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 of unknown function. Downregulation of *Lifeguard* disrupts cellular homeostasis and disease by sensitizing neurons to FasL-mediated apoptosis. We used bioinformatic analyses to identify CG3814, a putative homologue of Lifeguard, and knocked down *CG3814/LFG* expression under the control of the *Dopa decarboxylase* (*Ddc-Gal4*) transgene in *Drosophila melanogaster* neurons to investigate whether it possesses neuroprotective activity. Knockdown of *CG3814/LFG* in *Ddc-Gal4*-expressing neurons resulted in a shortened lifespan and impaired locomotor ability, phenotypes that are strongly associated with the degeneration and loss of dopaminergic

neurons. Lifeguard interacts with anti-apoptotic Bcl-2 proteins and possibly pro-apoptotic proteins to exert its neuroprotective function. The co-expression of *Buffy*, the sole anti-apoptotic Bcl-2 gene family member in *Drosophila*, and *CG3814/LFG* by stable inducible RNA interference, suppresses the shortened lifespan and the premature age-dependent loss in climbing ability. Suppression of *CG3814/LFG* in the *Drosophila* eye reduces the number of ommatidia and increases disruption of the ommatidial array. Overexpression of *Buffy*, along with the knockdown of *CG3814/LFG*, counteracts the eye phenotypes. Knockdown of *CG3814/LFG* in *Ddc-Gal4*-expressing neurons in *Drosophila* diminishes its neuroprotective ability and results in a shortened lifespan and loss of climbing ability, phenotypes that are improved upon overexpression of the pro-survival *Buffy.*

Key words: Lifeguard; Bcl-2; Buffy; Neurons; Drosophila; CG3814

INTRODUCTION

Lifeguard (LFG) or Fas apoptotic inhibitory molecule 2, also known as Transmembrane Bcl-2 associated protein X (Bax) inhibitor motif 2, belongs to a diverse membrane-spanning protein family (Hu et al., 2009; Rojas-Rivera and Hetz, 2015) and inhibits apoptosis mediated by the Fas/CD95/Apo-1 receptor but not the closely related TNFR (Somia et al., 1999). LFG was first identified as neuronal membrane protein 35 when it was found to be differentially upregulated during rat postnatal development (Schweitzer et al., 1998) and predominantly localized to the endoplasmic reticulum (ER). A different nomenclature categorizes this protein into a family referred to as *LFG*, which is adopted from *Lifeguard* (Hu et al., 2009), and classifies LFG as LFG2. This conserved protein consists of seven transmembrane domains and is found in plants, insects, amphibians, fish, and mammals (Reimers et al., 2006). LFG regulates cell death by interfering with caspase 8 activation, but not its recruitment to the death-inducing signaling complex (Somia et al., 1999), 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 (Beier et al., 2005). This appears to occur through its regulation by PI3K. Another mechanism through which LFG regulates apoptosis is via interaction with Bcl-X_L and Bcl-2 at the ER to inhibit calcium release (Urresti et al., 2016). This interaction with Bcl-X_L is contrary to previous findings where LFG was shown to interact with Bax (Reimers et al., 2006). Dysregulation of *LFG* has been implicated in the disruption of cellular homeostasis involved in many cancers and neuronal diseases (Bucan et al., 2010; Reich et al., 2011). 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 contain anti-apoptotic and proapoptotic members (Siddiqui et al., 2015). These genes regulate life and death decisions at the cellular level by maintaining a delicate balance between pro-apoptotic and anti-apoptotic mediators. Homologues of *Bcl-2* family member in *Drosophila melanogaster* are limited to the anti-apoptotic *Buffy* and the pro-apoptotic *Debcl* (Quinn et al., 2003). In previous studies, overexpression of *Buffy* has been shown to confer survival advantages in response to external stimuli and under conditions of stress (Sevrioukov et al.,

2007; Tanner et al., 2011; Monserrate et al., 2012; M'Angale and Staveley, 2016d). A role for this gene in the mitochondrial pathway has been described during *Drosophila* oogenesis (Tanner et al., 2011). This suggests that this protein has an important role in aspects of cell death.

