Inhibition of *mitochondrial calcium uptake 1* in *Drosophila* neurons

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**ABSTRACT.** The mitochondrial calcium uptake 1 (MICU1) is a regulatory subunit of the mitochondrial calcium uniporter that plays an important role in calcium sensing. It contains two EF-hand domains that are well conserved across diverse species from protozoa to plants and metazoans. The loss of MICU1 function in mammals is attributed to several neurological disorders that involve movement dysfunction. The *CG4495* gene in *Drosophila melanogaster* was identified as a putative homolog of MICU1 in the HomoloGene database of the National Centre for Biotechnology Information (NCBI). In agreement with previous studies that have shown the development of neurological disorders and movement defects in MICU1 loss-of-function organisms, we attempted to identify the function of *CG4495/MICU1* in *Drosophila* neurons. We analyzed survival and locomotor ability of these flies and additionally performed biometric analysis of the *Drosophila* developing eye. The inducible RNA interference-mediated inhibition of *CG4495/MICU1* in the *Ddc-Gal4*-expressing neurons of *Drosophila* presented with reduction in survival coupled with a precocious loss of locomotor ability. Since the pro-survival Bcl-2 family genes have been shown to be protective towards mitochondria, and *CG4495/*
MICU1 has a mitochondrial targeting sequence, we attempted to rescue the phenotypes resulting from the inhibition of CG4495/MICU1 by overexpressing Buffy, the sole Bel-2 homologue in Drosophila. The co-expression of CG4495/MICU1-RNAi along with Buffy resulted in the suppression of the phenotypes induced by the inhibition of CG4495/MICU1. Subsequently, the inhibition of CG4495/MICU1 in the Drosophila developing eye, a neuron-rich organ, resulted in reduced number of ommatidia and a highly fused ommatidial array. These developmental eye defects were rescued by the overexpression of Buffy. Our study suggests an important role for MICU1 in the normal function of neurons in Drosophila.

**Key words:** MICU1; Buffy; Neurons; Ddc-Gal4; GMR-Gal4; Drosophila

### INTRODUCTION

The mitochondrial calcium uptake 1 (MICU1) encodes a mitochondrial EF-hand protein essential for the uptake of calcium (Perocchi et al., 2010). It is the regulatory component of the calcium uniporter that is proposed to regulate signaling, energy metabolism, and cell death (Perocchi et al., 2010; Baughman et al., 2011; De Stefani et al., 2011). The mitochondrial calcium uniporter (MCU) is the pore-forming subunit, whereas MICU1 together with other subunits form the regulatory components of the calcium uniporter (Foskett and Philipson, 2015). MICU1 is a peripheral membrane protein that possesses two EF-hand domains, which are used for calcium sensing. MICU1 is evolutionarily diverse being present in some protozoa, protists, plants, and metazoans (Bick et al., 2012). In most cases, it is found strongly co-expressed with MCU, and in some cases, it shares a potential promoter with MCU.

MICU1 is required for MCU-mediated calcium uptake (Perocchi et al., 2010), and plays an essential gatekeeping role to regulate cell survival (Mallilankaraman et al., 2012). The two MICU1 calcium-binding EF-hands are a key to its inhibitory functions. The knockdown of MICU1 inhibits the mitochondrial calcium uptake (Alam et al., 2012). Additionally, mutations that abrogate the functions of EF-hands result in MICU1 knockdown-like phenotypes (Mallilankaraman et al., 2012). Loss-of-function mutations in the MICU1 gene were found to cause a brain and muscle disorder that was linked to mitochondrial calcium signaling (Logan et al., 2014). Individuals suffering from this disorder suffered from proximal myopathy, learning difficulties, and a progressive extrapyramidal movement disorder. Thus, homozygous mutations in MICU1 cause myopathy with extrapyramidal signs (http://omim.org/entry/615673). Another 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 (Lewis-Smith et al., 2016), while the single nucleotide polymorphism analysis indicated the role of EF-hand in bipolar disorder.

