-mediated ROS production (Kim et al. 2012), reduction of cytochrome P450 2E1 activity and regulation of the ER membrane lipid

peroxidation (Kim et al. 2009). Undoubtedly BI-1 has significant cytoprotective roles and their abrogation lead to cellular homeostatic dysfunction and disease.

Drosophila melanogaster appears to possess most of the TMBIM protein family homologues: TMBIM6/BI-1 is represented by *BI-1/CG7188* (Attrill et al. 2015; Hu, Smith & Goldberger 2009; Rojas-Rivera & Hetz 2015). Drosophila has been used as a model organism in the study of gene expression and in human disease models, with very promising results in understanding Parkinson Disease (Staveley 2014). 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 (Chae et al. 2003), BI-1 as a negative regulator of the ER stress sensor IRE1α and its role in the UPR (Lisbona et al. 2009), and its modulation of autophagy (Castillo et al. 2011). 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 (Botella et al. 2009; Feany & Bender 2000; Park, Schulz & Lee 2007; Staveley 2014). The key elements of the original Drosophila model of Parkinson disease that utilizes the expression of a human *α-synuclein* transgene to induce the PD-like symptoms (Feany & Bender 2000); 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 (Auluck et al. 2002; Botella et al. 2009; Buttner et al. 2014; Feany & Bender 2000; Kong et al. 2015; Staveley 2014; Wang et al. 2015; Zhu et al. 2016). The spatio-temporal *UAS/GAL4* expression system (Brand & Perrimon 1993), 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

(Auluck et al. 2002; Botella et al. 2009; Buttner et al. 2014; Feany & Bender 2000; Kong et al. 2015; Staveley 2014; Wang et al. 2015; Zhu et al. 2016). Modelling PD and other neurodegenerative diseases in Drosophila provides a unique platform to explore the underlying mechanisms.

The *Bcl-2* family member homologues in Drosophila are limited to the single anti-apoptotic *Buffy* and the pro-apoptotic *Debcl* (Quinn et al. 2003). 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 (M'Angale & Staveley 2016; Monserrate, Chen & Brachmann 2012; Sevrioukov et al. 2007; Tanner et al. 2011). These experiments 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 if 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 amino acid sequences of the proteins were acquired from the National Center for Biotechnology Information (NCBI; http://www.ncbi.nlm.nih.gov/protein/) and the domains were ascertained using the NCBI Conserved Domain Database (CDD; http://www.ncbi.nlm.nih.gov/cdd) (Marchler-Bauer et al. 2015) and the Eukaryotic Linear Motif (ELM; http://elm.eu.org/) (Dinkel et al. 2016) which mediates the 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/) (Goujon et al. 2010; Sievers et al. 2011) to show conservation of the domains in the selected proteins. The prediction of the nuclear export signal (NES) was by NetNES (http://www.cbs.dtu.dk/services/NetNES/) (la Cour et al. 2004). Further analysis of protein sequences was performed with Phyre2 (Kelley et al. 2015), 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 (Blum, Briesemeister & Kohlbacher 2009) (https://abi.inf.uni-tuebingen.de/Services/MultiLoc2). Transmembrane domains were identified using TMpred (Artimo et al. 2012), 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 a standard cornmeal/molasses/yeast/agar media which had been treated with propionic acid and methylparaben to inhibit fungal growth. Stocks were maintained at room temperature (~20° C) while all crosses and the ageing and climbing determinations 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* (Quinn et al. 2003) was provided by Dr. L. Quinn (University of Melbourne), *Ddc-Gal4*

flies (Li et al. 2000) by Dr. J. Hirsch (University of Virginia) and *UAS-α-synuclein* (Feany & Bender 2000) by Dr. M. Feany (Harvard Medical School). *GMR-Gal4* (Freeman 1996) 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* compound lines, used to overexpress *α-synuclein* or *Buffy* in neurons and the developing eye, were generated through standard homologous recombination and marker selection methods as previously described (M'Angale & Staveley 2016a; M'Angale & Staveley 2016c). Standard PCR and gel electrophoresis was used to generate DNA fragments to identify some recombinant lines.

