

# Molecular chaperones protect against JNK- and Nmnat-regulated axon degeneration in *Drosophila*

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## Summary

Axon degeneration is observed at the early stages of many neurodegenerative conditions and this often leads to subsequent neuronal loss. We previously showed that inactivating the c-Jun N-terminal kinase (JNK) pathway leads to axon degeneration in *Drosophila* mushroom body (MB) neurons. To understand this process, we screened candidate suppressor genes and found that the Wallerian degeneration slow (Wld<sup>S</sup>) protein blocked JNK axonal degeneration. Although the nicotinamide mononucleotide adenyltransferase (Nmnat1) portion of Wld<sup>S</sup> is required, we found that its nicotinamide adenine dinucleotide (NAD<sup>+</sup>) enzyme activity and the Wld<sup>S</sup> N-terminus (N70) are dispensable, unlike axotomy models of neurodegeneration. We suggest that Wld<sup>S</sup>-Nmnat protects against axonal degeneration through chaperone activity. Furthermore, ectopically expressed heat shock proteins (Hsp26 and Hsp70) also protected against JNK and Nmnat degeneration phenotypes. These results suggest that molecular chaperones are key in JNK- and Nmnat-regulated axonal protective functions.

**Key words:** Axon degeneration, Nmnat, JNK, Heat shock proteins, *Drosophila*

## Introduction

Axonal loss is detected at the early stages of many neurodegenerative pathologies (Coleman, 2005; Luo and O'Leary, 2005; Saxena and Caroni, 2007; Wang et al., 2012) and some studies show blocking axonal degeneration can reduce progressive degenerative phenotypes, such as in motoneuron loss (Ferri et al., 2003; Pun et al., 2006). Understanding the basis of axon degeneration and how it can be prevented could provide rational bases to treat common or unique neurodegenerative conditions.

It is important to note selective degeneration of axons, dendrites and synapses also occurs naturally during development and plasticity (Luo and O'Leary, 2005). Such pruning events share common features with neurodegenerative events. However, neurons are not lost during developmental pruning. Whether blocking all known neurodegenerative mechanisms is equally effective in preventing axon degeneration, under natural or pathological conditions, is still unclear. Nonetheless, present studies reveal cell death, axonal and dendritic degenerative phenotypes are distinct, despite some mechanistic overlaps (Sagot et al., 1995; Finn et al., 2000; Coleman, 2005; Luo and O'Leary, 2005; Hooper et al., 2006; Kuo et al., 2006; Williams et al., 2006; Beirowski et al., 2008; Nikolaev et al., 2009; Schoenmann et al., 2010; Vohra et al., 2010).

Cell-protective mechanisms that control molecular chaperones, autophagy, the ubiquitin-proteosome system (UPS), oxidative stress and mitochondrial functions are also key. These are not only cell-essential but their manipulations can alleviate neurodegenerative pathologies (Watts et al., 2003; Zhai et al.,

2003; Lin and Beal, 2006; Voisine et al., 2010; Wong and Cuervo, 2010; Bingol and Sheng, 2011). These can act by detecting and responding to potentially degenerative stimuli, by promoting neuronal integrity and initiating repair upon nerve damage.

The JNK pathway is a central regulator of diverse neuropathologies. Aberrant JNK signalling is implicated in Alzheimer's (Morishima et al., 2001; Okazawa and Estus, 2002), Parkinson's (Peng and Andersen, 2003), and Huntington's (Morfini et al., 2009; Perrin et al., 2009) diseases, where its activation leads to neuronal cell death. JNK also induces neurodegeneration in response to stress stimuli, such as toxins and excitotoxicity (Yang et al., 1997; Kuan et al., 2003; Brecht et al., 2005; Waetzig et al., 2006), growth factor deprivation (Xia et al., 1995; Eilers et al., 1998; Maroney et al., 1998; Ghosh et al., 2011) and acute physical injury (Miller et al., 2009). Axon fragmentation is evident in many of these cases.

Despite its central pro-degenerative and pro-apoptotic activities, JNKs are also neuroprotective (Brecht et al., 2005) and involved in neuronal patterning during axonal outgrowth (Oliva et al., 2006), dendritogenesis (Rosso et al., 2005) and in synaptic plasticity (Sanyal et al., 2002) and transmission (Thomas et al., 2008). We, and others, previously showed JNK signals have conserved functions in maintaining axonal stability and we showed that sustained JNK activities throughout development are essential, prior to the onset of degenerative events (Chang et al., 2003; Rallis et al., 2010). Physical injury paradigms in *C. elegans* and *Drosophila* show JNK is also required post-injury during axonal regeneration (Ayaz et al., 2008; Nix et al., 2011). This

may also be conserved in mammals (Herdegen et al., 1998; Raivich et al., 2004; Barnat et al., 2010). Therefore, determining how JNK promotes axonal stability and regeneration (while avoiding its pro-degenerative effects) can be useful in defining the strategies required to prevent neurodegenerative pathologies and promote repair programs upon nerve damage.

Here we show that inactivating the JNK gene (*basket*; *bsk*) in *Drosophila* neurons induces an age-dependent, Wallerian-like axon degeneration phenotype. This is not due to aberrant developmental pruning and cannot be suppressed by neuroprotective molecules linked to apoptosis, autophagy, the Ubiquitin-Proteosome (UPS) pathway, mitochondrial function or translational repression. Instead, we find JNK axonal degeneration (in this study defined as those caused by JNK inactivation) is linked to the axonal-protective effects of Wld<sup>S</sup>.

Wld<sup>S</sup> was discovered from the molecular cloning of spontaneously generated *slow Wallerian degeneration* (*Wld<sup>S</sup>*) mutant mice that showed a strong capacity to promote axonal survival following acute physical lesion (Lunn et al., 1989; Coleman and Freeman, 2010). The Wld<sup>S</sup> protein has neuroprotective effects across different species and in different neurodegeneration models (Coleman and Freeman, 2010; Feng et al., 2010; Vohra et al., 2010; Barrientos et al., 2011; Ali et al., 2012; Bhattacharya et al., 2012; Fang et al., 2012). The Wld<sup>S</sup> gene product results from the fusion of first 70 residues of the UBE4B gene (N70), that is involved in polyubiquitination, with the entire nicotinamide mononucleotide adenyltransferase protein sequence (Nmnat1) that is involved in nicotinamide adenine dinucleotide (NAD<sup>+</sup>) biosynthesis (Conforti et al., 2000; Mack et al., 2001). Different portions of Wld<sup>S</sup> can confer neuroprotective function (Coleman and Freeman, 2010). However, Wld<sup>S</sup> function remains unclear. For example, despite its predominant nuclear localisation, it is axonal localisation that appears to be key to neuroprotection, even though Wld<sup>S</sup> and different Nmnat isoforms have subtle and distinct subcellular locations (Berger et al., 2005; Conforti et al., 2007; Beirowski et al., 2009; Babetto et al., 2010; Sasaki and Milbrandt, 2010; Yahata et al., 2009) (supplementary material Fig. S2). Also, while in many neurodegenerative paradigms the Nmnat enzyme activity is essential, it is unclear how the NAD<sup>+</sup> pathway contributes to axonal protection (Araki et al., 2004; Wang et al., 2005; Kaneko et al., 2006; Conforti et al., 2007; Avery et al., 2009; Sasaki et al., 2009; Coleman and Freeman, 2010). Furthermore, some studies suggest Nmnat neuroprotective functions are enzyme-independent (Zhai et al., 2006; Zhai et al., 2008; Wen et al., 2011). To date, the relationship between Wld<sup>S</sup> function(s) and axon-neuronal damage and repair also remains unclear, although recent data suggest Wld<sup>S</sup>-Nmnat regulation of mitochondrial motility and calcium buffering functions may underlie key neuroprotective responses to physical injury in *Drosophila* and mouse axons (Avery et al., 2012). A further report suggests *Drosophila* Nmnat (*dNmnat* or *nmnat*) also controls axonal mitochondria levels and their availability is key to neuroprotection following acute injury (Fang et al., 2012). Previous data suggest Wld<sup>S</sup>-Nmnat localisation within mitochondria may also be the underlying basis of axonal neuroprotection (Yahata et al., 2009).