The *Drosophila* homologue was initially reported to be NMDARA1 by Schweitzer et al. (1998), although a previous study by Pellicena-Pallé and Salz (1995) reported this transcript to be NMDARA1 and a homologue of the rat glutamate-binding protein. It was originally listed as CG3798 in FlyBase and is currently listed as Nmda1, and is also known as NMDA receptor-associated protein (Reimers et al., 2006). The protein sequence used for bioinformatic comparisons in the present study was Nmda1 polypeptide C (http://flybase.org/reports/FBpp0088816.html). The accession number of the protein sequence used by Reimers et al. (2006) (NP_610824.1), has since been updated in both FlyBase and National Center for Biotechnology Information the (NCBI) and currently represents the CG3814 isoform A (Polypeptide Dmel\CG3814-PA) (http://flybase.org/reports/FBpp0086927.html). Interestingly, CG3814 and Nmda1 are adjacent to 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 have made comparisons between *Drosophila* CG3814 and/or CG9722, and human LFG (Hu et al., 2009; Rojas-Rivera and Hetz, 2015). The existence of homologues of this protein family that are implicated in the regulation of FasL-mediated apoptosis may underpin their evolutionary importance in cytoprotection. *Drosophila* is used as a model organism to study changes in gene expression and to model human diseases (Staveley, 2015). We have previously used *Ddc-Gal4*-expressing neurons 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 can be used as rapid assays to assess the organismal effects of altered gene expression and to identify genetic interactions. In the present study, we investigated the effect of *CG3814/LFG* knockdown, which has a wider expression pattern than the testes-specific *CG9722* (Hu et al., 2009), under the control of the *Dopa decarboxylase* transgene in neurons of *Drosophila*. We further determined whether there is interaction with Bcl-2 proteins by overexpressing the pro-survival *Bcl-2* homologue *Buffy*.

MATERIAL AND METHODS

Bioinformatic analysis

The protein sequences for *D. melanogaster*: NP_610824.1, *Homo sapiens*: NP_036438.2, *Xenopus tropicalis*: NP_001072357.1, and *Mus musculus*: NP_082500.2 were sourced from 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; (Marchler-Bauer et al., 2015) and the Eukaryotic Linear Motif (ELM) resource (http://elm.eu.org/) (Dinkel et al., 2016), which focuses on the annotation and detection of ELMs, also known as short linear motifs. 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 identify conservation of the Bax inhibitor-1 domain. Additional analysis were performed using Phyre2 (http://www.sbg.bio.ic.ac.uk/phyre2/html/page.cgi?id=index) (Kelley et al., 2015), a web portal for protein modeling, prediction, and analysis.

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° 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 the 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/) and was obtained from Bloomington *Drosophila* Stock Center at Indiana University, USA. Further information about these constructs is available online. *GMR-Gal4* (Freeman, 1996) and *UAS-lacZ* flies were obtained from the Bloomington *Drosophila* Stock Center. The *UAS-Buffy* flies (Quinn et al., 2003) were a gift from Dr. L. Quinn (University of Melbourne), and *Ddc-Gal4* flies (Li et al., 2000) 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 methods for homologous recombination and marker selection as previously described (M'Angale and Staveley, 2016a,c).

Survival assay

Several crosses of each genotype were performed and a cohort of male flies were collected upon eclosion (Todd and Staveley, 2012; M'Angale and Staveley, 2016d). For each genotype, at least 200 flies were aged and scored every 2 days for the presence of deceased adults (Staveley et al., 1990). Longevity data were analyzed 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 (Todd and Staveley, 2004). A climbing analysis was performed and climbing indices were analyzed by GraphPad Prism version 5.04. The climbing curves were fitted using non-linear regression and data were compared using 95% confidence intervals with a P value of 0.05 or less considered as 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 as previously described (M'Angale and Staveley, 2016d). Ten different scanning electron micrographs of each genotype were analyzed using the National Institutes of Health ImageJ software (Schneider et al., 2012) and biometric analysis was performed using GraphPad Prism version 5.04. The area of disruption of the ommatidial array was determined as detailed previously (M'Angale and Staveley, 2012). Statistical comparisons comprised one-way analyses of variance (ANOVA) and the Dunnett multiple comparison tests. P values less than 0.05 were considered significant.