The CG4495 gene in Drosophila melanogaster was identified as a putative homologue of MICU1 using HomoloGene, an automated system for constructing putative homology groups from the complete gene sets of a wide range of eukaryotic species, on the website of National Centre for Biotechnology Information (NCBI; https://www.ncbi.nlm.nih.gov/homologene?Db=homologene&Cmd=Retrieve&list_uids=4431). These genes were found to be conserved, and their protein sequences were used for sequence comparison and identification.
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of the conserved domain architecture. The Online Mendelian Inheritance in Man database (http://omim.org/entry/615673) lists homozygous mutations in MICU1 as causative for myopathy with extrapyramidal signs, a disorder characterized by the early-childhood onset of proximal muscle weakness and learning disabilities (Logan et al., 2014). Following the studies that show the development of neurological disorders and movement defects in MICU1 loss-of-function organisms, we attempted to identify a role for CG4495/MICU1 in Drosophila. The CG4495/MICU1 gene in Drosophila neurons was inhibited by stable inducible RNA interference, and the flies were assayed for changes in survival and locomotor ability. The developing compound eye in Drosophila is a neuron-rich organ and a powerful experimental model system to study the functions of a gene or biological process (Thomas and Wassarman, 1999). Importantly, a vast number of genes are required for the assembly and neuronal connectivity of the eye and therefore, altered expression or inhibition of a key gene, such as CG4495/MICU1, in the developing eye should present with quantifiable phenotypes; even the subtlest phenotypes are easy to detect and score.

The Bcl-2 family genes are the key regulators of cell death and survival in animals; they regulate cell fate decisions following developmental or stress signals (Kollek et al., 2016). The Bcl-2 family is classified into pro-death and pro-survival members, with the latter described as the “guardians of the mitochondria”. The Bcl-2 family member homologues in Drosophila are limited to a single anti-apoptotic Buffy gene (Quinn et al., 2003) and a pro-apoptotic Debcl gene (Colussi et al., 2000). The overexpression of Buffy is reported to confer survival benefits in response to external stimuli and stress (Sevrioukov et al., 2007, Tanner et al., 2011, Monserrate et al., 2012, M’Angale and Staveley, 2016a). Since the pro-survival Bcl-2 family genes have been shown to be protective towards the mitochondria, and CG4495/MICU1 has a mitochondrial targeting sequence, we attempted to rescue the phenotypes that resulted from the inhibition of CG4495/MICU1 by overexpressing Buffy, the sole pro-survival Bcl-2 homologue in Drosophila. Further, Arvizo et al. (2013), while working with cancer cells, showed that the short interfering RNA-mediated silencing of MICU1 resulted in the increased levels of pro-apoptotic Bax and decreased levels of pro-survival Bcl-2. This, in turn, increased the levels of cytochrome c, a potent apoptogenic factor, and caspase-3 activity, thereby initiating apoptosis. To determine the pro-survival effects of the Bcl-2 homologue Buffett and CG4495/MICU1, we attempted to suppress the CG4495/MICU1-induced phenotypes by overexpressing Buffy along with CG4495/MICU1 in the Ddc-Gal4-expressing neurons and the developing eye in Drosophila.

We propose that CG4495 is the MICU1 homologue in Drosophila. The bioinformatic evidence specifically found in the Orthologs section of FlyBase renders CG4495 as the homologue of MICU1 (http://flybase.org/reports/FBgn0031893.html). Additionally, we showed that the inhibition of CG4495/MICU1 in Drosophila neurons under the direction of the Dopa decarboxylase transgene results in the shortened lifespan and locomotor dysfunction. Interestingly, these phenotypes are counteracted by the overexpression of Buffy.

MATERIAL AND METHODS

Bioinformatic analysis

The MICU1 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) (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 or short linear motifs (SLiMs). The Clustal Omega multiple sequence alignment (http://www.ebi.ac.uk/Tools/msa/clustalo/) (Sievers et al., 2011) was performed to indicate the conservation of domains. The transmembrane domains were identified by TMpred (http://www.ch.embnet.org/software/TMPRED_form.html) (Artimo et al., 2012). Further analysis of protein domains, protein modeling, and structure prediction and analysis were performed with Phyre2 (http://www.sbg.bio.ic.ac.uk/phyre2/html/page.cgi?id=index) (Kelley et al., 2015). More information on this protein is available on http://www.uniprot.org/uniprot/A2VEI2.