When tested ectopically, many Nmnat isoforms and homologs show axonal-protective effects even though some appear to be weaker, possibly due to labile effects (Sasaki et al., 2009; Gilley and Coleman, 2010). However, apart from *Drosophila* Nmnat

(Wen et al., 2011; Fang et al., 2012), currently only mouse Nmnat2 has an endogenous role in promoting axonal stability (Gilley and Coleman, 2010; Hicks et al., 2012). It is important to note, beyond their neuronal roles, Nmnats also have obligate roles in NAD<sup>+</sup> metabolism and multiple cellular processes across species (Zhai et al., 2009; Lin et al., 2010). Very recent reports show Nmnat1 mutations cause Leber congenital amaurosis (LCA), highlighting its importance in retinal degenerative diseases in humans (Chiang et al., 2012; Falk et al., 2012; Koenekoop et al., 2012; Perrault et al., 2012).

Here we show that the Wld<sup>S</sup> protein protects against axon degeneration triggered by JNK inactivation. Contrary to previous models, while the Nmnat1 region is sufficient, we find that its enzyme activity is dispensable for Wld<sup>S</sup> neuroprotection. The results suggest that Nmnat and JNK axonal-protective functions occur through molecular chaperones.

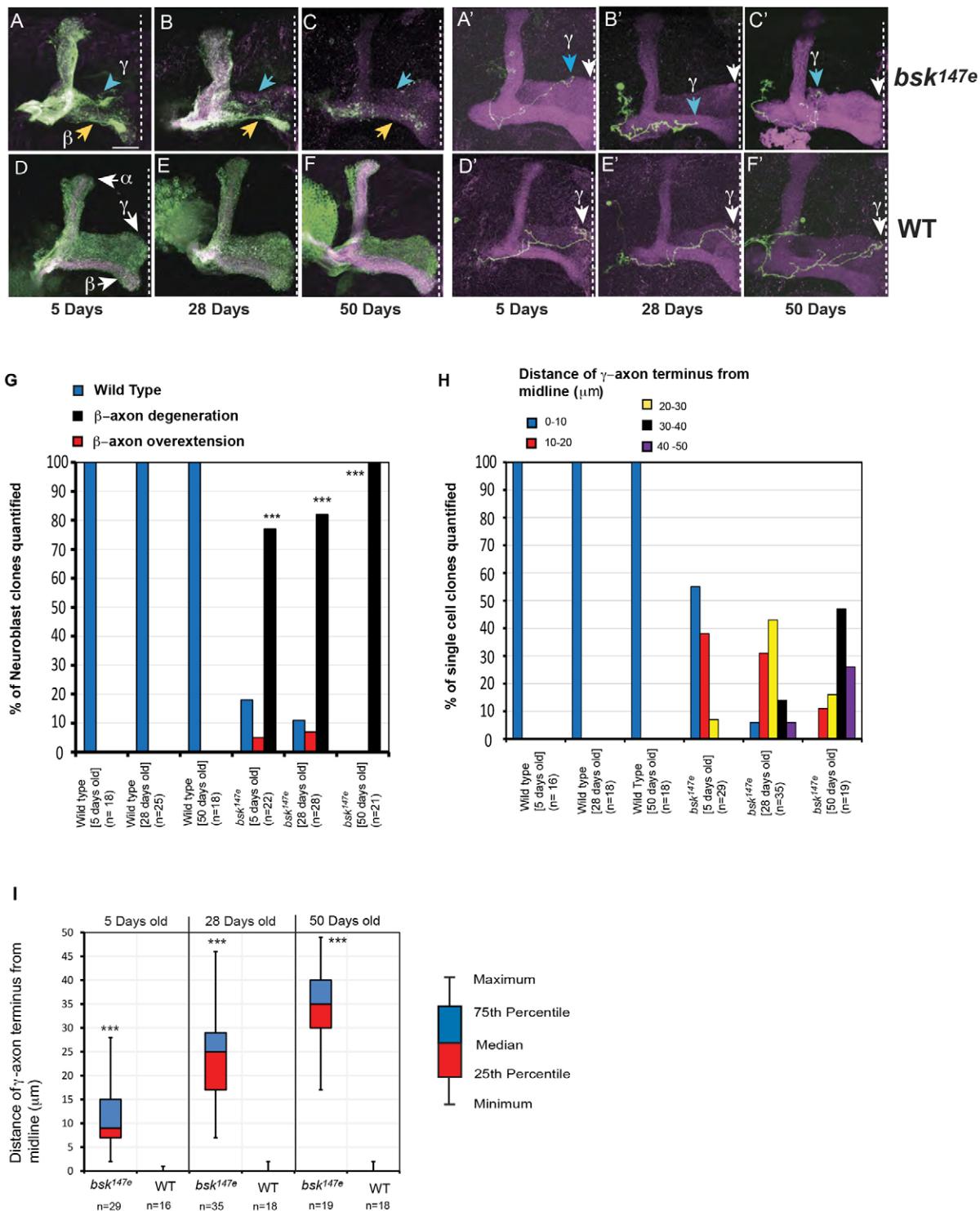
## Results

### JNK inactivation causes age-dependent axonal degeneration in *Drosophila* MB neurons

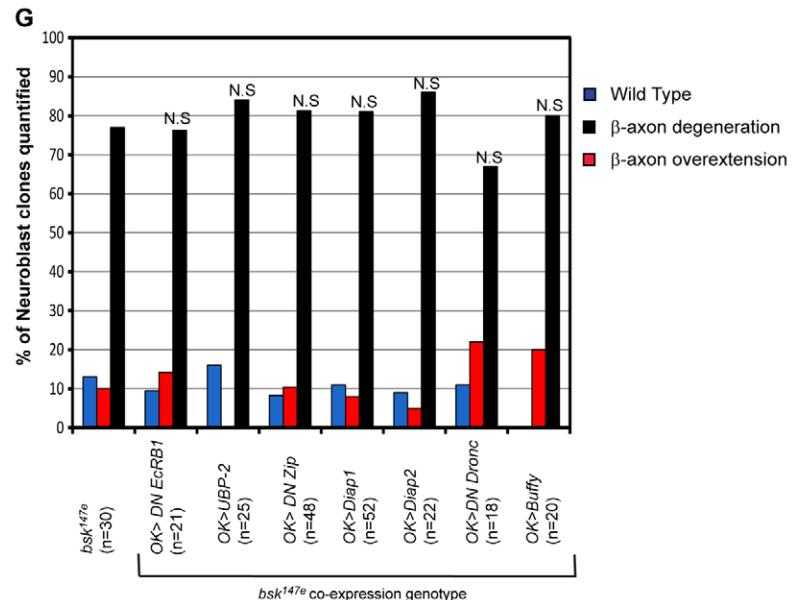
We previously showed that *Drosophila* JNK mutant mushroom body (MB) neurons have axon degeneration phenotypes (Rallis et al., 2010). These are not a consequence of axon growth defects. Also, a small fraction of axons have overextension phenotypes, which is distinct from axon degeneration. This study focused only on the axon degeneration phenotype in the β-lobe in neuroblast clones and in γ-single clones. To examine how this occurs, we generated JNK mutant clones (using the *bsk*<sup>147E</sup> null allele) using MARCM (Lee and Luo, 1999) and analyzed this phenotype at different adult stages. At 5 days post-eclosion, although many *bsk* axons did show some degenerative phenotypes, most MB axons were still visible (Fig. 1A,G). This degenerative phenotype becomes more severe at 28 days and 50 days post-eclosion (Fig. 1B,C,G). By generating single-cell clones, we could measure the extent to which each axon reaches the wild-type termination point. We found a greater terminal loss in aged axons (Fig. 1A'-C',H,I), suggesting that a 'dying back' (Wallerian-like) degeneration is progressing with age.

### Axon pruning and myosin-II based retraction are not involved in JNK axonal degeneration

To determine whether developmental regulated axon pruning is involved in *bsk* axonal degeneration, we expressed a dominant-negative (DN) form of the ecdysone receptor EcR-B1 (Cherbas et al., 2003) in *bsk*-null MB neuroblast clones. During *Drosophila* metamorphosis, the steroid hormone ecdysone initiates local axonal degeneration in MB γ-neurons through a nuclear receptor complex composed of ultraspirel (USP) and EcR-B1 (Lee et al., 2000). With EcR-B1 DN, even though pruning was blocked (not shown; Hooper et al., 2006), it failed to suppress the *bsk* degeneration phenotype (Fig. 2A,G). The UPS pathway is also required in axon pruning and ectopic expression of the ubiquitin protease UBP-2 blocks MB axon pruning (not shown; Watts et al., 2003). However, UBP-2 does not block the *bsk* phenotype (Fig. 2B,G). These results suggest the *bsk* degeneration phenotype is not due to ectopic axon pruning. Given the 'dying-back' phenotype, we tested whether myosin II based retraction is involved. Myosin II activity causes axonal retraction *in vitro* (Wylie and Chantler, 2003; Gallo, 2004; Gallo, 2006) and *in vivo* in MB neurons (Billuart et al., 2001) by



