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

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

Key words
Parkinson disease, *Drosophila melanogaster*, dopaminergic neurons, locomotion, ageing, *HtrA2*, *PARK13*, *Buffy*
Introduction
Loss of function mutations in High temperature requirement A2 (HtrA2; also known as Omi), a mitochondrial localized serine protease, are linked to Parkinson disease (PD) (Strauss et al. 2005). HtrA2 encodes a nuclear protein with a mitochondrial targeting signal at the amino-terminus, that appears to be related to the Drosophila Reaper, Hid and Grim pro-apoptotic proteins due to the presence of the inhibitor of apoptosis (IAP) inhibitory Reaper-like motif (Martins et al. 2002). The HtrA2 protein is released into the cytosol from the mitochondria during apoptosis to bind IAPs and thus block the inhibition of caspases (Suzuki et al. 2001). The binding of HtrA2 to IAPs activates the protease function.

A common facet of Parkinson disease (PD) is mitochondrial dysfunction and the loss of mitochondrial complex I function in the midbrain of PD patients highlights the importance of the mitochondria in the pathology (Antony et al. 2013; Ryan et al. 2015; Subramaniam and Chesselet 2013). A role for HtrA2 in neurodegeneration has been established through an association with the autosomal dominant early onset Alzheimer disease gene presenilin (Gupta et al. 2004). As well, the amyloid β protein interacts with the HtrA2 protein (Park et al. 2004). The motor neuron degeneration (mnd2) disorder in mice is caused by a loss-of-function mutation in the protease-domain encoding region of HtrA2 (Jones et al. 2003). Further studies in mice showed a link between altered expression of HtrA2 and PD-like neurodegeneration (Martins et al. 2004). This protein seems to play an important role in cellular protection in response to stress.

In Drosophila melanogaster, the HtrA2 serine protease has been shown to function in apoptosis through the cleavage of IAP1 in the vicinity of the mitochondria (Challa et al. 2007; Guo 2012; Igaki et al. 2007). Modelling of PD in Drosophila is robust and has been insightful in understanding the role of several PD-linked genes in disease...
pathology (Botella et al. 2009; Lu and Vogel 2009; Staveley 2014). The links between
HtrA2 and other PD-linked genes has been demonstrated in both mammals and the
fly, including the Pink1 kinase (Plun-Favreau et al. 2007; Whitworth et al. 2008) and
the E3 ubiquitin ligase, parkin (Tain et al. 2009; Whitworth et al. 2008). The link
between HtrA2 and the Pink1/Parkin pathway has been challenged by an in vivo study
that relied on a loss of function (Yun et al. 2008). Whether HtrA2 functions
downstream of the Pink1/Parkin pathway or if it affects mitochondrial homeostasis,
the loss of its protease activity is known to lead to neuronal degeneration.

We demonstrate that an age-dependent loss in locomotor function, accompanied by a
reduction in lifespan, results when HtrA2 is inhibited in the DA neurons of
Drosophila, which corroborates previous studies (Tain et al. 2009). In addition, the
overexpression of the anti-apoptotic Bcl-2 homologue Buffy has been shown to restore
healthspan in a disease model (M’Angale and Staveley 2016). Here, we report
inhibition of HtrA2 along with overexpression of the sole pro-survival Bcl-2
homologue Buffy.

Materials and methods

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

Drosophila stocks and derivative lines
UAS-Buffy (Quinn et al. 2003) was provided by Dr. Leonie Quinn (University of
Melbourne) and Ddc-Gal4 (Li et al. 2000) by Dr. J. Hirsch of University of Virginia.

w^{1118}; P(GD13932)cv24104 referred to as HtrA2-RNAi was obtained from Vienna
Drosophila Resource Center, *GMR-Gal4* (Freeman 1996) and *UAS-lacZ* flies were acquired from the Bloomington Drosophila Stock Center at Indiana University. The *GMR-Gal4; UAS-Buffy/CyO; Ddc-Gal4* and *UAS-Buffy/CyO; GMR-Gal4* were generated using standard homologous recombination methods and were used for overexpression of *Buffy* in DA neurons using the *Ddc-Gal4* transgene or in the developing eye using the *GMR* response elements. PCR reactions and gel electrophoresis were used for analysis of recombination events.

