Protein turnover of the Wallenda/DLK kinase regulates a retrograde response to axonal injury

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Regenerative responses to axonal injury involve changes in gene expression; however, little is known about how such changes can be induced from a distant site of injury. In this study, we describe a nerve crush assay in Drosophila melanogaster to study injury signaling and regeneration mechanisms. We find that Wallenda (Wnd), a conserved mitogen-activated protein kinase (MAPK) kinase homologous to dual leucine zipper kinase, functions as an upstream mediator of a cell-autonomous injury signaling cascade that involves the c-Jun NH2-terminal kinase MAPK and Fos transcription factor. Wnd is physically transported in axons, and axonal transport is required for the injury signaling mechanism. Wnd is regulated by a conserved E3 ubiquitin ligase, named Highwire (Hiw) in Drosophila. Injury induces a rapid increase in Wnd protein concomitantly with a decrease in Hiw protein. In hiw mutants, injury signaling is constitutively active, and neurons initiate a faster regenerative response. Our data suggest that the regulation of Wnd protein turnover by Hiw can function as a damage surveillance mechanism for responding to axonal injury.

Introduction

Regenerative responses to axonal injury require a transcriptional reprogramming of the injured neuron, and there is great interest in understanding how this reprogramming is activated and controlled (Richardson et al., 2009; Sun and He, 2010). Because axons are long (as long as 1 m for human motoneurons), a key element of an injury response mechanism is the ability to signal to the nucleus that the axon has been damaged. Several studies suggest that signaling molecules are physically transported in axons via microtubule-based motors, such as the minus end–directed motor dynein (for reviews see Hanz and Fainzilber, 2006; Abe and Cavalli, 2008). The transported molecules include transcription factors, intermediate filaments, and activated MAPKs ERK and JNK. How such molecules are orchestrated to detect and respond to axonal damage is poorly understood.

Recent findings have brought attention to a conserved MAPK kinase kinase, dual leucine zipper kinase (DLK), as a candidate regulator of axonal damage signaling. DLK localizes to axons (Hirai et al., 2005; Eto et al., 2010) and is functionally required for regeneration after axotomy in Caenorhabditis elegans and mice (Hammarlund et al., 2009; Itoh et al., 2009; Yan et al., 2009). Interestingly, DLK is also required for Wal1erian degeneration of the distal stump after injury (Miller et al., 2009). The dual function in degeneration and regeneration suggests that DLK may be acutely activated by axonal injury to mediate these various downstream injury responses.

In Drosophila melanogaster, we have previously found that the homologue of DLK, Wallenda (Wnd), regulates synaptic bouton growth and morphology at the larval neuromuscular junction (NMJ) via a nuclear signaling pathway that requires JNK and the transcription factor Fos (Collins et al., 2006). Wnd and its homologue in C. elegans are regulated by a conserved E3 ubiquitin ligase, named Highwire (Hiw) in Drosophila (Nakata et al., 2005; Collins et al., 2006). Mutations in hiw lead to increased levels of Wnd protein in axons (Collins et al., 2006), and this misregulation of Wnd leads to increased branching and bouton growth at the motoneuron nerve terminus (Wan et al., 2000).

In this study, we test the hypothesis that Hiw and Wnd function to regulate an injury response pathway. For this, we
developed an axon injury and regeneration assay in *Drosophila* motoneurons. Importantly, a downstream molecular reporter, whose induction coincides with the initiation of regeneration, allows us to dissect the steps required for neurons to mount a transcriptional response to injury. Our findings indicate that Wnd acts as a key upstream mediator of a nuclear signaling pathway, which is activated by axonal damage and promotes axonal sprouting after injury. Furthermore, transport and destruction of Wnd in axons are important elements of a damage surveillance mechanism. By regulating the levels of Wnd in axons, the Hiw ubiquitin ligase plays a key role in regulating a retrograde injury signaling pathway.

**Results**

A nerve crush injury assay in *Drosophila*

To study axon injury response in the *Drosophila* system, we established a nerve crush assay for larval segmental nerves (see Materials and methods; Fig. 1 A and Fig. S1 A), which contain motoneuron and sensory neuron axons. Because they run close to the ventral midline, the segmental nerves can be visualized through the cuticle in third instar larvae under a standard dissection stereomicroscope. After anesthetization with CO₂, the nerves and surrounding cuticle are pinched tightly with number 5 forceps. The injury leads to paralysis in the posterior segments; however, the animal is still able to feed and, remarkably, survives pupation and ecloses as a fully motile adult. Fig. 1 B shows an example segmental nerve 24 h after nerve crush. The nerve becomes stretched and thin, and vesicular cargoes accumulate at the crush site but do not pass through. Staining for MAP1B (Futsch) indicates a loss of microtubule structure at the injury site 1, 24 h after the injury (Fig. S1 C). Distal to the crush site, nerves and synaptic NMJs degenerate within 24 h after the injury (Fig. S1 D).