**Fig. 1. Drosophila JNK loss results in age-dependent axon degeneration.** (A–C) Adult MB JNK (*bsk<sup>147e</sup>*) neuroblast clones exhibit axon degeneration with axon thinning and terminal fragmentation in  $\beta$ - (yellow arrows) and  $\gamma$ -axons (blue arrows). This phenotype worsens with age, as seen between 5- (A), 28- (B) and 50-day-old (C) flies (post-eclosion). (D–F) Aged-matched wild-type (WT) controls show no degeneration. Arrows label the  $\alpha$ -,  $\beta$ - and  $\gamma$ -MB subsets with axon defects, as shown. (A'–C') Adult *bsk*  $\gamma$ -single neuron clones show age-dependent axonal loss from the distal terminal ends (white arrows on the right). Note the increasing distance away from the midline (dashed line) as axon degeneration proceeds in aged animals. (D'–F') Aged-matched wild-type axons do not display any terminal loss. All clonal generated neurons were labeled with CD8-GFP (green). FasII immunostaining (magenta) labels all  $\gamma$  and  $\alpha/\beta$  subset of MB axons. As FasII labels both mutant and wild-type axons, this provides a useful marker to compare the MB axon terminal zones between mutant and wild-type axons. Confocal images are z-stacks of serial sections taken at 1  $\mu\text{m}$  intervals. Scale bar: 20  $\mu\text{m}$ . (G) Quantification of  $\beta$ -axon degeneration in neuroblast clones at indicated stages. The neurodegenerative phenotypes are extremely significant ( $***P<0.0001$ ; Fisher's exact test) in *bsk<sup>147e</sup>* compared with wild-type (WT) age-matched neuroblast clones; *n* indicates the number of clones analyzed. (H) Quantification of the  $\gamma$ -axon terminal loss in single cell clones by measuring the distance of a single cell axon terminus from the midline. (I) A box-and-whisker plot representation (by measuring the distance of a single cell axon terminus from the midline) showing that dying-back degeneration increases with age. The *P*-values ( $***P<0.001$ ; Mann-Whitney U-test) were highly significant between age-matched *bsk<sup>147e</sup>* and wild-type axon terminals.



**Fig. 2. JNK axon degeneration is not caused by aberrant axonal pruning or myosin-based retraction or affected by apoptotic inhibitors.** (A–C) Overexpressing a dominant-negative (DN) form of EcR-B1 (A) or the ubiquitin protease UBP-2 (B) in *bsk*<sup>147e</sup> MB neuroblast clones fails to suppress the JNK axon degeneration phenotype in either  $\gamma$ - or  $\beta$ -neurons (blue and yellow arrows, respectively). MB neuroblast clones were analyzed at 14 days post-eclosion. (C) Dominant-negative Zipper (DN Zip) expression also fails to rescue the *bsk* degeneration phenotype. (D–F) Overexpression of anti-apoptotic regulators including Diap1 (D), a dominant-negative (DN) form of Dronc (E) or Buffy (F) does not suppress JNK degeneration phenotype. Green, CD8-GFP (neurons); magenta, FasII (axons). Scale bar: 20  $\mu$ m. (G) Quantification of  $\beta$ -axon degeneration in JNK-null genotypes in the presence of the indicated transgenes. n indicates the number of MB neuroblast clones analyzed. No significant differences were found between age-matched *bsk*<sup>147e</sup> mutant and *bsk*<sup>147e</sup> UAS coexpression genotypes ( $P>0.05$ ; using Fisher's exact test).

generating actin-based contractile forces. However, dominant-negative *Zipper* expression, which encodes *Drosophila* non-muscle Myosin II (Dawes-Hoang et al., 2005), did not suppress the axon degeneration phenotype (Fig. 2C,G), suggesting that myosin-II is not the key effector in the *bsk* phenotype.

