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Models of axon regeneration in *Drosophila*

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Abstract

Maintaining neuronal connectivity in the face of injury and disease is a major challenge for the nervous system. The great length of axons makes them particularly vulnerable to insult with dire consequences for neuronal function. In the peripheral nervous system there is a program of axonal regeneration that can reestablish connectivity. In the mammalian central nervous system, however, injured axons have little or no capacity to regenerate. The molecular mechanisms that promote axon regeneration have begun to be identified and many of the implicated pathways are evolutionarily conserved. Here we discuss *Drosophila* models of axonal regrowth, describe insights derived from these studies, and highlight future directions in the use of the fly for dissecting the mechanisms of axonal regeneration.

Keywords

Drosophila; Axon regeneration; DLK; Wallenda; Axon injury; Degeneration; Dendrite; JNK

1. *Drosophila* as a model for the study of axon injury

When peripheral axons are injured, the distal axon degenerates while the proximal axon launches a regenerative response. *Drosophila* has been used more extensively to study the process of axon degeneration, and recently several key pathways promoting axon loss have been discovered in flies, underscoring the power of *Drosophila* to characterize axonal injury signaling (Bhattacharya et al., 2012; Fang et al., 2012; Gerdts et al., 2015; Hoopfer et al., 2006; Keller et al., 2011; Miller et al., 2009; Mishra et al., 2013; Neukomm et al., 2014; Osterloh et al., 2012; Xiong and Collins, 2012; Xiong et al., 2012). We expect studies in *Drosophila* to be as fruitful for elucidating mechanisms regulating axon regeneration. Here we discuss fly models of axon regeneration, highlighting the attributes of each model and the insights obtained.

In mammals, injured axons of the peripheral nervous system (PNS) can regenerate after injury, while axons of the central nervous system (CNS) fail to regenerate due to a combination of environmental factors and low intrinsic growth potential. Peripheral axon regeneration after injury involves several distinct processes (Christie and Zochodne, 2013; Lu et al., 2014; Rishal and Fainzilber, 2014; Scheib and Höke, 2013; Tedeschi, 2011). To

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regenerate, injured axons must (1) sense the injury (2) activate a local regenerative response that leads to cytoskeletal rearrangements and formation of a growth cone (3) traffic a retrograde injury signal back to the nucleus to initiate a pro-regenerative transcriptional program, (4) elongate and navigate toward the correct target, and (5) form stable and functional synaptic connections upon arriving at the target. Although PNS axons can regenerate, there are therapeutic situations where enhancing the speed of regeneration would be beneficial. Indeed, peripheral regeneration is often too slow, leading to degeneration of the target cell and impaired functional recovery (Gordon et al., 2011). Moreover, understanding the process of axon regeneration in the PNS may reveal targets for therapeutic interventions that increase CNS regeneration.

Studies in *Drosophila* have a long and storied history of identifying genes and elucidating pathways important for neurobiological functions including axon guidance and synapse formation. Excitingly, this methodology is now being utilized to dissect the molecular response to axonal injury. With the advent of several new models of axon regeneration in *Drosophila*, a new round of discovery may be imminent. Loss and gain of function studies as well as spatial and temporal control of gene expression in an *in vivo* model are easily accessible in the fly. Most importantly, at least some of the essential molecular mechanisms of axon regeneration are conserved between mammals and *Drosophila*, validating the utility of using *Drosophila* to identify new molecules and mechanisms that control the regenerative potential of axons.

Drosophila genetics offer several advantages for the study of axon regeneration. First, *Drosophila* genetics are well developed, with the widespread availability of traditional loss-of-function mutants for most genes. Moreover, the arrival of Cas9/CRISPR greatly simplifies the generation of such mutants for those genes currently lacking traditional mutants. In addition, stock centers maintain multiple independent transgenic RNAi lines per gene for more than 90% of *Drosophila* genes, allowing for conditional and tissue-specific loss-of-function studies. For gain-of-function and rescue studies, transgenic fly lines are available for more than 2000 genes, and the generation of such transgenes for a gene of interest is straightforward. Second, the *GAL4*-UAS system allows for both spatial and temporal control of gene expression (Brand and Perrimon, 1993). Thousands of *GAL4* lines are available that express the yeast transcription factor *GAL4* in specific cells or tissues including many that express in only a subset of neurons. *GAL4* binds to the UAS sequence and drives expression of transgenes that are regulated by UAS promoters. This allows for transgene expression only in the subset of cells or tissues where *GAL4* is expressed. Beyond your gene of interest, the *GAL4*-system allows expression of GFP in sparse neuronal populations that can provide single axon resolution of regenerating axons in distinct neuronal classes that may have different regenerative properties. This rich palette of tools has been invaluable for the study of neural development and function in the fly, and we expect it to be similarly useful for the analysis of neuronal injury and regeneration.