**Drosophila media, stocks, and derivative lines**

Fly stocks and crosses were cultured on a standard medium composed of cornmeal, molasses, yeast, and agar, and treated with propionic acid and methylparaben to inhibit fungal growth. The stocks were raised at 23° ± 2°C, while the crosses and experiments were performed at 25° and 29°C, respectively. The CG4495 RNA interference lines, w^{1118}; P(GD4927)v49349 and w^{1118}; P(GD4927)v49350, hereafter 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 (Quinn et al., 2003) was kindly provided by Dr. L. Quinn (University of Melbourne, Melbourne, Australia) and Ddc-Gal4 flies (Li et al., 2000) by Dr. J. Hirsch (University of Virginia, USA). GMR-Gal4 (Freeman, 1996) and UAS-lacZ flies were obtained from the Bloomington Drosophila Stock Center (Indiana University, USA). The UAS-Buffy/CyO; Ddc-Gal4 and UAS-Buffy/CyO; GMR-Gal4 derivative lines were generated by using the 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 Glass Multiple Reporter (GMR) response elements following a standard protocol described previously (M’Angale and Staveley, 2016b, c).

**Survival analysis**

For each genotype evaluated, multiple crosses were performed, and a cohort of male flies was collected upon eclosion. The male flies were typically used as they do not suffer from the reproductive stress. Survival analysis was performed using a standard protocol (Staveley et al., 1990; Todd and Staveley, 2012; M’Angale and Staveley, 2016a). At least two hundred flies were assayed per genotype; they were observed every 2 days for the presence of deceased adults. Longevity data were analyzed 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 ≤ 0.05 with Bonferroni correction).

**Climbing analysis**

A cohort of male flies was collected upon eclosion and scored for their ability to climb every 7 days (Todd and Staveley, 2004). The climbing analysis was performed for a total of 50 male flies that had been divided into 10 flies per vial. The climbing indices were
evaluated using GraphPad Prism version 5.04. The climbing curves were fitted using non-linear regression, and compared using 95% confidence intervals. A P value less than 0.05 was considered statistically significant.

**Scanning electron microscopy of the Drosophila eye**

Crosses prepared for each genotype were analyzed at 29°C. A batch of male flies was collected upon eclosion, and was prepared for scanning electron microscopy following a standard protocol described previously (M’Angale and Staveley, 2016a). A minimum of 10 different eye images per genotype were assessed using the ImageJ software (National Institutes of Health, USA) (Schneider et al., 2012), and the biometric assays performed using GraphPad Prism version 5.04. The disruption area of the eye was calculated as described previously (M’Angale and Staveley, 2012). Statistical comparisons comprised one-way analyses of variance and Dunnett’s multiple comparison tests. P values less than 0.05 were considered statistically significant.

**RESULTS**

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

The *Drosophila* CG4495 gene encodes two isoforms, A and B, which are 97% identical and 98% similar. The *Drosophila* CG4495 isoform B and the human MICU1 homologue contain two EF-hand domains that are closely related and evolutionarily conserved as determined by the NCBI Conserved Domain Database (CDD) (Marchler-Bauer et al., 2015). The CG4495 protein is composed of 525 amino acids (524 amino acids in isoform A), and shows 57% identity and 75% similarity to the 476-amino acid human MICU1 protein (Figure 1). The *Drosophila* homologue has 2 EF-hand domains located 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 ELM (Dinkel et al., 2016). Two to three transmembrane (TM) domains in the *Drosophila* and human transcripts were predicted using TMpred (Artimo et al., 2012). A nuclear-localization signal was detected in both CG4495 (390 to 404) and human MICU1 (87 to 105) using ELM. A multiple sequence alignment of protein sequences using Clustal Omega (Sievers et al., 2011) showed high conservation of the EF-hand domains (Figure 1A), CG4495/ MICU1 is localized to the mitochondria, and has a mitochondrial targeting peptide with a presequence cleavage site at amino acid 115. The human MICU1 has a presequence length of 33 amino acids as predicted using TargetP (Emanuelsson et al., 2000). A 3D modeling of both proteins using Phyre2 (Kelley et al., 2015) is shown (Figure 1B).