#### JNK axonal degeneration is not due to ectopic caspase activities

Previous *Drosophila* studies show developmental pruning in dendrites requires non-apoptotic, caspase activity (Kuo et al., 2006; Williams et al., 2006; Schoenmann et al., 2010; Tao and Rolls, 2011). Caspase activity is also triggered in response to Alzheimer's disease-causing forms of the Beta-amyloid precursor protein (APP) resulting in axonal fragmentation and cell death (Nikolaev et al., 2009). Various caspase inhibitors were tested. *Drosophila* IAP1 (DIAP1) is an E3 ubiquitin ligase that promotes the ubiquitination of caspases, thereby preventing caspase activation (Muro et al., 2002). DIAP1 expression also blocks dendrite pruning (Schoenmann et al., 2010; Tao and Rolls, 2011) in *Drosophila* mechanosensory neurons and neurodegeneration in MB neurons overexpressing mutant ataxin-3 (Ghosh and Feany, 2004). However, using two copies of UAS-DIAP1 (2X), DIAP1 overexpression did not alter the *bsk* degeneration phenotype

(Fig. 2D,G). We also tested a related protein, DIAP2. Like DIAP1, DIAP2 also suppresses naturally occurring cell death as well as by cell death activators *reaper* (Rpr) or *head involution defective* (Hid) (Hay et al., 1995). Interestingly, DIAP2 does show some differences in caspase-inhibitory preferences (Ribeiro et al., 2007). Nonetheless, DIAP2 overexpression did not rescue the *bsk* phenotype (data not shown; Fig. 2G). We also tested a dominant-negative (DN) form of Dronc, an initiator caspase, which blocks cell death induced by cell death activators (Hawkins et al., 2000; Meier et al., 2000). Dronc also controls dendritic pruning in *Drosophila* neurons (Kuo et al., 2006). However, expressing two copies of DN Dronc (2X) transgenes failed to suppress the *bsk* degeneration (Fig. 2E,G). Bcl-2 family proteins are known inhibitors of caspase-dependent events and required for mitochondria integrity and function (Vander Heiden et al., 1997). Buffy, the sole *Drosophila* anti-apoptotic Bcl-2 homologue, can suppress cell death phenotypes associated with caspase activity (Quinn et al., 2003) and mitochondrial dysfunction in the *Drosophila* PINK1 model of an early onset form of Parkinson's disease (PD) (Park et al., 2006). Nonetheless, ectopic Buffy did not alter the *bsk* phenotype (Fig. 2F,G). These results suggest caspase activities or Bcl-2 functions are unrelated to the JNK axon degeneration phenotype.

### **Wld<sup>S</sup> is a long-term suppressor of JNK axonal degeneration**

Other candidate genes were tested but none of these suppressed the *bsk* phenotype (supplementary material Fig. S1). For example, we also test various regulators (TOR, HDAC6, 4EBP and Parkin) associated with autophagy, protein translational inhibition and mitochondrial quality control that were previously linked to neurodegenerative phenotypes.

TOR inhibition has been demonstrated to promote neuroprotection by inducing autophagy and reducing levels of translational activity (Ravikumar et al., 2002). However, TOR overexpression, which also causes a TOR loss-of-function effect in *Drosophila* (Hennig and Neufeld, 2002), did not modify the *bsk* axonal degeneration phenotype (supplementary material Fig. S1A,E). We also examined the effect of HDAC6 overexpression in *bsk* MB neuroblast clones. HDAC6 has been shown to act at an intersection between the UPS, the principle non-lysosomal degradative pathway of ubiquitinated proteins, and autophagy, a lysosomal degradative pathway. HDAC6 expression rescues neurodegeneration in an autophagy-dependent manner when the UPS system is impaired in a *Drosophila* model of spinobulbar muscular atrophy (Pandey et al., 2007). Furthermore, HDAC6 has recently been found to regulate the autophagosome-lysosome fusion step during autophagy (Lee et al., 2010). However, overexpressed *Drosophila* HDAC6 failed to rescue the *bsk* axonal degeneration phenotype (supplementary material Fig. S1B,E). This potentially suggests that the *bsk* axonal degeneration phenotype might not be a consequence of UPS or autophagy dysfunction.

TOR can also confer neuroprotection via protein translational repression in *Drosophila* models of familial Parkinson's disease (PD) (Tain et al., 2009; Liu and Lu, 2010). In these models, PD neurodegeneration is associated with mitochondrial dysfunction, aberrant protein synthesis and degradation and oxidative stress (Abou-Sleiman et al., 2006; Farrer, 2006). Ectopic expression of the TOR effector pathway, the 4EBP translation inhibitor is sufficient to suppress neurodegeneration and other pathological phenotypes that occur in PD gene mutants, *parkin*, *Pink1* and in *LRRK2* transgenic animals (Imai et al., 2008; Tain et al., 2009). Nonetheless, overexpression of 4EBP (*Drosophila* Thor) in a *bsk*-null genetic background failed to rescue the axonal degeneration phenotype (supplementary material Fig. S1C,E), suggesting that JNK inactivation results in a distinct form of degeneration from that observed in the fly PD models.

In PD models, the E3 ubiquitin ligase Parkin acts as part of a mitochondrial quality control system through its recruitment to dysfunctional mitochondria, where it ubiquitinates outer mitochondrial membrane proteins and promote autophagy of defective mitochondria (Narendra et al., 2010; Narendra and Youle, 2011). Additionally, Parkin also mediates Beclin-dependent mitophagy in a mouse Alzheimer's model and in the autophagic clearance of ubiquitinated A $\beta$  in vivo (Khandelwal et al., 2011). Nonetheless, Parkin overexpression had no effect on the *bsk* phenotype (supplementary material Fig. S1D,E), suggesting that the JNK inactivation phenotype is unrelated to defective mitophagy. Wld<sup>S</sup> is a promising candidate given its wide-ranging neuroprotective effects (Coleman and Freeman, 2010). However, Wld<sup>S</sup> does not suppress all forms of axon degeneration (Coleman, 2005), such as developmental pruning of *Drosophila* MB axons or the reorganisation of mouse retinotectal axons (Hoopfer et al., 2006). We tested two different Wld<sup>S</sup>

transgenic lines (1 and 2), both of which fully suppressed the *bsk* axonal phenotype (Fig. 3). Furthermore, Wld<sup>S</sup> maintained axonal integrity in aged *bsk* clones (Fig. 3A,C,D) and the single-axon analyses showed it blocked the dying-back phenotype in aged clones (Fig. 3B,E,F,Q). Therefore, the Wld<sup>S</sup> protective effect was sustained, and not transient, as previously reported in other contexts (Mack et al., 2001; Beirowski et al., 2008; Beirowski et al., 2010).