2. Larval models of regeneration

The embryonic and larval *Drosophila* PNS is a favorite model for studying many aspects of neuronal development and function including dendritic tiling, axon guidance, synapse

formation, synaptic terminal growth, active zone development, and mechanisms of neurotransmitter release (Collins and DiAntonio, 2007; Corty et al., 2009; Sigrist and Schmitz, 2011). In addition, the larval PNS is accessible for studies of axonal cell biology, including the analysis of axonal transport and mechanisms controlling microtubule dynamics. Recently, *Drosophila* geneticists have adapted assays of axonal development and function to study axon regeneration in *Drosophila*. Models of both sensory and motor neurons have been developed, allowing for the identification of common regenerative pathways as well as cell type specific mechanisms.

3. Larval peripheral nerve crush model

The Collins lab has developed a simple yet elegant *Drosophila* nerve crush model (Xiong et al., 2010) that recapitulates many aspects of sciatic nerve crush injuries in rodent models of PNS regeneration (Cho et al., 2013; Perry et al., 2012). The segmental nerve runs just under the cuticle on the ventral side of the larva, allowing for a gentle crush of the nerve with forceps while maintaining viability of the animal. The segmental nerve in *Drosophila* contains both sensory and motor axons, however these axons are oriented in opposite directions within the nerve. The motor neuron cell bodies are located in the ventral nerve cord (VNC) and project axons to the periphery, while sensory neurons such as Class IV ddaC (dendritic arborization) neurons have cell bodies in the periphery and project axons into the VNC (Fig. 1A). Individual axons can be visualized and monitored by expression of GFP under the control of *GAL4* lines that express in only a few axons per nerve (*m12-GAL4*: for motor neurons (Ritzenthaler et al., 2000); or *ppk-GAL4*: for ddaC sensory neurons (Kuo et al., 2006)). At the injury site, it is possible to visualize individual axons that have been transected as well as a build-up of vesicular cargo. Although we will focus on insights into regeneration, the distal axon is easily visualized and degenerates over the course of 24 h, so this assay has also been used to study degeneration of both sensory and motor axons (Brace et al., 2014; Mishra et al., 2013; Xiong et al., 2012). This underscores that the crush paradigm for axon regeneration can easily be adapted to study sensory axon regeneration by simply expressing GFP with the sensory specific *GAL4* line (*ppk-GAL4*) since both the motor neurons and sensory neurons are damaged during the crush injury.

Upon axonal injury, retrograde signals are sent to the motor neuron cell bodies leading to a transcriptional response that is essential for regeneration (Rishal and Fainzilber, 2014). The regenerative response of these axons is apparent via the formation of a growth cone that forms within hours of axon injury, although this growth cone does not successfully navigate through the injury site (Xiong et al., 2010). The crush assay has been used to study the role of the MAP Kinase pathway comprising Wallenda (Wnd)/Dual Leucine Zipper Kinase (DLK), a MAPKKK, and the MAP kinase JNK (Jun N-terminal kinase) in axon injury and regeneration. The Collins lab demonstrated that axon injury activates a JNK signaling pathway (Xiong et al., 2010). Within 6 h of axonal injury, phosphorylated JNK (p-JNK) accumulates in the cell bodies of injured motor neurons, where it regulates injury-induced transcriptional changes. The magnitude of the injury signal can be monitored in the cell body by expression of *puckered*, a JNK phosphatase that is upregulated in response to JNK signaling as part of a negative feedback loop. *Puckered* expression is assayed via the expression from the *puckered* locus of a β -galactosidase (*lacZ*) transcriptional reporter.

There is also a decrease in expression of the *Drosophila Vesicular Glutamate Transporter* (*DVGLUT*), suggesting that injury may downregulate genes needed for neuronal function while activating genes that promote regeneration (Xiong et al., 2010).