Nuclear and cell body responses to the nerve crush injury

To study the mechanism of nuclear injury response, we first needed to identify a reporter for cellular changes induced by injury. The JNK phosphatase *puckered*, whose expression can be reported via a lacZ enhancer trap (Martín-Blanco et al., 1998), is an attractive candidate because it has been previously observed to be induced around sites of traumatic brain injury in *Drosophila* (Leyssen et al., 2005). *Puc-lacZ* is expressed at very low levels in uninjured neurons (Fig. 1 D); however, nerve crush induces a dramatic increase in *puc-lacZ* expression in injured motoneurons (Fig. 1 E). The induction of *puc-lacZ* by injury is cell autonomous. This is shown by varying the location of injury (Fig. 1 C) to different segments in the animal, which induces *puc-lacZ* only in the motoneurons whose axons have been injured (Fig. 1, E1–E3).

To quantify the change in *puc* promoter activity, the mean intensity of lacZ staining was measured in the neurons that lie along the dorsal midline, most of which are motoneurons (Sanyal, 2009). When segmental nerves are crushed at injury site 1, *puc-lacZ* expression begins to increase within 8 h after injury (at 25°C) and continues to increase linearly until pupation (Fig. 1 F).

A well-documented response to injury in vertebrate neurons is a reduction in synaptic transmission (Brumovsky et al., 2007). In *Drosophila* motoneurons, the *Drosophila* vesicular glutamate transporter (DVGLUT) plays a critical role in neurotransmission (Daniels et al., 2006) and is robustly detected by antibody staining in motoneuron cell bodies (Daniels et al., 2008). We found that injury induces a dramatic reduction in the cell body levels of DVGLUT (Fig. 1 G).

Wnd and downstream signaling via JNK and Fos mediate the nuclear response to axonal injury

With *puc-lacZ* and DVGLUT as downstream molecular responses to axonal injury, we could use these markers as reporters to dissect the mechanism of injury signaling. We first tested the role of the *Drosophila* DLK homologue Wnd in injury response because DLK has recently been implicated in both regenerative and degenerative injury responses in other organisms (Hammarlund et al., 2009; Itoh et al., 2009; Miller et al., 2009; Yan et al., 2009). Because of the chromosomal location of *wnd* (near the centromere), we were unable to recombine *wnd* mutations with the *puc-lacZ* reporter. Therefore, we used a transgenic RNAi knockdown strategy to reduce *wnd* transcript (Dietzl et al., 2007). An upstream activation sequence (UAS)-*wnd*-RNAi transgene was coexpressed with *Dcr2* using the neuronal BG380-Gal4 driver line, which drives strong expression in many neurons, including motoneurons (Sanyal, 2009). This reduced the total levels of Wnd protein in whole brain extracts (Fig. S2 A) and was sufficient to recapitulate other loss of function phenotypes for *wnd* in motoneurons, such as disruption of axonal transport (Fig. S2 B; Itoh et al., 2007) and suppression of synaptic overgrowth caused by mutation in *hiw* (Fig. S2 C; Collins et al., 2006). Expression of *wnd*-RNAi completely inhibited the induction of *puc-lacZ* by injury (Fig. 2 B). In addition, *wnd* heterozygotes partially inhibited the effect of injury on *puc-lacZ* (Fig. S2 D). In *wnd*/+ loss of function mutants, the effect of injury upon DVGLUT was abolished (Fig. 2 B and Fig. S2 E). Conversely, overexpression of *wnd* in uninjured neurons causes a dramatic increase in *puc-lacZ* and decrease in DVGLUT (Fig. 2, D and F), mimicking the effect of the nerve crush. We conclude that Wnd is a critical upstream regulator of the cellular responses to injury.

Because Wnd was previously found to promote synaptic overgrowth via JNK and Fos (Collins et al., 2006), we tested the requirement of JNK and Fos in the injury signaling mechanism. Expression of dominant-negative (DN) transgenes JNK*DN* and Fos*DN* inhibited the injury-induced effects upon both *puc-lacZ* and DVGLUT (Fig. 2, C and E). In contrast, Jun*DN* had no effect (Fig. 2 E), which parallels previous observations that Fos*DN* but not Jun*DN* inhibits synaptic overgrowth (Collins et al., 2006).

Two controls further address the specificity of Wnd’s role and effect. First, the other mixed lineage kinase family member in *Drosophila*, Slpr, has no effect on *puc-lacZ* in neurons either when it is mutant or overexpressed (Fig. 2 F) despite the fact that Slpr regulates *puckered* in epithelial cells during dorsal closure (Stronach and Perrimon, 2002). Second, mutations in bone
Similarly to puc-lacZ, p-JNK appears only in the cell bodies of injured neurons. However, its accumulation is more transient, beginning around 6 h (for injury site 1), peaking around 12 h, and decreasing thereafter, concomitant with the induction of the puckered phosphatase (Fig. 1 F). Importantly, p-JNK did not appear in the cell body in wnd mutants after injury (Fig. 3, C and D), which indicates that Wnd is required for JNK activation in injured neurons. Because wnd mutants did not have reduced p-JNK staining in the neuropil (Fig. 3 C) or segmental nerves (not depicted), we infer that Wnd is only one of the regulators of JNK in axons. However, Wnd is a critical upstream regulator of JNK in response to axonal injury.