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

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

**Figure 1.** Drosophila CG4495/MICU1 has two evolutionarily conserved EF-hand motifs. The two EF-hand domains identified by using the NCBI CDD (Marchler-Bauer et al., 2015) and the nuclear localization signal identified by using the ELM resource (Dinkel et al., 2016) were highly conserved in Drosophila CG4495/MICU1. The transmembrane domains were identified using TMpred (Artimo et al., 2012) and Phyre2 (Kelley et al., 2015). The mitochondrial targeting sequence in both human and Drosophila MICU1 were identified by using TargetP (Emanuelsson et al., 2000). Clustal Omega multiple sequence alignment (Sievers et al., 2011) 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 two EF-hand domains. The asterisk indicates the residues that are identical; the colon indicates the conserved substitutions; and the dot indicates the semi-conserved substitutions. Different colors indicate the chemical nature of amino acids. Red: small hydrophobic (including aromatic); blue: acidic; magenta: basic; and green: basic with hydroxyl or amine groups. A 3D modeling of both proteins using Phyre2 is shown in the bottom panel.
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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 neurons under the control of the Dopa decarboxylase transgene results in the reduced lifespan, with a median survival of 42 days and 46 days in comparison to 70 days for the control flies that express the benign lacZ transgene. The genotypes are Ddc-Gal4/UAS-lacZ (control), 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 ≥ 200].

B. Directed suppression of CG4495/MICU1 in the Ddc-Gal4-expressing neurons resulted in the premature loss of climbing ability as determined by the nonlinear fitting of the climbing curves and significance was determined by comparing 95% confidence interval (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.

C. 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 as 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 the log-rank (Mantel-Cox) test with N ≥ 200). D. Inhibition of MICU1 along with the overexpression of Buffy in these neurons results in the suppression of the age-dependent loss of 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.

**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 resulted in a significant increase in the lifespan and climbing ability. The co-expression of Buffy with MICU1-RNAi resulted in increased median survival of 70 and 71 days in comparison to Buffy control flies with a median survival of 74 days as determined by the Log-rank test (Figure 2C). The climbing ability of the MICU1-RNAi flies was improved as determined by comparing the climbing curves at 95% CI, with 0.039 to 0.052 as compared with 0.039 to 0.051 which was not significant (Figure 2D). These results suggest a protective role for Buffy as it improves the lifespan and locomotor function in MICU1-deficient neurons.
Inhibition of \textit{MICU1} in the developing eye decreases ommatidia number and increases the disruption of the ommatidial array

The inhibition of both \textit{MICU1} RNAi lines in the eye under the direction of the \textit{GMR-Gal4} transgene decreases ommatidia number, and results in a significant disruption of the ommatidial array (Figure 3B, C, and G) when compared to the controls (Figure 3A) as determined by one-way analysis of variance (ANOVA) $P < 0.0001$. The overexpression of \textit{Buffy} along with the inhibition of \textit{MICU1} restored the number of ommatidia and the percentage disruption (Figure 3E, F, and H) to control levels (Figure 3D) as determined by ANOVA, a $P$ value greater than 0.50 was obtained, indicating insignificant results. Taken together, these results suggest that \textit{MICU1} might play a developmental role in the \textit{Drosophila} eye, and that \textit{Buffy} suppresses the developmental eye defects that result from the inhibition of \textit{MICU1}.