Ageing assay

A number of single vial crosses to generate each genotype was performed and a cohort of male flies of each critical class were collected upon eclosion and assayed using a standard protocol that we have described before (M'Angale & Staveley 2016; Todd & Staveley 2012). For each critical class genotype greater than 200 flies were aged and scored every 2 days for the presence of recently deceased adults until extinction of each cohort (Staveley, Phillips & Hilliker 1990). Survival data was analysed employing the statistical software 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 the 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 an established method that was described by our research group (Todd & Staveley 2004). Climbing analysis was accomplished through the use of 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

Critical class male flies were collected upon eclosion and aged for three to five days and then prepared for scanning electron microscopy using a standard protocol (M'Angale & Staveley 2016). For each cross at least 10 different eye images were analysed using the National Institutes of Health (NIH) ImageJ software (Schneider, Rasband & Eliceiri 2012) and biometric analysis performed using GraphPad Prism version 5.04. The disrupted area of the eye was calculated according to a methodology that we have established (M'Angale & Staveley 2012). Statistical comparisons consisted of one-way analyses of variance (ANOVA) and Dunnett's multiple comparison tests. P-values less than 0.05 are 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 (Marchler-Bauer et al. 2015). An alignment of the protein sequences using Clustal Omega (Goujon et al. 2010; Sievers et al. 2011) 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) (Dinkel et al. 2013) and TMpred (Artimo et al. 2012). An analysis of membrane-spanning domains by Phyre2 (Kelley et al. 2015) 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 (la Cour et al. 2004) 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.

Inhibition of *BI-1* in 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 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 intensified 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 5A – C and 5J) 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 and the percentage disruption to control levels as determined by a one-way analysis of variance with p = 0.2439 and 0.2342 (Figure 5D – F and 5K). 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 5G – I and 5L). 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 *Bax Inhibitor-1 (BI-1)* via stable inducible RNAi transgenes in the *Ddc-Gal4*-expressing neurons of Drosophila results in decreased survival and impaired climbing ability over time. Although there is no known version of *Bax* in Drosophila, the only *Bcl-2* homologue that has been demonstrated to possess pro-apoptotic functions is *Debcl* (Brachmann et al. 2000; Colussi et al. 2000; Igaki et al. 2000; Zhang et al. 2000). The Drosophila *BI-1* is able to block *Bax*-induced cell death in yeast (Chae et al. 2003), and loss of *BI-1* function induces cell death (Xu & Reed 1998). 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 (Xu & Reed 1998) 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 (Chae et al. 2004). It seems to do this by the regulation of calcium ions (Lisak et al. 2015; Xu et al. 2008) and ROS (Kim et al. 2009; Lee, Kim & Chae 2012). *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 may result in neuronal dysfunction, degeneration and death. The only pro-survival *Bcl-2* homologue in Drosophila is *Buffy* (Quinn et al. 2003) and the overexpression of *Buffy* is known to confer survival advantages to cells under normal conditions and under conditions of stress (Clavier et al. 2014; M'Angale & Staveley 2016; M'Angale & Staveley 2016b; M'Angale & Staveley 2016c; Monserrate, Chen & Brachmann 2012; Quinn et al. 2003; Sevrioukov et al. 2007). 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 (Feany & Bender 2000), 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 (Li et al. 2014). 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 (Kramer & Staveley 2003). 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 developing Drosophila eye can reduce the number of ommatidia and can generate a highly disrupted ommatidial array (Feany & Bender 2000). The *α-synuclein*-induced developmental defects in the eye provides a model 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 results in a 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 (Chinta et al. 2010). 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 (Li et al. 2014). 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 a reduction in lifespan and an age-dependent loss in climbing ability. These phenotypes are associated with the degeneration and loss of dopaminergic neurons that have been observed in Drosophila models of Parkinson Disease. The co-expression of the pro-survival *Buffy* with *BI-1-RNAi* results in the rescue of the phenotypes observed and it is possible that the Buffy and BI-1 protein interact to promote anti-apoptotic mechanisms. Finally, *BI-1* appears to be neuroprotective as its inhibition along with *α-synuclein* expression result in enhanced phenotypes.