Figure 1. Axon injury induces transcriptional changes in the Motoneuron cell body. (A) Schematic of the nerve crush assay. The segmental nerves within a third instar larva are crushed by pinching the ventral cuticle with forceps. (B) Injured segmental nerves 24 h after nerve crush. Synaptic vesicle precursors detected by staining for DVGLUT staining (green) accumulate at the proximal side of the crush site (arrow). (C) Cartoon of neuron cell bodies (blue dots) and segmental nerves (blue lines). Different crush sites (dashed red lines) injure a predictable number of motoneurons. Crush site 1 injures more cells than crush sites 2 and 3. (D) In uninjured animals, puc-lacZ expression is barely detectable. A nuclear localization signal on lacZ (green) localizes the reporter to the nucleus, and neuronal nuclei are detected by staining for the ElaV (red) marker. (E1–3) Injury induces puc-lacZ expression. 24 h (at 25°C) after injury at sites 1–3 induces expression of puc-lacZ in a defined subset of motoneurons as predicted by the anatomy cartooned in C. (F) Time course quantitation of puc-lacZ. The mean intensity of puc-lacZ is measured as described in Materials and methods for the dorsal midline neurons. 24 h after injury, puc-lacZ intensity is increased 3.5-fold compared with uninjured animals (n > 15). (G) Axon injury leads to a decrease of DVGLUT protein in motoneuron cell bodies. Error bars indicate mean ± SEM. Bars, 25 µm.

TheWnd pathway is required for axon regeneration after injury

What is the physiological role of the nuclear and cell body response to injury? An attractive model is that this injury signaling pathway morphogenetic protein signaling, which also affect NMJ morphology and development via a nuclear signaling pathway (Keshishian and Kim, 2004; Marqués and Zhang, 2006), do not affect the puc-lacZ induction by injury (Fig. 2 F). Furthermore, injury has no effect upon levels of nuclear phospho-MAD (unpublished data).

The puc-lacZ reporter is regulated by JNK signaling in other developmental contexts (Dobens et al., 2001; Stronach and Perrimon, 2002; Galko and Krasnow, 2004; Bosch et al., 2005; McEwen and Peifer, 2005). To address directly whether JNK is activated by axon injury and whether this activation requires Wnd, we probed larvae with an antibody against phosphorylated JNK (p-JNK). In uninjured animals, p-JNK is prominent in the neuropil of the larval ventral nerve cord (VNC; Fig. 3 A). This staining is specific for JNK because transgenic RNAi knockdown of the Drosophila JNK homologue bsk in neurons abolishes the p-JNK staining (Fig. 3 B). Dramatically, nerve crush induces the appearance of p-JNK in injured motoneuron cell bodies (Fig. 3 A).
induces a regenerative response in the injured neuron. To test whether larval motoneuron axons regenerate after injury, we used the Gal4/UAS system to label single-motoneuron axons within each segmental nerve by expression of UAS–mCD8-GFP. Two different driver lines were used for this purpose. m12-Gal4 (Ritzenthaler et al., 2000) drives strong expression in only two axons per segmental nerve, which are tightly fasciculated with one another. RRa(eve)-Gal4 (Fujioka et al., 2003) drives specific expression, albeit more weakly, in RP2 and aCC motoneurons in third instar larvae.

With either subset of labeled motoneurons, extensive branching is observed from the proximal stump within 14 h of injury (Fig. 4 A). Within 24 h, branches can be longer than 100 µm (Fig. 4 D). Because the lesion is a great distance (>1 mm) from the target muscles and the time before pupation is limited (<3 d), we do not observe functional reconnection of the injured axons to their targets. Likewise, reconnection to the distal stump also does not occur because the distal stump degenerates. Nonetheless, the axonal branching represents new axonal growth in response to injury, which can be considered as an attempted regenerative response. To quantify this response, we counted the fraction of injured nerves that showed more than five branches of at least 10 µm in length at the injury site (Fig. 4 A, arrows) while blinded to genotype. Because only two axons per nerve are labeled by each Gal4 driver, the new branches must have arisen from new remodeling/growth of these injured axons. By this criteria, 70–80% of the injured axons (in a wild-type [WT] genetic background) show signs of regenerative growth within 14 h after injury (Fig. 4, C and E).