\textbf{Figure 3.} Inhibition of \textit{MICU1} in the eye results in decreased ommatidia and increased disruption of the ommatidial array. A.-F. Scanning electron micrographs of the inhibition of \textit{CG4495/MICU1} in the \textit{Drosophila} developing eye and its co-expression along with \textit{Buffy}. The genotypes are \textit{GMR-Gal4/UAS-lacZ} (A), \textit{GMR-Gal4/UAS-MICU1-RNai (1)} (B), \textit{GMR-Gal4/UAS-MICU1-RNai (2)} (C), \textit{UAS-Buffy}; \textit{GMR-Gal4/UAS-lacZ} (D), \textit{UAS-Buffy}; \textit{GMR-Gal4/UAS-MICU1-RNai (1)} (E), and \textit{UAS-Buffy}; \textit{GMR-Gal4/UAS-MICU1-RNai (2)} (F). G. Biometric analysis of the loss of \textit{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 \textit{Buffy} with \textit{MICU1-RNai} results in the suppression of the phenotypes with developmental eye defects. Comparisons were determined by one-way analysis of the variance (ANOVA); $P < 0.05$; error bars indicate standard errors of the mean; asterisks indicate statistical significance, and $N = 10$.  

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DISCUSSION

The importance of MICU1 as a regulatory subunit of MCU is exemplified by the disorders resulting from its misexpression or mutations that alter the EF-hand function (Logan et al., 2014; Xu, 2015; Lewis-Smith et al., 2016; Safari et al., 2016). The bioinformatic analysis reveals highly conserved EF-hand domains in MICU1; this, in turn, might point to an evolutionarily conserved cellular pathway involved in calcium uptake and possibly in calcium signaling. Further analysis showed the presence of transmembrane domains and a nuclear localization signal motif containing 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 the lifespan and a profound early-onset loss of climbing ability. The loss of MICU1 function in humans alters mitochondrial calcium signaling and results in the proximal skeletal muscle weakness and brain disorder (Logan et al., 2014). The MICU1-RNAi flies exhibited erratic climbing behavior and a complete lack of climbing at a very early time; 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 of climbing ability was not matched by the decreased survival. From Figure 2A and B it is evident that at 40 days, when most of the flies in both the control and RNAi lines were alive, the MICU1-RNAi flies had lost their climbing ability in an age-dependent manner, which was indicative of neuronal degeneration. We established that the suppressed activity of MICU1 in Drosophila results in a significant reduction in survival and a precocious loss of climbing ability. We co-expressed the Buffy gene along with MICU1-RNAi, and observed that this counteracted the induced phenotypes. This was an important finding as it elucidates the role of Buffy in calcium signaling and possibly in mitochondrial calcium uptake. The pro-survival role of Buffy in conditions of stress is well documented (M’Angale and Staveley, 2016a, b, c, d). However, this new role of Buffy, which might point to the conserved role of Bcl-2 proteins in the regulation of calcium signaling (Vervliet et al., 2016), deserves further investigation. Additionally, the rescue of defective phenotypes 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. Additional studies are required to determine the molecular pathways precisely attributed to MICU1 dysfunction and Buffy rescue.

In complimentary experiments on neuron-rich Drosophila eyes, the inhibition of MICU1 by stable inducible RNA interference under the direction of the GMR-Gal4 transgene resulted in a reduction in the number of ommatidia and an increase in the eye area with fused ommatidia. MICU1-RNAi (1) showed a greater penetrance of the eye phenotypes by exhibiting significantly lower numbers of ommatidia and greater disruption of the ommatidial array than MICU1-RNAi (2). This indicates that the 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 the Drosophila Ddc-Gal4-expressing neurons and developing eye.

CONCLUSIONS

The bioinformatic candidate for the MICU1 gene in Drosophila appears to be CG4495. Experimental evidence shows that the inhibition of this gene in neurons results in the 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. Further studies are required to dissect the role of CG4495/MICU1 and calcium signaling in these neurons. Molecular pathways that are involved in Drosophila, specifically the role of Buffy, need to be identified and correlated with those implicated in mammalian systems.

Conflicts of interest

The authors declare no conflict of interest.

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