### **Wld<sup>S</sup> neuroprotection requires Nmnat1 but is independent of enzyme activity**

Different portions of Wld<sup>S</sup> are thought to confer neuroprotective function (Coleman and Freeman, 2010). When the mouse *Nmnat1* gene (mNmnat1) was expressed, this robustly rescued the *bsk* degeneration phenotype, surpassing the Wld<sup>S</sup> effect (Fig. 3G,H,L,R). Single axon studies show many of these neurons had wild-type termination points (Fig. 3L,M,R). This suggests that the mNmnat1 region is fully sufficient and that the Wld<sup>S</sup> N70 region is dispensable for neuroprotection here.

Other Nmnat isoforms were subsequently tested. While mNmnat2 failed to suppress the *bsk* phenotype ( $P>0.05$ ; Fig. 3G,I,L,N,R), mNmnat3 expression provided some rescue (Fig. 3G,J,L,O,R), although not to the same extent as with Wld<sup>S</sup> or mNmnat1. Taken together, these data suggest that Nmnat1 and, to some extent, mNmnat3 provide the greatest axonal protective functions in *bsk*-dependent degeneration. This closely parallels other reports that show Nmnats 1 and 3 are potent suppressors of axonal degeneration induced by physical injury (Avery et al., 2009; Coleman and Freeman, 2010; Avery et al., 2012; Fang et al., 2012). As all Wld<sup>S</sup> and Nmnat transgenes used here are myc tagged, we could verify their expression by immunohistochemistry. Their expression levels were broadly similar in MB neurons (supplementary material Fig. S2). This suggests the differences in Nmnat neuroprotective actions are unlikely to be due to differences in expression levels.

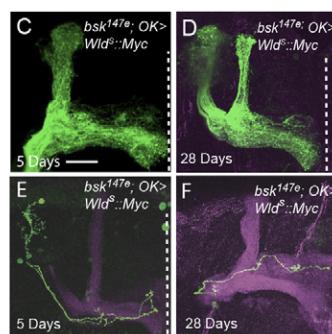
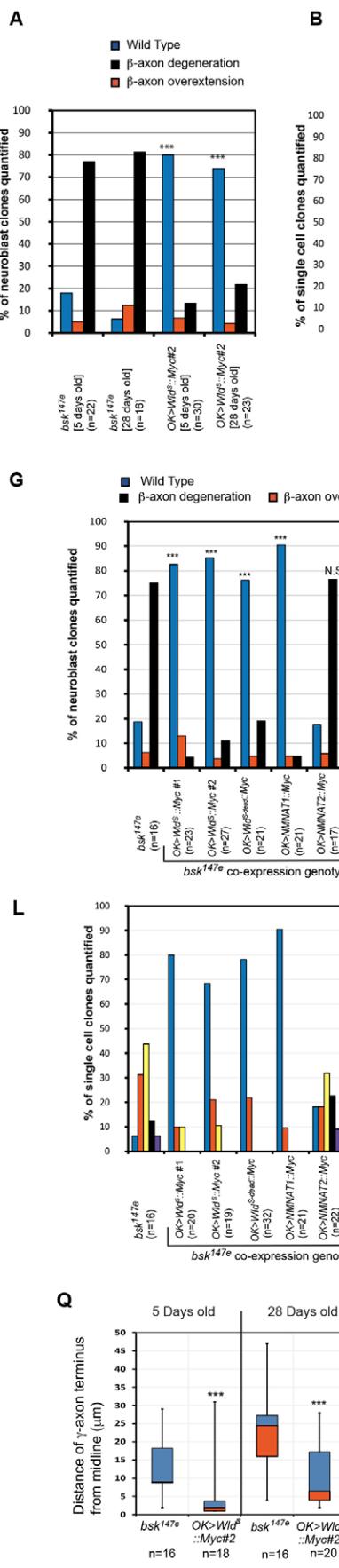
We tested whether Nmnat enzyme activity is important by expressing Wld<sup>S-dead</sup>, an enzyme-inactive form of mNmnat1 (Avery et al., 2009). Wld<sup>S-dead</sup> also suppressed *bsk* axon degeneration to a degree similar to Wld<sup>S</sup> and mNmnat1 (Fig. 3G,K,L,P,R). This suggests while the mNmnat1 portion confers axonal protection, its enzyme activity is dispensable. This is in contrast to *Drosophila* and mouse axotomy models, where its enzyme activity is essential (Araki et al., 2004; Avery et al., 2009; Conforti et al., 2009; Yahata et al., 2009).

### ***Drosophila* Nmnat inactivation results in axon degeneration and neuronal loss**

To test the endogenous function of Nmnat, we made mutant clones of *Drosophila Nmnat* (Zhai et al., 2006). *nmmat<sup>l</sup>* loss-of-function clones showed an axonal phenotype similar to *bsk* and mutants of the upstream JNK regulators, *hep* and *Mkk4*, where the  $\beta$ -lobe was lost (Fig. 4A-C, respectively, quantified in Fig. 4E) (Rallis et al., 2010). Interestingly, in *nmmat<sup>l</sup>* clones, earlier-born neurons ( $\gamma$  and  $\alpha'\beta'$ ) were not visible, suggesting that apart from axonal maintenance it is also required for neuronal viability. Some neuronal cell loss was also evident in *nmmat<sup>l</sup>*  $\alpha\beta$  neurons.

### **Evidence of molecular chaperone involvement in JNK and Nmnat degeneration**

One previous report showed that *Drosophila* Nmnat has a non-enzyme function that involves molecular chaperone activity



**Fig. 3.** *Wld<sup>S</sup>* confers sustained protection against JNK axon degeneration that is Nmnat enzyme-independent.

(A,B) Graphs quantifying JNK axon degeneration in neuroblast (A) or single cell clones (B) expressing *Wld<sup>S</sup>* in 5- and 28-day-old adults. *P*-values were highly significant comparing age-matched *bsk<sup>147e</sup>* neuroblast clones with those in the presence of *Wld<sup>S</sup>::Myc*.

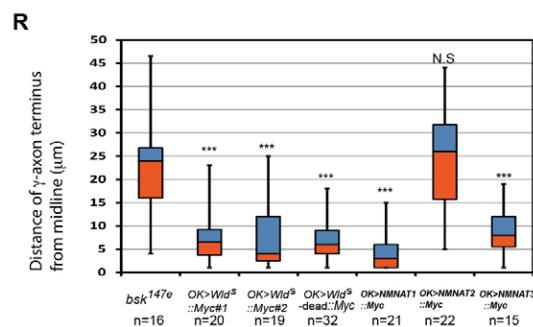
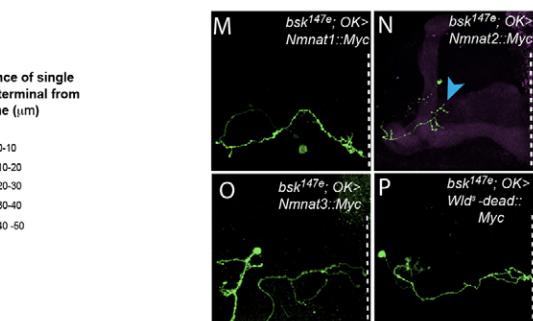
(C–F) *Wld<sup>S</sup>* expression blocks JNK axon degeneration and terminal loss phenotypes in *bsk* neuroblast (C,D) and single cell clones (E,F), respectively.