RESULTS

Human *LFG* is closely related to *Drosophila CG3814* and has seven transmembrane domains (TMDs)

To characterize the *D. melanogaster* homologue of LFG, we performed bioinformatic analysis on the CG3814 isoform A, which is composed of 239 amino acids and shares 45% identity and 65% similarity with the 316-amino acid human LFG. The *Drosophila* homologue contains seven TMDs (Figure 1) and a tumor necrosis factor receptor-associated factor-binding consensus motif at amino acids 55-58 and 208-211, which may be involved in TNFR signaling as determined by the NCBI CDD (Marchler-Bauer et al., 2015), ELM resource (Dinkel et al., 2016) and Phyre2 (Kelley et al., 2015). Alignment of protein sequences in Clustal Omega multiple sequence alignment (Goujon et al., 2010; Sievers et al., 2011) revealed there is high conservation of the Bax inhibitor-1-like domain. Three-dimensional modeling of these proteins using Phyre2 (Kelley et al., 2015) shows there is close similarity in the structure and orientation of the transmembrane domains.

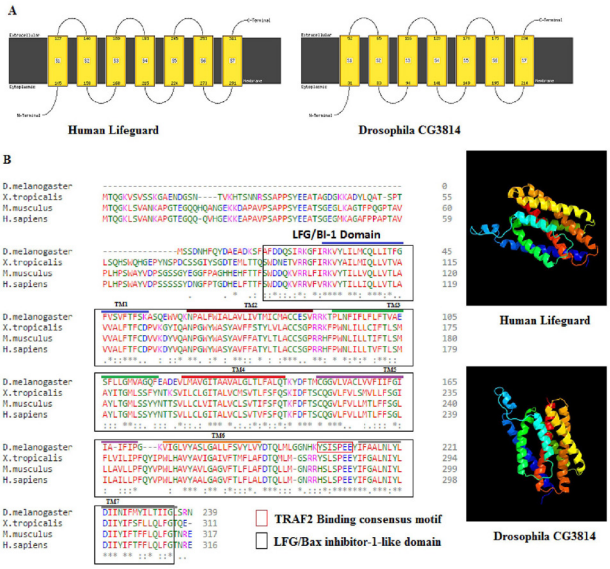

Figure 1. *Drosophila* CG3814 contains seven transmembrane domains that are evolutionarily conserved. **A.** Seven membrane-spanning regions and the 3D protein models of *Drosophila* CG3814 and the human homologue LFG were generated by Phyre2 (Kelley et al., 2015). These show the similarity between human LFG and *Drosophila* CG3814. **B.** *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) (Marchler-Bauer et al., 2015) and the Eukaryotic Linear Motif (Dinkel et al., 2016). Clustal Omega multiple sequence alignment (Goujon et al., 2010; Sievers et al., 2011) 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. Asterisk indicates the residues that are identical, colon indicates the conserved substitutions, dot indicates the semi-conserved substitutions. Colors denote 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.

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

We investigated the neuroprotective role of *CG3814/LFG* in *Drosophila*, and found that suppression of *CG3814/LFG* in the *Ddc-Gal4*-expressing neurons resulted in decreased lifespan and impaired locomotor ability. We employed two different RNAi lines to determine the specificity of the knockdown and compared the results with a control line. The median lifespan of *LFG-RNAi* flies was 42 and 46 days compared to 70 days for the *lacZ* controls as compared by Log-rank (Mantel-Cox) test with a $P < 0.0001$ (Figure 2A). Knockdown of *CG3814* in these neurons produces flies with significantly impaired climbing ability as determined by the nonlinear fitting of the climbing curves and by comparing the CI at 95% (Figure 2B). The *LFG-RNAi* flies had a CI of 0.069-0.091 and 0.050-0.071 when compared to 0.030-0.048 for the controls that express the benign *lacZ* transgene. Taken together, these results suggest a neuroprotective role for *CG3814/LFG* in *Drosophila*.