To test whether the Wnd/JNK/Fos signaling pathway is required for this regenerative response, we used the strong m12-Gal4 driver to express wnd-RNAi, JNKDN, or FosDN in the labeled motoneurons (Fig. 4 B). We find that disruption of each component of the pathway significantly inhibits the formation of new axonal branches at the injury site (Fig. 4, B and C). Because we were unable to isolate recombinants between

**Figure 2.** The nuclear and cell body response to axonal injury specifically requires Wnd and downstream signaling components. (A–D) puc-lacZ expression (left) and staining for DVGLUT (right) in VNCs uninjured and 24 h after injury. (A) The nerve crush injury induces an increase in puc-lacZ expression (left) and decrease in staining for DVGLUT (right) in motoneuron cell bodies. (B) The response to injury requires Wnd function. No obvious change in puc-lacZ (left) and DVGLUT (right) is observed after injury when Wnd is disrupted. (C) The response to injury is inhibited by FosDN. (D) Overexpression (OE) of wnd in neurons is sufficient to activate the injury response, including induction of puc-lacZ and reduction in DVGLUT staining. (E and F) Quantification of the puc-lacZ expression level before (white bars) or 24 h after (black bars) injury in different genotypes. The BG380-Gal4 driver is used to drive expression of all UAS lines [wnd RNAi, JNKDN, FosDN, JunDN, and Slpr]. P > 0.05 was not significant. **, P < 0.001; ***, P < 0.0001. Error bars indicate mean ± SEM. Bars, 25 µm.
which begins to appear \( \sim 8 \) h after injury, is also similar to the timing of \( \text{puc-lacZ} \) induction (Fig. 1F). We conclude that the nuclear response to injury, which is mediated by Wnd signaling, induces new axonal growth/branching of the injured neuron.

Because Wnd is an upstream mediator of an injury response pathway, an attractive hypothesis is that Wnd is locally activated in

Figure 3. Injury induces p-JNK accumulation in the cell bodies and nuclei of injured neurons. (A–C) VNCs are costained for p-JNK (green) and the nuclear marker Elav (magenta). In both uninjured and injured animals, p-JNK stains the neuropil (A, asterisks and dotted lines), which can be seen surrounding the cell bodies (A, yellow bracket). (A) p-JNK appears in motoneuron cell bodies within 12 h after injury. Bar, 25 \( \mu \)m. (B) This p-JNK staining is abolished by expression of \( \text{bsk-RNAi} \) [Dietzl et al., 2007] in neurons. (C) Mutations in \( \text{wnd} \) inhibit the cell body accumulation of p-JNK after injury. The bright dots in the nuclei are fixation artifacts. (D) Quantification of p-JNK intensity in the cell body before (white bars) and 12 h after (black bars) injury. *, \( P < 0.05 \); **, \( P < 0.001 \); ***, \( P < 0.0001 \). Error bars indicate mean ± SEM.
axons. Consistent with this hypothesis, the vertebrate homologue of Wnd, DLK, can be detected in axons, growth cones, and synapses (Mata et al., 1996; Hirai et al., 2005; Eto et al., 2010). Therefore, we tested whether Wnd localizes in axons in *Drosophila*.

Endogenous Wnd is barely detectable by immunocytochemistry in a WT background (Fig. 5 A). However, when axonal transport was inhibited by overexpressing a truncated form of the dynactin subunit p150/Glued (GluedDN) in motoneurons (Allen et al., 1999), cargo for axonal transport accumulate in axonal swellings (Martin et al., 1999), and Wnd protein can be detected in these swellings (Fig. 5 A). No such axonal accumulations are observed when transport is disrupted in a *wnd* mutant (Fig. 5 A) when the Wnd antibody epitope is absent (Collins et al., 2006). Also consistent with transport in axons, Wnd protein accumulates at both sides of the site of nerve crush within 2 h of axonal injury (Fig. 5 B), similarly to other cargo for axonal transport (Horichii et al., 2005; Barkus et al., 2008). To directly assay the transport of Wnd protein, we used the Gal4/UAS system to drive expression of a UAS–GFP- wndKD transgene exclusively in motoneurons using OK6-Gal4 (Sanyal, 2009). The GFP- wndKD transgene contains a mutation in the kinase domain (kinase dead [KD]), which allows the protein to be expressed at detectable levels for live imaging without causing lethality. Importantly, the GFP- wndKD transgenic protein can be visualized in live dissected third instar larvae by rapid time-lapse imaging. Fig. 5 C shows a single frame from a representative video (Video 1) and the kymograph generated from a single-axon tract. The GFP-WndKD protein localizes to discrete puncta, many of which move anterogradely and/or retrogradely with mean segment velocities of 0.83 ± 0.02 µm/s (n = 342) and 0.62 ± 0.02 µm/s (n = 271), respectively. These velocities are comparable with other cargo for axonal transport machinery in *Drosophila* axons (Miller et al., 2005; Haghnia et al., 2007; Barkus et al., 2008).