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Figures Legends

Figure 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 (Marchler-Bauer et al. 2015). They have six transmembrane-spanning regions as predicted by the Eukaryotic Linear Motif (ELM) (Dinkel et al. 2013) and TMpred (Artimo et al. 2012). 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 (Goujon et al. 2010; Sievers et al. 2011) 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 (Kelley et al. 2015) 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).

Figure 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 the *lacZ* transgene. 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 $N=50$.

Figure 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 inhibition 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.

Figure 4 – Loss of *BI-1* in neurons complements the *α-synuclein*-induced phenotypes

A) The 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.

Figure 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 1,* and I) *UAS-α-synuclein; GMR-Gal4/ UAS-BI-1-RNAi 2.* 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 *** $p<0.001$).

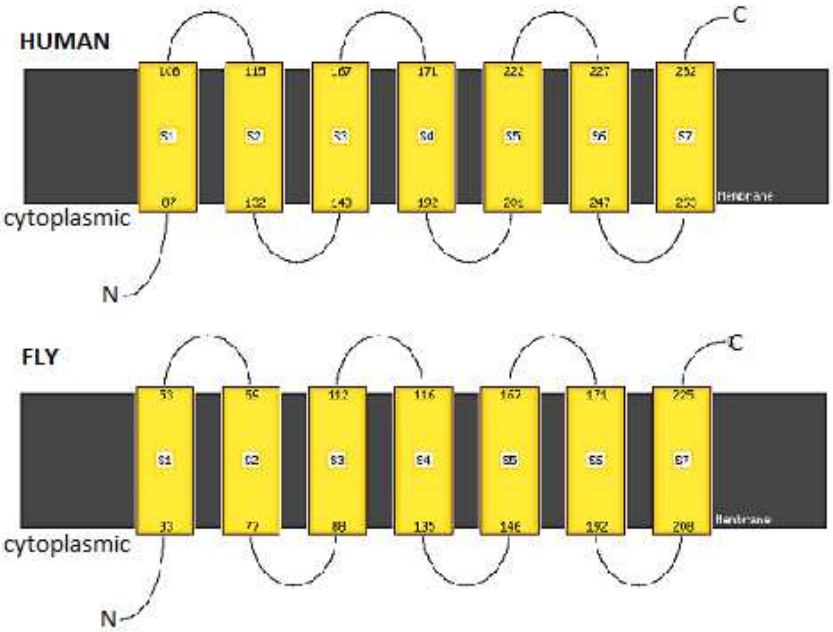
Figure 1(on next page)

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 (Marchler-Bauer et al. 2015) . They have six transmembrane-spanning regions as predicted by the Eukaryotic Linear Motif (ELM) (Dinkel et al. 2013) and TMpred (Artimo et al. 2012) . 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 (Goujon et al. 2010 ; Sievers et al. 2011) 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 (Kelley et al. 2015) 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).

A.

B.



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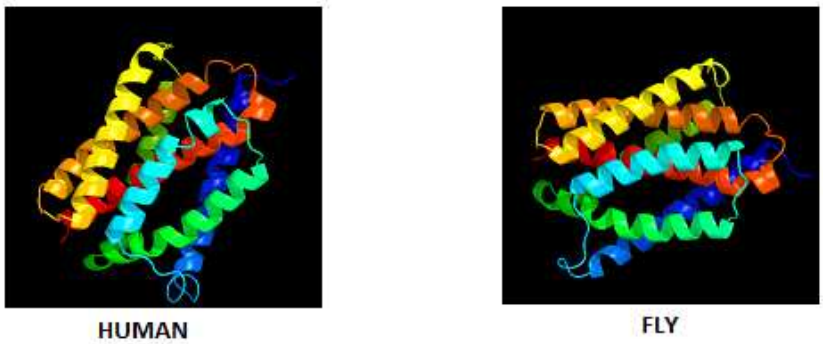

Figure 2 (on next page)