**Ageing assay**
Several single-vial matings were carried out to produce a cohort of critical class male flies that were collected upon eclosion. At least two hundred flies were aged per genotype at a density of 20 or fewer flies per vial to avoid crowding on fresh media replenished every other day. Flies were observed and scored every two days for the presence of deceased adults. Flies were considered dead when they did not display movement upon agitation (Staveley et al. 1990). Longevity data was analysed using the GraphPad Prism version 5.04 and survival curves were compared using the log-rank (Mantel-Cox) test. Significance was determined at 95%, at a P-value less than or equal to 0.05 with Bonferroni correction.

**Climbing assay**
Cohort of critical class male flies was collected upon eclosion and scored for their ability to climb during their lifetime (Todd and Staveley 2004; Todd and Staveley 2008). Every 7 days, 50 males from every genotype were assayed for their ability to climb 10 centimetres in 10 seconds in a clean climbing apparatus in ten repetitions. Analysis was performed using the GraphPad Prism version 5.04 and climbing curves were fitted using non-linear regression and compared using 95% confidence interval with a 0.05 P-value.
Scanning electron microscopy of the drosophila eye
Several single-vial matings were made at 29°C, a cohort of adult male flies collected
upon eclosion and aged for three days before being frozen at -80°C. Whole flies were
mounted on scanning electron microscope stubs, desiccated overnight and
photographed with a FEI Mineral Liberation Analyzer 650F scanning electron
microscope. For each cross at least 10 eye images were analysed using the National
Institutes of Health (NIH) ImageJ software (Schneider et al. 2012) and biometric
analysis performed using GraphPad Prism version 5.04. The percent area of eye
disruption was calculated as previously described (M'Angale and Staveley 2012).

Results

The HtrA2 protease and PDZ domains are highly conserved
The Drosophila and human protein sequences have a 52% identity and 70% similarity
along their full length as determined by NCBI BLAST comparison, the two domains,
Trypsin and PDZ are highly conserved as determined by an NCBI conserved domain
search (Marchler-Bauer et al. 2015) (Figure 1). An ELM resource search for
functional sites (Dinkel et al. 2016) indicates the presence of an inhibitor of apoptosis
binding motif (IBM) that function in the abrogation of caspase inhibition by IAPs,
three different di-Arginine ER retention motifs, an Atg8 binding motif and a possible
NLS. TargetP (Emanuelsson et al. 2000) shows a pre-sequence cleavage site at the
17th amino acid in Drosophila and the 45th amino acid in the human version.

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

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

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

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

The inhibition of HtrA2 in the eye under the control of the GMR-Gal4 transgene, results in decreased ommatidia number and significant disruption of the ommatidial array (Figure 3A, II and 3B, I) as determined by an unpaired T-test p<0.0001. The overexpression of Buffy along with the inhibition of HtrA2 restored the number of ommatidia and the percentage disruption to control levels as determined by an unpaired T-test, p>0.50 (Figure 3A, III and 3B, II). Taken together, these results suggest that loss of HtrA2 activity is detrimental to normal development of the Drosophila eye and that Buffy suppresses the developmental eye defects that result from this inhibition.
Discussion
The loss of protease function (Strauss et al. 2005) or mutations in the PDZ domain (Bogaerts et al. 2008) of PARK13/ HtrA2 confers susceptibility to PD, and disruption of HtrA2 was found to cause neurodegeneration and PD-like phenotypes in mice (Martins et al. 2004). In corroborative experiments (Tain et al. 2009), when we inhibited HtrA2 in DA neurons of Drosophila using the *dopa decarboxylase* transgene, it resulted in a marked decline in survival and led to premature loss in locomotor function. HtrA2 is involved in cell death, the deletion of this gene in mice did not alter cell death rates, instead, suffered loss of a population of neurons in the striatum leading to neurodegeneration (Martins et al. 2004). Studies suggest that the primary role of HtrA2 in neurons is protection against stress (Bogaerts et al. 2008; Tain et al. 2009). The inhibition of HtrA2 in DA neurons resulted in flies with poor healthspan, they had shortened lifespans and lost their climbing ability prematurely. HtrA2 seems to have a special neuroprotective function since loss in its protease or PDZ domain activity, or its deletion or inhibition seems to lead to neurodegeneration. Interestingly, the inhibition of HtrA2 in the eye resulted in fewer number of ommatidia and worsening of the rough eye phenotype. It seems the neuroprotective functions of this protein are not limited to DA neurons specifically, but general neuroprotection.