The localization of Wnd to discrete motile particles suggests that Wnd associates with vesicles. Wnd and its homologues in vertebrates have no transmembrane domains and are predicted to be cytoplasmic proteins. However, biochemical characterization of DLK from mouse brain homogenates indicates that a significant fraction of the protein cofractionates with membranes (Mata et al., 1996). Differential centrifugation and sucrose floatation assays from *Drosophila* head extracts indicate that Wnd behaves biochemically like a membrane-associated protein (Fig. S3). We conclude that Wnd is transported in axons while associated with vesicles.

### Injury signaling and regenerative response via Wnd requires axonal transport machinery

If the axonal localization of Wnd is functionally relevant for the injury signaling mechanism, a prediction is that mutations that disrupt axonal transport machinery would inhibit both the nuclear and downstream regenerative responses to injury. We disrupted axonal transport using mutations in either the minus end–directed motor dynein (Fig. S4) or the dynactin complex (Fig. 6), which plays a critical role in dynein cargo binding (Schroer, 2004). Dyactin was disrupted by expressing a DN-truncated subunit, p150/Glued (GluedDN; Allen et al., 1999), in larval motoneurons. This resulted in a strong inhibition to the regenerative response (Fig. 6, A and B). However, this was not particularly informative alone because axonal transport may also be required for steps downstream of the nuclear injury signal, such as the transport of new material into the regenerating axon. The *puc-lacZ* reporter allowed us to directly test the requirement for dynactin and dynein in injury signaling independent of downstream events. Both GluedDN (Fig. 6, C and E) and dynein (sw/; Fig. S4) mutations cause a near-complete block to the induction of *puc-lacZ* by injury. The cell body/nuclear accumulation of p-JNK is also inhibited (unpublished data). We conclude that dynein and dynactin are required for the transduction of the injury signal to the nucleus.

Interestingly, the requirement for dynactin in injury signaling cannot be simply bypassed by overexpressing *wnd* in neurons. That is, although overexpression of *wnd* is sufficient to activate *puc-lacZ* and down-regulate DVGLUT (Fig. 2, D and E), it is not sufficient when axonal transport is inhibited by GluedDN (Fig. 6, D and E). We interpret that localization of Wnd alone to the cell body is not enough to induce the signaling pathway. Rather, Wnd may need to be transported into axons to become activated or to encounter a necessary cofactor or substrate. It is
difficult to distinguish between these possibilities because mutations in components of axonal transport machinery inhibit both anterograde and retrograde transport in *Drosophila* motoneurons (Allen et al., 1999; Haghnia et al., 2007; Barkus et al., 2008; unpublished data). Furthermore, additional cellular processes may be disrupted by the GluedDN and *sw1* mutations (Levy and Holzbaur, 2006). Nonetheless, these findings imply that axonal localization and transport is an important element of Wnd’s function in regulating an axon to nucleus signaling cascade.

Injury regulates Wnd protein turnover

The aforementioned observations suggest that injury signaling involves local activation of Wnd in axons. A previous study in vertebrate cells suggests that DLK protein is activated via dimerization (Nihalani et al., 2000). Because overexpression of *wnd* alone can activate downstream signaling (Fig. 2, D and F), a potential mechanism of activation is to increase local levels of Wnd so that it can dimerize and self-activate. Previous studies suggest that Wnd protein levels are tightly regulated by protein turnover (Nakata et al., 2005; Collins et al., 2006; Wu et al., 2007). The Hiw E3 ubiquitin ligase plays an important role in this regulation because mutations in *hiw* lead to increased levels of Wnd protein in axons (Collins et al., 2006). Therefore, we tested whether axonal injury induces changes in Wnd protein levels.

VNCs and connected segmental nerves from uninjured or injured larvae were microdissected and processed for Western blotting at different time points after injury. Intriguingly, we observed an 80% increase in Wnd protein within 4 h of injury (Fig. 7, A and B). This short time frame (much shorter than the time required for *puc*-lacZ induction) suggests that increased Wnd levels may be an earlier event in the injury signaling mechanism.

To test whether the effect of injury upon Wnd protein level is posttranscriptional, we used the Gal4/UAS system to drive expression of GFP-*wnd* in motoneurons. Because expression from the OK6-Gal4 driver is not affected by injury (unpublished data), any changes to GFP-WndKD protein levels should be posttranscriptional, reflecting altered localization, translation, or protein turnover. We find that injury induces a dramatic increase in the amount of GFP-WndKD in axons. This can be detected both in axonal segments within the VNC (Fig. 7 C1, arrows) and segmental nerve (Fig. 7 C2), where a greater than threefold increase in mean intensity (Fig. 7 D) and particle density (Fig. 7 E) was observed 4 h after injury. In contrast, we measured no significant change in the intensity of GFP-Wnd in cell bodies (Fig. 7 C1 and Fig. S5 A). Because the total levels of Wnd increase after injury (Fig. 7 A) and transgenic Wnd levels also increase in axons, we hypothesize that Wnd levels increase via an alteration in stability or protein turnover.