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

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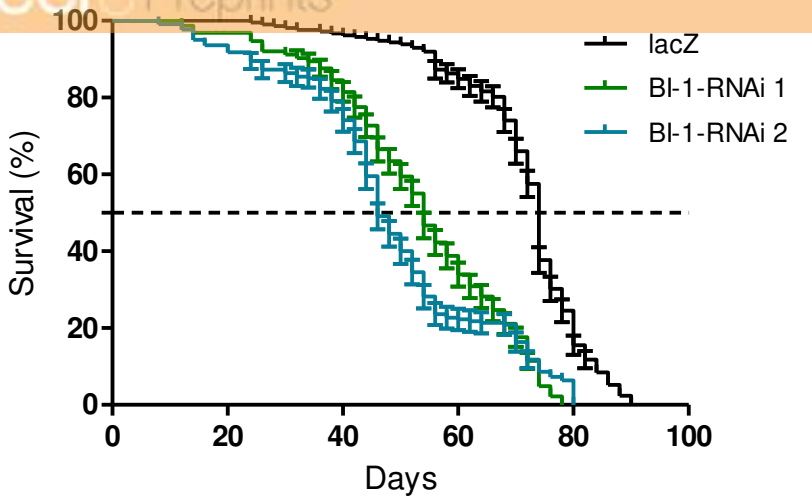
A.



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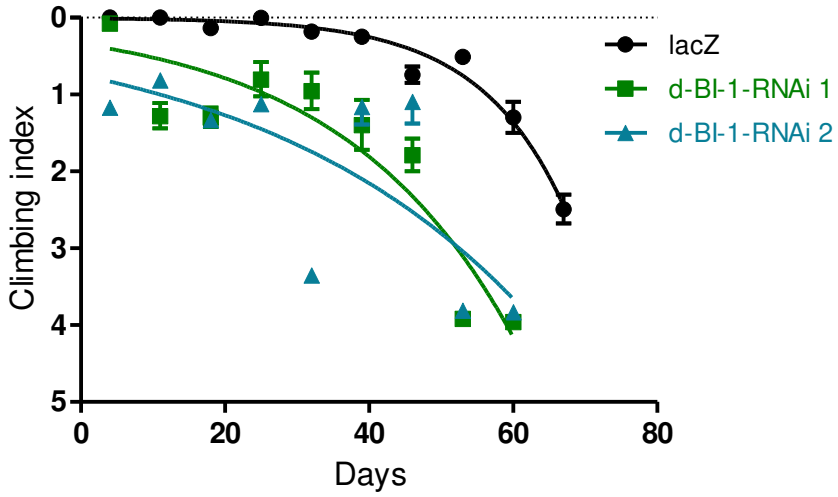

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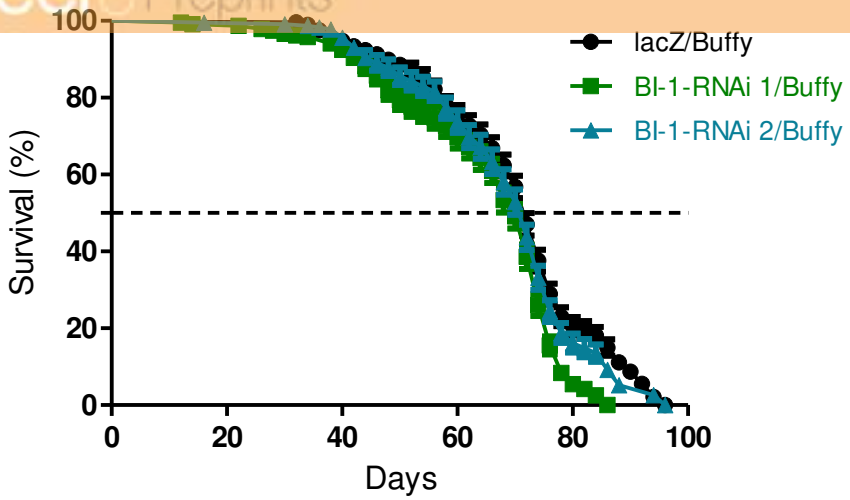
A.