The functional HtrA2 acts in the vicinity of the mitochondria in Drosophila (Igaki et al. 2007), further showing the importance of the mitochondria in cell death and aetiology of PD. In mammals HtrA2 is involved in a p53-dependent cell death pathway (Jin et al. 2003), but its role in Drosophila is an elusive one. In our experiments, the overexpression of the sole pro-survival *Bcl-2* homologue in Drosophila, *Buffy* (Quinn et al. 2003), along with the inhibition of HtrA2 counteracted the loss of HtrA2-induced phenotypes. These flies had an increase in lifespan that was
augmented by improved locomotor ability suggesting a protective role for Buffy. The
*HtrA2* null mutants in Drosophila do not exhibit mitochondrial morphological defects
(Yun et al. 2008), though this does not exclude mitochondrial dysfunction. The study
also did not find DA neuronal loss or muscle degeneration. Though the loss of DA
neurons in Drosophila has remained a contentious issue as regards models of
Parkinson disease (Botella et al. 2009; Staveley 2014), there is consensus on PD-like
phenotypes such as loss in locomotor ability. A lack of loss in DA neurons does not
exclude degeneration and the progression of PD-like phenotypes in Drosophila. We
have previously shown that the overexpression of Buffy in the α-synuclein-induced
PD model rescues the phenotypes (M'Angale and Staveley 2016). Therefore, the
alleviation of *HtrA2*-induced phenotypes by overexpression of the pro-survival *Buffy*
may be a general survival pathway signal or may be a specific *HtrA2*-dependent
response. We obtained similar results when *Buffy* was overexpressed in the eye along
with *HtrA2-RNAi*, with restored ommatidia number and decreased disruption of the
ommatidial array. It seems that this serine protease functions in survival since its
inhibition results in compromised healthspan, and the overexpression of the pro-
survival *Buffy* rescues the phenotypes.

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

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Competing interests
The authors declare that no competing interests exists.

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**Figures**

**Figure 1 - The protease and PDZ domains are conserved in human and Drosophila HtrA2**

The Drosophila HtrA2 isoform B gene encodes a protein comprised of 422 amino acids and contains a presequence cleavage site, trypsin and PDZ domains. Domains are highly conserved in the organisms compared as determined by the NCBI Conserved Domain Database Search (CDD) (Marchler-Bauer et al. 2015) and the Eukaryotic Linear Motif resource search (Dinkel et al. 2013). A Clustal Omega multiple sequence alignment (Goujon et al. 2010; Sievers et al. 2011) show conservation of the trypsin and PDZ domains (Hsap is *Homo sapiens* NP_037379.1, Mmus is *Mus musculus* NP_062726.3, Dmel is *Drosophila melanogaster* NP_001262565.1 and Agam is *Anopheles gambiae* XP_310886.5). "**" indicate the residues that are identical, ":." indicate the conserved substitutions, ":." indicate the
semi-conserved substitutions. Colours show the chemical nature of amino acids. Red is small hydrophobic (including aromatic), Blue is acidic, Magenta is basic, and Green is basic with hydroxyl or amine groups.

**Figure 2 - Loss of HtrA2 activity in DA neurons shortens lifespan and retards climbing ability, phenotypes that are suppressed by overexpression of Buffy**

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

**Figure 3 – HtrA2-induced eye phenotypes are suppressed upon Buffy overexpression**

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

B.

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