An attractive model is that injury induces Wnd by inhibiting its regulation by Hiw. Therefore, we investigated whether injury altered the levels or localization of Hiw protein. UAS–GFP-*hiw* was expressed in motoneurons using the OK6-Gal4 driver. This transgenic protein, which is capable of rescuing the *hiw* mutant phenotype of synaptic overgrowth (Wu et al., 2005), localizes to both axons and cell bodies (Fig. 7 F1 and F2). Although injury does not induce significant changes in the levels of GFP-Hiw in cell bodies.
would be constitutively activated when hiw is absent. Two observations support this model. First, when hiw is mutant, puc-lacZ is increased more than fivefold above basal levels, whereas DVGLUT expression is dramatically decreased in motoneuron cell bodies (Fig. 8, A and B), resembling neurons that have been injured (Fig. 1). These effects can be suppressed by removing a single copy of wnd (Fig. 8, A and B). The induction of puc-lacZ by mutation of hiw also requires Fos and Glued (Fig. 8 B). We conclude that when hiw is mutant, the retrograde injury signaling pathway is ectopically active.

Second, when hiw is mutant, injured neurons require less time to initiate a regenerative response (Fig. 8, C and D). Most neurons exhibit new branching within 7 h of injury in hiw mutants, whereas it takes >12 h for WT neurons to exhibit the effects.
We use this new assay to reach several insights into the function of a conserved MAPK kinase kinase, Wnd/DLK, and its mechanism of activation during injury signaling and regeneration. First, we show that Wnd functions as an upstream regulator of a nuclear signaling pathway that is activated by axonal injury. Second, we show that activation of this signaling pathway promotes axonal growth and branching of the injured neuron. Third, we demonstrate that Wnd is transported in axons, peripherally associated with membrane-bound vesicles, and the injury signaling mechanism requires functional axonal transport machinery. Fourth, we find that injury signaling is regulated by the Hiw E3 ubiquitin ligase and that protein turnover of Wnd in axons may be a surveillance mechanism for detecting axonal damage. Injury induces a rapid increase in Wnd protein, concomitant with a decrease in Hiw protein. In hiw mutants, the nuclear response to injury is constitutively active, and neurons require less time to initiate a regenerative response to injury. We conclude that the regulation of Wnd by Hiw in axons comprises an important mechanism for detecting and responding to axonal injury.

Discussion

In this study, we describe an axonal injury and regeneration assay in Drosophila larval motoneurons, which creates a powerful paradigm for studying the molecular events and requirements for axonal regeneration of defined neurons within an intact living animal. Importantly, a molecular reporter (the JNK phosphatase puckered) whose expression is induced by axonal injury in a cell-autonomous fashion allows us to dissect the steps required for neurons to mount a transcriptional response to injury.

We use this new assay to reach several insights into the function of a conserved MAPK kinase, Wnd/DLK, and its mechanism of activation during injury signaling and regeneration.

First, we show that Wnd functions as an upstream regulator of a nuclear signaling pathway that is activated by axonal injury. Second, we show that activation of this signaling pathway promotes axonal growth and branching of the injured neuron. Third, we demonstrate that Wnd is transported in axons, peripherally associated with membrane-bound vesicles, and the injury signaling mechanism requires functional axonal transport machinery. Fourth, we find that injury signaling is regulated by the Hiw E3 ubiquitin ligase and that protein turnover of Wnd in axons may be a surveillance mechanism for detecting axonal damage. Injury induces a rapid increase in Wnd protein, concomitant with a decrease in Hiw protein. In hiw mutants, the nuclear response to injury is constitutively active, and neurons require less time to initiate a regenerative response to injury. We conclude that the regulation of Wnd by Hiw in axons comprises an important mechanism for detecting and responding to axonal injury.
The Wnd/DLK kinase regulates a regenerative response to axonal injury

Our findings indicate that an important component of Wnd/DLK’s role in regeneration is to regulate a retrograde signaling pathway, linking injury, which activates the Wnd pathway in axons, to a downstream transcriptional response in the nucleus. In the vertebrate nervous system, not all neurons are capable of regenerating after axonal injury. Conditioning lesion studies in dorsal root ganglion neurons suggest that the capacity to regenerate in the central nervous system is linked to the ability to mount a cellular and transcriptional response to injury (Hannila and Filbin, 2008; Hoffman, 2010). Therefore, DLK is an attractive candidate regulator of injury signaling in vertebrate axons. It will be interesting to determine the function of DLK in different neuronal types.