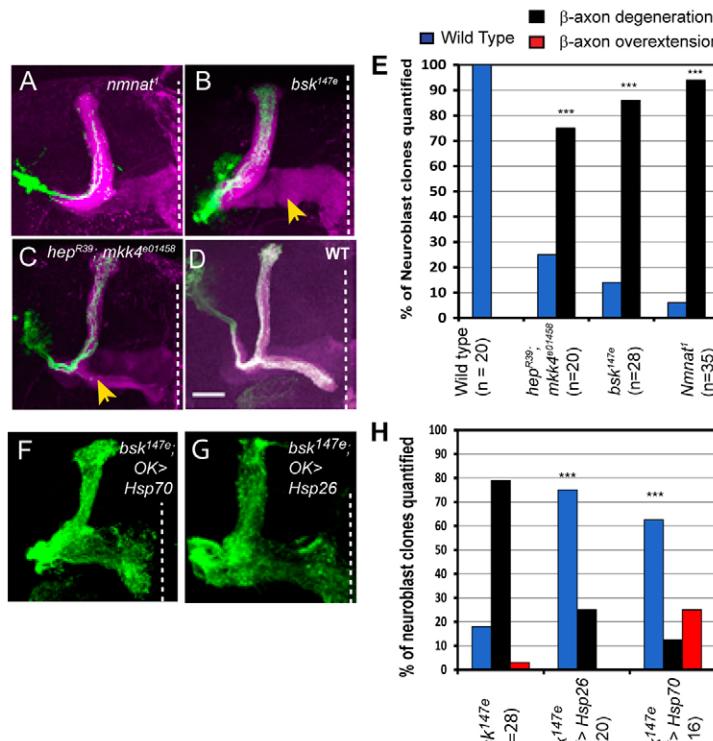
(G) Quantification of axon degeneration in 28-day-old *bsk* neuroblast clones expressing mNmnat1, mNmnat2, mNmnat3 or *Wld<sup>S</sup>:dead*. Fisher's exact test between age-matched *bsk<sup>147e</sup>* neuroblast clones and those in the presence of the *Wld<sup>S</sup>:Myc* lines (#1 and #2), *Wld<sup>S</sup>:dead*, Nmnat1::Myc and Nmnat3::Myc were highly significant, but not significant for Nmnat2::Myc.

(H–K) Representative images of genotypes. Yellow and blue arrows indicate axonal degeneration in β- and γ-axons, respectively.

(L) Quantification of the terminal loss in 28-day-old JNK single-cell clones expressing *Wld<sup>S</sup>*, *Wld<sup>S</sup>:dead*, mNmnat1, mNmnat2 and mNmnat3. (M–P) Representative images of clones. Blue arrow indicates where axon terminal loss has occurred. Green, CD8-GFP; magenta, FasII. Scale bar: 20 μm.

(Q) Box-and-whisker representation of the axon degeneration phenotype, measuring the distance of single axon terminus from the midline. Significant differences were found between 5- and 28-day-old age-matched *bsk<sup>147e</sup>* single γ-axons compared with *bsk* clones with *Wld<sup>S</sup>::Myc*. (R) Similar plots with other Nmnat lines, including *Wld<sup>S</sup>:dead*, Nmnat1::Myc and Nmnat3::Myc, also showed significant differences compared with *bsk<sup>147e</sup>* mutants alone. By contrast, *bsk<sup>147e</sup>* clones expressing Nmnat2::Myc exhibited no significant difference. \*\**P*<0.01; \*\*\**P*<0.001; N.S., *P*>0.05.





**Fig. 4. Nmnat inactivation results in axon degeneration.**

(A–D) Images of 28-day-old *nmnat<sup>l</sup>* (A), *bsk<sup>147e</sup>* (B), *hep/Mkk4* double mutant (C) and wild-type (D) MB  $\alpha/\beta$  neuroblast clones. These loss-of-function mutations all result in  $\beta$ -axon loss (yellow arrows). (E) Quantification from these genotypes indicates highly significant differences compared with wild-type clones. (F,G) Hsp26 or Hsp70 expression blocks axonal degeneration. (H) Quantification of axon degeneration and overextension in neuroblasts expressing Hsp26 or Hsp70. Scale bars: 20  $\mu\text{m}$ . Green, CD8-GFP; Magenta, FasII. \*\*\* $P < 0.001$ .

(Zhai et al., 2008). *Drosophila* Nmnat was recruited together with the molecular chaperone, Heat shock protein (Hsp) Hsp70 to polyglutamine expanded *spinocerebellar ataxin-1* (SCA-1) containing aggregates. Non-enzyme Nmnat functions were involved in regulating protein folding and blocking SCA-1 neurotoxicity. Very recent results show non-enzyme Nmnat also functions to clear tau oligomers *in vivo* (Ali et al., 2012). We tested the effect of Heat shock proteins (Hsps) on the *bsk* phenotypes in two ways. In *bsk*-null neuroblast clones, we found that, like Wld<sup>S</sup> and Nmnats1 and 3, ectopic Hsp70 or Hsp26 also blocked the *bsk* axon degeneration (Fig. 4F–H).

As shown previously (Rallis et al., 2010), compared to wild-type axons, *bsk* axons showed more abnormal protrusions and swellings along the axons and terminals (Fig. 5A,B,B', respectively; quantified in Fig. 5F). When Hsp70, Wld<sup>S</sup>, Nmnat and Nmnat enzyme-inactive forms were expressed in these clones, these were reduced suggesting that this phenotype is also linked to Hsps and non-enzyme Nmnat activities (Fig. 5C–E; quantified in Fig. 5F).