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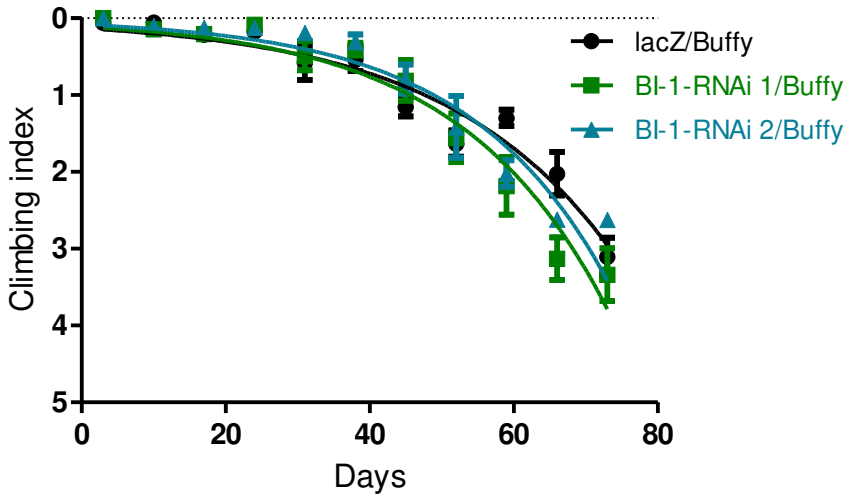
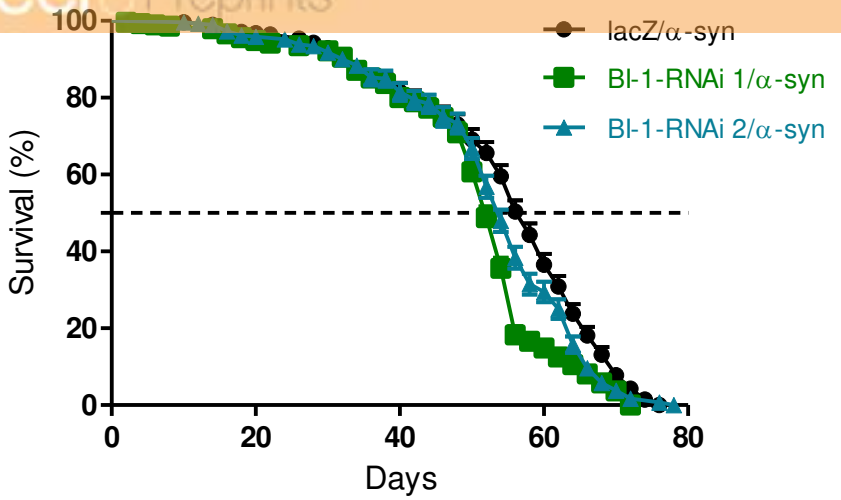


Figure 4(on next page)

Loss of *BI-1* in neurons complements the *α-synuclein*-induced phenotypes

A) The 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.

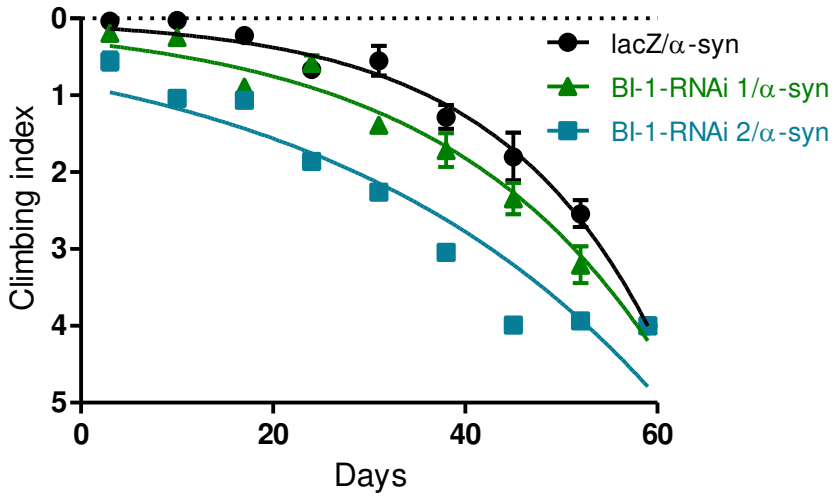


Figure 5(on next page)

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 1,* and I) *UAS-α-synuclein; GMR-Gal4/ UAS-BI-1-RNAi 2.* 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 *** $p<0.001$).