The role for Wnd in activating a nuclear injury response does not rule out additional functions for Wnd in the axon. Recent studies in C. elegans suggest that DLK regulates translation in axons (Yan et al., 2009), which is important for both injury signaling and regeneration (Hanz et al., 2003; Yudin et al., 2008; Gumy et al., 2010). Wnd/DLK may also regulate local cytoskeletal changes because JNK signaling is known to regulate microtubules in axons (Gelderblom et al., 2004; Bogoyevitch and Kobe, 2006; Barnat et al., 2010; Stone et al., 2010). An attractive hypothesis, which is consistent with growth cone phenotypes for vertebrate homologues of hiw (Lewcock et al., 2007; Hendricks and Jesuthasan, 2009), is that Wnd signaling alters cytoskeleton to form or modify growth cones after injury. In addition to these roles in regeneration, we should also consider the involvement of JNK in cell death and degeneration (Johnson and Nakamura, 2007). It is possible that the activation of Wnd/DLK by injury could have negative consequences in some scenarios.

Mechanism of activation of axonal injury signaling

Wnd is transported in axons. Wnd is transported in axons, in the form of particles that move at a speed similar to other cargoes for fast axonal transport. Because endogenous Wnd protein cofractionates with membranes, this cytoplasmic kinase may associate peripherally with vesicles. An interesting future pursuit is to determine the molecular nature of these vesicles. Studies in the vertebrate sciatric nerve demonstrate that the JNK scaffolding protein JIP3/Sunday Driver associates with large, multivesicular organelles that travel retrogradely in response to injury (Cavalli et al., 2005; Abe et al., 2009). Because vertebrate DLK also associates with a membrane compartment (Mata et al., 1996), it is possible that DLK associates with a retrograde signaling cargo or a precursor to such cargo. Future tools are needed to follow the transport and associations of kinase-active and endogenous Wnd after injury.

Wnd is activated in axons. Previous studies of the role of DLK in regeneration in C. elegans (Hammarlund et al., 2009; Yan et al., 2009) and degeneration in cultured dorsal root ganglion neurons (Miller et al., 2009) suggest that DLK functions acutely at the time of injury. The most attractive model for both observations is that DLK/Wnd is locally activated in axons by injury to mediate different responses in different contexts.

Our findings strongly support this model. Both endogenous and GFP-tagged Wnd localize to axons, and functional axonal transport machinery is required for transduction of the injury signaling to the nucleus. Even if Wnd is ectopically overexpressed, when presumably some protein could localize to the cell body, the downstream response requires functional axonal transport machinery. We interpret that the axonal localization and transport of Wnd is functionally relevant for its signal transduction mechanism. It may need to be localized in axons to become activated or to encounter a necessary cofactor or substrate.

Injury regulates protein turnover of Wnd. Injury leads to an increase in the total levels of endogenous Wnd protein. Furthermore, the levels of transgenically expressed GFP-WndKD are also increased, particularly in axons. Because the OK6-Gal4 driver is not affected by injury, the increase in GFP-WndKD must take place posttranscriptionally either as increased protein synthesis or decreased protein turnover. Several observations favor the model that injury activates Wnd by inhibiting its turnover in axons. First, the GFP-WndKD transgene that the Wnd signaling pathway, which is regulated by Hiw in axons, is down-regulated by the Hiw E3 ubiquitin ligase (Nakata et al., 2005; Collins et al., 2006), which decreases in axons after injury. Third, regulation of the levels of Wnd is a viable mechanism for regulating its activation because overexpression of Wnd is sufficient to activate this signaling pathway.

Our data suggest that down-regulation of Hiw could be part of the injury signaling mechanism. However, further studies are required to understand the mechanism of Hiw regulation after axonal injury. Intriguingly, a recent study suggests that Hiw is regulated by autophagy (Shen and Ganetzky, 2009), which could potentially be induced by axonal injury.

Rescue experiments indicate that Hiw function is required throughout the larval stage to down-regulate Wnd (Wu et al., 2005); thus, Wnd is constantly made, transported, and destroyed in axons. It has previously been perplexing that despite the amount of energy required to regulate this signaling pathway, there was not an obvious function of Wnd in synaptic development or function. We propose that the regulation of Wnd by Hiw in axons could be part of a damage surveillance mechanism for the cell to detect and respond to axonal injury.

Connections between injury response, synaptic growth, and synaptic maintenance

A previous study in Drosophila suggested that Hiw functions to regulate nerve terminal growth at the NMJ (Wan et al., 2000). The fivefold increase in number of presynaptic boutons in hiw mutants is one of the most dramatic phenotypes described at the larval NMJ and is caused by the misregulation of Wnd protein. This synaptic overgrowth may simply be the outcome of a misregulated regenerative response within an intact, uninjured circuit.

However, it is also interesting to consider the possibility that the Wnd signaling pathway, which is regulated by Hiw in larval axons, normally performs other functions. An injury, which disrupts an axon’s connections with its target, may share some similarity with other insults that affect the functional connections
at the synapse. Along this line, a recent study found that Hwi-regulated signaling can counteract synaptic retraction caused by loss of the spectrin cytoskeleton and that loss of spectrin induces expression of puckered (Massaro et al., 2009). Although the role of Wnd in this remains to be addressed, an intriguing possibility is that loss of synaptic adhesion induces the injury signaling/regeneration pathway. This could counteract the loss of synaptic contacts by promoting more growth. It is of great interest to understand in more detail the mechanism by which this molecular pathway is regulated and the downstream consequences of its activation. Our data, combined with others’ results in Drosophila and other model organisms, point to Hwi and Wnd as important upstream regulators of a therapeutically interesting cellular response in neurons.