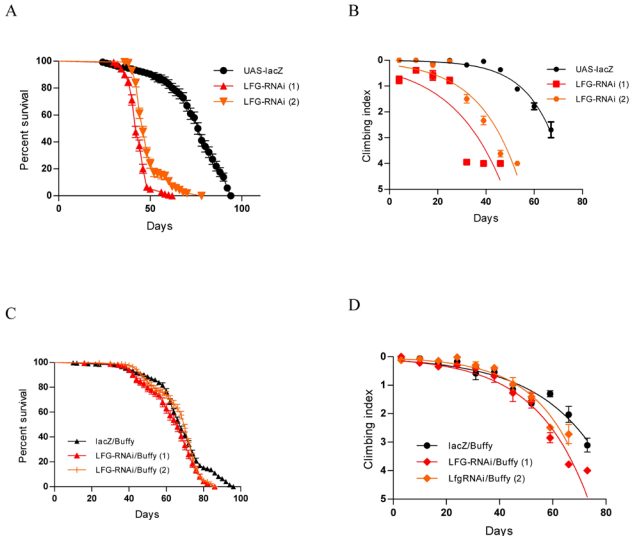

Figure 2. Loss of *CG3814/LFG* activity decreases survival, impairs climbing ability, and is rescued upon *Buffy* overexpression. **A.** Knockdown of *CG3814/LFG* in neurons under the control of the *Ddc-Gal4* transgene leads to reduced lifespan, with a median survival of 42 and 46 days compared with 70 days for control flies that express 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 presented as percent survival [$P < 0.05$, determined by the log-rank (Mantel-Cox) test and $N \geq 200$]. **B.** Directed suppression of *CG3814/LFG* in the *Ddc-Gal4*-expressing neurons resulted in the premature loss of climbing ability as determined by nonlinear fitting of the climbing curves and comparison of the 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 the standard error of the mean and N = 50. **C.** 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 presented as percent survival [$P < 0.05$, determined by log-rank (Mantel-Cox) test with $N \geq 200$]. **D.** Knockdown of *CG3814/LFG*, along with the overexpression of *Buffy* in these neurons, suppresses 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)*. Analyses was performed by nonlinear fitting of the climbing curves, and significance was determined by comparing the 95%CI. Error bars indicate a standard error of the mean and N = 50.

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

We investigated the pro-survival role of *Buffy* and *CG3814/LFG* in Drosophila neurons. Co-expression of the pro-survival *Bcl-2* homologue *Buffy*, and knockdown of *CG3814/LFG* significantly increased the lifespan and improved climbing ability. When *Buffy* was co-expressed with *LFG-RNAi*, the results indicated 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 2C). This is an increase in survival when compared to the flies documented in Figure 2A, in which *CG3814/LFG* expression was reduced by RNAi. The climbing ability of the *LFG-RNAi* flies was improved, as determined by comparing the climbing curves at 95%CI (Figure 2D). The *LFG-RNAi* constructs had a CI of 0.049-0.068 and 0.043-0.057 compared to the controls, which had 0.035-0.050, which indicates that the climbing curves were not significantly different. These results suggest a pro-survival role for *CG3814/LFG* in *Drosophila* neurons since phenotypes that result from insufficient levels of *CG3814/LFG* can be abrogated by overexpression of the pro-survival *Buffy*.

Knockdown of *CG3814* in the eye decreases ommatidia number and increases degeneration; phenotypes that are rescued upon *Buffy* overexpression

Biometric analysis of the neuron-rich *Drosophila* compound eye can reveal subtle differences in phenotypes that result from altered gene expression. Directed suppression of *CG3814/LFG* in the developing eye using the *GMR-Gal4* transgene results in a decreased number of ommatidia and higher disruption of the ommatidial array (Figure 3A and B) as determined by ANOVA at a value less than 0.050.