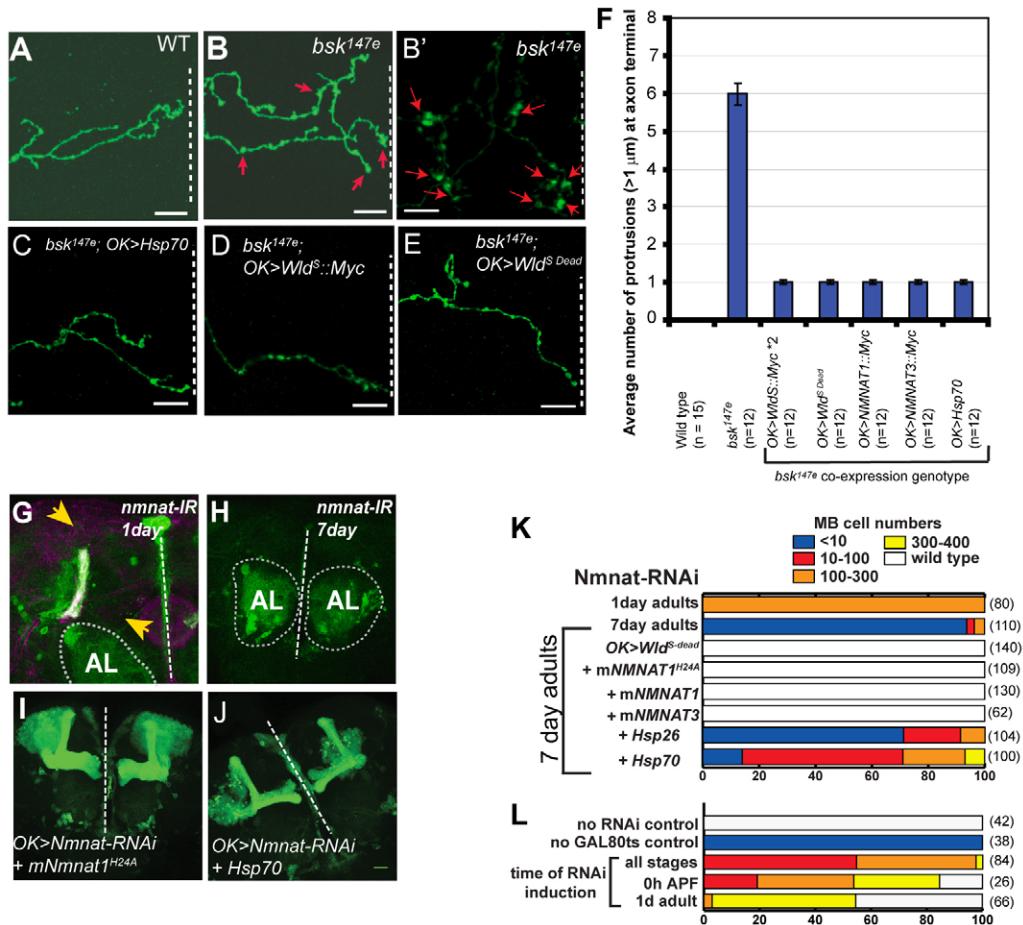
To further test the neuroprotective activity of Hsps, we turned to Nmnat RNAi assays (Fig. 5G–L). When Nmnat RNAi was expressed in MB neurons, this resulted in a  $\beta$ -axon loss phenotype similar to *nmnat<sup>l</sup>* loss-of-function clones above. Some neuronal loss was visible in newly eclosed adults (1-day-old adults). However, almost all neurons were lost in 7-day-old adults (Fig. 5G,H, respectively; quantified in Fig. 5K), suggesting that Nmnat is an obligate maintenance factor, consistent with previous reports (Zhai et al., 2006). We found the Nmnat RNAi axon and neuronal cell loss was rescued by enzyme-inactive forms of mNmnat1 (H24A) and Wld<sup>S-dead</sup> (Fig. 5I; quantified in Fig. 5K; not shown). Furthermore, Hsp26

and Hsp70 expression also partially suppressed the Nmnat RNAi phenotype (Fig. 5J,K; not shown). Together, these results suggest non-enzyme Nmnat and chaperone activities are linked to JNK axonal functions.

Using the GAL80ts system (McGuire et al., 2003) to control JNK temporal expression, we previously showed that JNK activity is required throughout development, even though the axon degeneration phenotype occurs mainly at adult stages (Rallis et al., 2010; this study). To determine Nmnat's temporal requirements, we coupled Nmnat RNAi to GAL80ts control and induced the loss-of-function phenotype at various stages of development (Fig. 5L). We found that RNAi throughout the development and adult phase caused the strongest neuronal loss phenotype. RNAi induction at pupal or adult stages also caused neuronal loss, albeit at a weaker levels. These results suggest Nmnat is required throughout development as well as adult stages. Even though the Nmnat RNAi phenotype is more severe in adults, as in *bsk* mutants, unlike *bsk*, Nmnat's genetic requirements extend beyond the developmental stages and are essential at adult stages. This suggests *Drosophila* Nmnat may have additional roles at adult stages that may be independent of JNK activity.

## Discussion

Here we reveal a ‘Wallerian-like’ degeneration occurs in JNK mutant axons, which progressively worsens with age. This ‘dying-back’ degeneration is prevalent in many neurodegenerative conditions and closely linked to Wld<sup>S</sup>-Nmnat activities (Finn et al., 2000; Sajadi et al., 2004; Mi et al., 2005; Hasbani and O’Malley, 2006; Kaneko et al., 2006; Howell et al., 2007; Beirowski et al., 2008; Wang et al., 2012). However,



**Fig. 5. Enzyme-inactive Nmnats and Hsps can block JNK and Nmnat inactivation phenotypes.** (A–B') Images of 28-day-old wild-type (A) and *bsk*<sup>147e</sup> single cell  $\gamma$ -neuron axon terminals (B,B'). Enlarged and supernumerous axonal protrusions (red arrowheads) are evident in *bsk* mutant axons along the axon shaft (B) and terminals (magnified image in B'). (C–E) Magnified images of 28-day-old *bsk* clones expressing Hsp70 (C), Wld<sup>S</sup> (D) or Wld<sup>S-dead</sup> (E). These large protrusions were reduced by Hsp70, Wld<sup>S</sup> or Wld<sup>S-dead</sup> expression. Green, CD8-GFP. Scale bars: 20  $\mu\text{m}$  (A,B), 10  $\mu\text{m}$  (B'–E). (F) Quantification of enlarged protrusions ( $>1 \mu\text{m}$  in diameter) within 20  $\mu\text{m}$  of axonal terminals, scored from the above genotypes, including the effects of Nmnats 1 and Nmnats 3. *n* indicates number of single cell clones analyzed. (G,H) Nmnat RNAi transgene in MB neurons resulted in axonal loss, visible in newly eclosed adults (1 day old) (G). In aged adults (7 days old), MB neurons were no longer visible (H), suggesting that Nmnat is an obligate cell maintenance factor. Only the labeled antennal lobes (AL), ventral to the MB lobes, were visibly labeled. The midline is indicated by a dashed white line. (I,J) Nmnat RNAi axonal and neuronal loss phenotype was blocked with mNmnat1(H24A) and Hsp70 expression at the 7-day-old adult stage (I and J, respectively). (K) The quantified neuronal loss phenotype shows that Nmnats and enzyme inactive forms of mNmnat1(H24A) and Wld<sup>S-dead</sup> are more potent at blocking Nmnat RNAi neuronal loss than Hsp26 and Hsp70. (L) Using the GAL80ts system to control Nmnat RNAi expression, flies were raised at 18°C (off-state) or at 29°C (on-state). By transferring flies to 29°C, RNAi transgene expression was induced either throughout the life cycle (all stages, beginning at embryogenesis) or at defined pupal (0 hours after pupal formation; APF) or adult stages (1 day old). As controls, sibling genotypes that contained no GAL80ts or Nmnat RNAi transgenes (but raised throughout at 29°C) were also analyzed for phenotypes. All flies were dissected and analyzed at the 7-day-old stage. Note that the cell number for the no GAL80ts control suggests that, even at the restrictive temperature of 29°C, the presence of UAS-GAL4-mediated expression.

unlike the prevalent model of Nmnat function, our studies show non-enzyme Nmnat and chaperone functions are key interactors with the JNK pathway in controlling axonal stability.