Materials and methods

Genetics

The following strains were used in this study: Canton-S [WT], puc-lacZ<sup>69</sup> (Martín-Blanco et al., 1998), BG380-Gal4 (Budnik et al., 1996), OK6-Gal4 (Aberle et al., 2002), m12-Gal4 (PJGal4)<sup>5037</sup> (Ritzenhaler et al., 2000), RReve(Gal4 (Fujiko et al., 2003), hwi<sup>109</sup> (Wu et al., 2005), hwi<sup>1050</sup> (Wan et al., 2000), wnd<sup>1</sup>, wnd<sup>2</sup>, and UAS-wnd (Collins et al., 2006), UAS-Fos<sub>2</sub> (FzB) and UAS-Jun<sub>2</sub> (FzB; Eresh et al., 1997), slp<sup>190</sup> and UAS-slr<sub>WT</sub> (Polaski et al., 2006), UAS-Bx[NK]<sup>190</sup> (Weber et al., 2000), UAS-p150(Flag)<sup>50</sup>, 968 (Allen et al., 1999), UAS-GFP-Hiw (Wu et al., 2005), sw<sup>1</sup> (Boylan and Hays, 1997), khc<sup>h7</sup> (Hurd and Saxton, 1996), khc<sup>27</sup> (Brendza et al., 1999), wtf<sup>119</sup> (Aberle et al., 2002), and wtf<sup>138</sup> (Aberle et al., 2002), which was recombined with puc-lacZ<sup>69</sup>. UAS-GFP-wnd<sup>20</sup> was generated directly from UAS-wnd<sup>20</sup> (Collins et al., 2006), which contains the K188A mutation in kinase domain. UAS-wnd-RNAi and UAS-bsk-RNAi were acquired from the Vienna RNAi center (Dietzel et al., 2007).

Immunocytochemistry

Wandering third instar larvae were dissected in PBS and fixed in either 4% paraformaldehyde in PBS or Bouin’s fixative for 15–30 min, depending on the antibodies used. Antibodies were used at the following dilutions in PBS with 5% normal goat serum: rabbit anti–Wnd A1, 1:100 (Collins et al., 2006); rabbit anti-DVGLUT, 1:5,000 (Daniels et al., 2004); rat anti-eLaV, 1:50 (7E8A10; Developmental Studies Hybridoma Bank); mouse anti-lacZ, 1:100 (40-1a; Developmental Studies Hybridoma Bank); Cy3 goat anti-HRP, 1:1,000 (Jackson ImmunoResearch Laboratories, Inc.); A488 rabbit anti–GFP, 1:2,000 (Invitrogen); and mouse anti-p-JNK, 1:1,000 (Cell Signaling Technology). For secondary antibodies, Cy3- and A488-conjugated goat anti-rabbit and A488–mouse (Invitrogen) were used at 1:1,100. All samples were mounted and imaged in 70% glycerol.

Imaging and quantification

Confocal images were collected at room temperature on a spinning-disk confocal system (PerkinElmer) consisting of a scanner (Nipkow CSU10; Cargile Instruments) and an electron microscopy charge-coupled device camera (C9100-50; Hamamatsu Photonics) mounted on an inverted microscope (Axio Observer; Carl Zeiss, Inc.) with 25× 0.8 NA multi and 40× 1.3 NA, 63× 1.5 NA, and 100× 1.46 NA oil objectives. Similar settings were used to collect all compared genotypes. All imaging and quantification were conducted with Velocity software (PerkinElmer).

To quantify the mean intensity of puc-lacZ expression, the neuronal nuclei that lie along the dorsal midline of the nerve cord in segments A3–A7 were selected based on staining for eLaV. Because of an NLS sequence fused to lacZ, the reporter protein also localized to nuclei. The mean lacZ intensity per nucleus was measured for at least eight animals for each genotype and normalized against analogous measurements in the control (WT) genetic background. A similar strategy was used to quantify the intensity of staining for phosphorylated JNK in motoneuron nuclei.

The total intensity GFP-wnd<sup>20</sup> (Fig. 7 D) was measured in 100 µm of each segmental nerve adjacent to the site of exit from the VNC. To measure the density of GFP-wnd<sup>20</sup> (Fig. 7 E) and GFP-Hiw (Fig. 7 G) particles in this area, we used Velocity software to select and count the number of particles above a threshold pixel intensity and size (GFP-wnd<sup>20</sup>, intensity, >2,000; size, >1 µm<sup>2</sup>; GFP-Hiw, intensity, >2,000; size, >2.5 µm<sup>2</sup>).
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