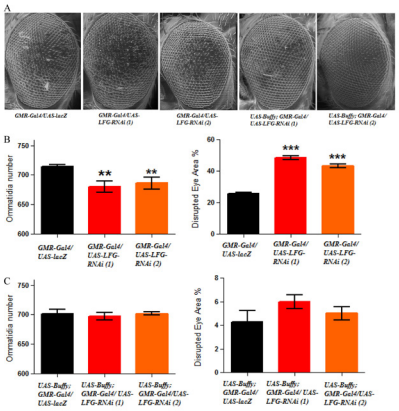

Figure 3. Knockdown of *CG3814/LFG* in the developing eye results in phenotypes that are counteracted by *Buffy* overexpression. **A.** Scanning electron micrographs were obtained following *CG3814/LFG* suppression in the eye and co-expression with *Buffy.* The genotypes are *GMR-Gal4/UAS-lacZ*; *GMR-Gal4/UAS-LFG-RNAi (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 following *CG3814/LFG* suppression in the eye revealed a decreased number of ommatidia and a higher percentage of ommatidial disruption when compared to the control. **C.** Co-expression of *Buffy* with *LFG-RNAi* suppressed the eye phenotypes, ommatidia number, and disruption of the eye were restored to control levels as determined by biometric analysis. Comparisons were determined by one-way analysis of the variance (ANOVA), $P < 0.05$, error bars represent the standard error of the mean, asterisks represent statistical significance and $N = 10$.

The reduced number of ommatidia is largely due to the high degree of ommatidial fusion. Co-expression of *LFG-RNAi* with *Buffy* restored the mean number of ommatidia and the percentage of disruption to control levels as determined by ANOVA, $P > 0.50$ (Figure 3A and C). 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 showed CG3814 to be the strongest candidate for *Drosophila* LFG, with a sequence identity of 45% and similarity of 65%. However, we do not exempt CG9722; CG3814 was widely expressed when compared to CG9722, which is predominantly expressed in the testis (Hu et al., 2009). Therefore, we propose that *CG3814* is the putative *Drosophila* homologue of *LFG*.

The conditional knockdown of *CG3814* by stable inducible RNA interference in *Drosophila* neurons under the control 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 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 (Schweitzer et al., 1998; Fernández et al., 2007; Hurtado de Mendoza et al., 2011; Reich et al., 2011), is highly expressed in 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 the result of neuronal loss, since compared to control flies at around the same age and at the same time point, there is a significant difference in climbing abilities. In our study, we did not perform any cell death assays and as such our conclusions are mostly based on behavioral phenotypes that are comparable to the controls. This does not negate the evidence we obtained following the knockdown of *CG3814/LFG*. In addition, RNAi does not exclude off-target regions that share homology with other BI-1 containing motifs, although data from the Vienna *Drosophila* Resource Centre show there are no off targets for these RNAi lines, which target the various isoforms present. Taken together, these results suggest a strong neuroprotective role for CG3814 in *Drosophila Ddc-Gal4*-expressing neurons.

The *CG3814*-induced phenotypes may occur through a mechanism that does not involve interaction with pro-survival Bcl-2 proteins at the ER membrane (Urresti et al., 2016), to regulate the release of calcium from the ER. Therefore, 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* (Quinn et al., 2003). Overexpression of *Buffy* is known to confer a survival advantage to cells under normal and stress conditions (Quinn et al., 2003; Sevrioukov et al., 2007; Monserrate et al., 2012; Clavier et al., 2014; M'Angale and Staveley, 2016b,c,d). Overexpression of *Buffy*, along with the knockdown of *CG3814*, suppressed the *CG3814*-induced phenotypes markedly, by significantly improving survival and locomotor function. This action of Buffy on "healthspan" may be specific to an interaction with CG3814, or it may be attributed to a general pro-survival signaling pathway that is initiated by Buffy in response to stress mediated by the loss of *CG3814* function. This 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 decreased number of ommatidia. The reduced ommatidium number was attributed to the high degree of fusion of the ommatidium and consequently resulted in ommatidium disarray. Knockdown of *CG3814* in the *Drosophila* eye seems to exacerbate the *Gal4*-induced apoptosis that manifests as the roughened eye phenotype (Kramer and Staveley, 2003). 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 being restored to control levels. Buffy seems to ameliorate this phenotype possibly via a general action on survival signals or through a concerted function that rescues *CG3814*-induced apoptosis.

In conclusion, knockdown of *CG3814/LFG* in the *Ddc-Gal4*-expressing neurons of *Drosophila* results in a severely shortened lifespan and a marked age-dependent loss in climbing ability, phenotypes that are strongly associated with the degeneration and loss of DA neurons. Overexpression of the pro-cell survival mediator *Buffy* along with the knockdown of *CG3814/LFG*, rescues the observed phenotypes, which suggests strong pro-survival and neuroprotective roles for *CG3814/LFG* in *Drosophila* neurons.

Conflicts of interest

The authors declare no conflict of interest.

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