Many experiments have addressed how Wld<sup>S</sup> confers neuroprotection (Coleman and Freeman, 2010). Previous axotomy models using primary neuronal cultures, mouse and *Drosophila* models show both the N70 and the Nmnat enzyme activity are essential (Conforti et al., 2007; Watanabe et al., 2007; Avery et al., 2009; Yahata et al., 2009). Here, we found that only the Nmnat portion is required to protect against JNK axonal degeneration. Furthermore, its enzyme activity is dispensable. Interestingly, this non-enzyme requirement was also reflected in the Nmnat RNAi rescue assays. Therefore, one possibility is that

the N70 and the Nmnat enzyme activity are differentially axon-protective; essential for transected axons but not for uncut (but degenerating) axons, such as in JNK and *Nmnat* loss-of-function or SCA-1 paradigms, where its non-enzyme chaperone activity has a greater role (this study; Zhai et al., 2006; Zhai et al., 2008). This also extends to a recent Tau neurotoxicity model in *Drosophila* where non-enzyme Nmnat also has a neuroprotective effect (Ali et al., 2012). Another recent report suggests Nmnat also has a role in dendritic maintenance that is enzyme-independent (Wen et al., 2011). All these studies put together suggest a growing involvement of non-enzyme Nmnat function in various neuropathological conditions that is intimately linked to neuronal maintenance and stability.

Neuroprotective effects of molecular chaperones in neurodegenerative disease models are well-documented (Cummings et al., 1998; Cummings et al., 2001; Auluck et al., 2002; Magrané et al., 2004; Gifondorwa et al., 2007; Fonte et al., 2008; Voisine et al., 2010). Recent studies show that increasing Hsp activity restores peripheral injured nerves to functional recovery by promoting axonal growth (Ma et al., 2011). Upon nerve damage, Hsps are upregulated and present in rat dorsal root ganglion axons (Willis et al., 2005). The Hsps tested here appear to be less neuroprotective than Nmnat1. One possibility is that chaperones require Nmnat to provide greater recruitment potential to sites of nerve damage. Alternatively, acting together, they provide stronger neuroprotection.

Two questions emerge from these results. First, how does Nmnat and chaperone activities interact with JNK signalling? One possibility is that Nmnats are JNK kinases substrates. While Nmnats are phosphorylation targets (Schweiger et al., 2001; Berger et al., 2007), we have yet to test if JNK is directly involved. Another possibility is that JNK signals via the AP-1 transcription program to modulate Nmnat expression. Using immunostaining protocols, no significant changes to Nmnat protein levels were found when either Bsk or AP-1 was altered (using gain- or loss-of-function paradigms) in MB neurons (unpublished observations). However, the interactions may also occur through extensive regulators. Given that the JNK pathway is highly tuned to stress responses and nerve damage (Brecht et al., 2005; Leyssen et al., 2005; Ayaz et al., 2008; Nix et al., 2011), JNK signals may act to indirectly coordinate a mechanism by which Nmnat and molecular chaperones function together to prevent axon degeneration and maintain neural integrity at sites of damage. Recent axonal injury paradigms suggest Nmnat responses reside in the mitochondria (Avery et al., 2012; Fang et al., 2012; Yahata et al., 2009). It would be interesting to determine whether non-enzyme Nmnat axonal-protective function similarly occur in mitochondria and whether this also applies to other neurodegenerative paradigms, such as those provoked by genetic mutations and toxicological stress.

Secondly, what are the protein substrates regulated by Hsps, Nmnat and JNK signals? Identifying these substrates and how they may be regulated may be key to determining how axonal stability and degeneration is controlled during neural development, maintenance and in neuropathological situations.

## Materials and Methods

### Drosophila strains

The *bsk<sup>147e</sup>*, *hep<sup>R39</sup>* and *nmnat<sup>l</sup>* flies are null strains (Glise et al., 1995; Sluss et al., 1996; Zhai et al., 2006). The *Mkk4<sup>01458</sup>* is a loss-of-function allele (Thibault et al., 2004; Rallus et al., 2010). Additional strains used: *UAS-EcRB1 DN* (Cherbas et al., 2003); *UAS-ZipDN::YFP* (Dawes-Hoang et al., 2005); *UAS-UBP2* (DiAntonio et al., 2001); *UAS-Diap1*, *UAS-Diap2* and *UAS-Dronc DN* ( $\Delta$ Ndrone.C>A) (Meier et al., 2000; Ribeiro et al., 2007); *UAS-Buffy* (Quinn et al., 2003); *UAS-mTOR* (Hennig and Neufeld, 2002); *UAS-HDAC6* (Pandey et al., 2007); *UAS-Thor (4E-BP)* (Miron et al., 2001); *UAS-Parkin* (Greene et al., 2003); *UAS-dNmnat RNAi* (Bloomington Drosophila Stock Center; 29402); *UAS-Hsp70* (Warrick et al., 1999); *UAS-Hsp26* (Wang et al., 2004); Myc tagged *UAS-Wld<sup>S</sup>*, *Nmnat*, and their variant strains (Avery et al., 2009). *Wld<sup>S</sup>* and *Nmnat* enzyme-inactive variants correspond to the H24A mutation that disrupts the ATP binding site, reducing the enzyme activity to ~0.3% of wild-type levels (Sasaki et al., 2009).

MARCM clones, GAL80ts experiments and immunohistochemical analyses were similarly performed as previously (Rallus et al., 2010). All UAS expression lines were verified either by immunofluorescence staining (using available antibodies), or by RT-PCR, using primer pairs that detect only UAS-derived mRNA transcripts (5'-CAAGCGCACGCTGAACAAAGCTAACAAATCTG-3' and gene-specific primers) (data not shown; (Wang et al., 2004). Overexpression of EcRB1 DN, UBP2 or ZipDN::YFP causes phenotypes which are consistent with previously reported defects in axon pruning [EcRB1 DN and UBP2 (Watts et al.,

2003; Hooper et al., 2006)] or in cytokinesis and axon overgrowth [ZipDN::YFP (Billuart et al., 2001); data not shown].

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