Mutants in *wnd/dlk* as well as the downstream signaling components *jnk* and *fos* block both the transcriptional changes in *puckered* and *DVGLUT* as well as the formation of the regenerative growth cone. Additionally, this injury response requires retrograde axonal transport—a dominant negative mutant in the dynactin subunit, p150^{Glued}, blocks the induction of *puc*-LacZ expression as well as the axonal regenerative response. Importantly, *Wnd/DLK* is not only essential for regeneration in *Drosophila*, but it is also required in *Caenorhabditis elegans* and mammals demonstrating the conserved nature of axon regeneration across species and the potential power of this assay to identify components of the axon regeneration machinery (Hammarlund et al., 2009; Shin et al., 2012; Xiong et al., 2010; Yan et al., 2009).

Wnd/DLK function is essential for axonal regeneration. Might activation of Wnd/DLK be sufficient to activate at least some aspects of the regeneration response? Overexpression of *Wnd/DLK* does lead to an increase in *puc*-LacZ expression suggesting that an “injury” signal has been activated and at least a partial transcriptional response has been mounted. In both flies and worm, the E3 ubiquitin ligase Highwire (Hiw)/rpm-1 targets Wnd/DLK for degradation (Collins et al., 2006; Nakata et al., 2005). Mutants in the ligase lead to an increase in Wnd/DLK levels and trigger an improved regenerative response that depends on the kinase, suggesting that increased MAPK signaling is sufficient to induce a regenerative response *in vivo* (Hammarlund et al., 2009; Xiong et al., 2010; Yan et al., 2009).

How might injury activate Wnd/DLK signaling? One attractive hypothesis is that injury modulates levels of the Highwire ligase. Collins and colleagues demonstrated that there is a decrease in Hiw levels in axons after injury and a subsequent increase in DLK levels (Xiong et al., 2010). How injury regulates Hiw levels is unknown, but studies on Rae1 and autophagy suggest potential links between Hiw regulation and injury (Shen and Ganetzky, 2009; Tian et al., 2011). Rae1 binds microtubules and physically interacts with Hiw (Tian et al., 2011). The interaction between Rae1 and Hiw prevents autophagy-mediated degradation of Hiw (Tian et al., 2011). In studies of synaptic development, loss of Rae1 or overexpression of the autophagy gene ATG1 leads to a decrease in Hiw levels and a subsequent rise in Wnd/DLK levels (Shen and Ganetzky, 2009; Tian et al., 2011). Putting these findings together, we speculate that axon injury may disrupt microtubules and impair the ability of Rae1 to bind Hiw, freeing Hiw to become a substrate for selective autophagy. This loss of Hiw would, in turn, lead to increased levels of Wnd/DLK and activation of the regenerative program. It will be interesting to determine if autophagy and the role of Rae1 in Hiw regulation extends to injury signaling or if it only functions to regulate Hiw during development.

A second mechanism to activate the DLK pathway was uncovered in *Drosophila* via studies of the gene *Short Stop* (*Shot*). Shot is a spectraplakin, a large multidomain protein that cross-links actin and microtubules and regulates cytoskeletal dynamics. Mutants in *shot* have an increase in *puc*-LacZ expression in motoneuron cell bodies, suggesting that these neurons

are sensing an injury signal even in the absence of a physical injury (Valakh et al., 2013). This “injury” signal is Wnd/DLK dependent since the increase in *puc-LacZ* is abrogated in *wnd/dlk* mutants (Valakh et al., 2013). Interestingly, this “injury” preconditions the axons to regenerate more robustly since *shot* mutants show an increase in regeneration after axonal crush that is Wnd/DLK dependent (Valakh et al., 2013). This phenotype is not specific to the loss of Shot, but instead appears to be a general response to cytoskeleton disruption. Knockdown of two additional genes required for cytoskeletal stability, TCP1 α or TCP1 β , also activate Wnd/DLK-dependent JNK signaling (Valakh et al., 2013). Demonstrating the power of *Drosophila* models to inform our ideas on mammalian regeneration, follow-up studies demonstrated that pharmacological agents that disrupt the actin or microtubule cytoskeleton activate the Wnd/DLK pathway and induce a pro-regenerative state leading to enhanced axon regeneration in mammalian sensory neurons both *in vitro* and *in vivo* (Valakh et al., 2015). The activation of DLK by microtubule disruption may also be conserved in *C. elegans* (Bounoutas et al., 2011; Chen et al., 2014). Other mechanisms regulating DLK have been identified in worms and mammals, including Ca⁺⁺-dependent regulation and JNK-dependent feedback regulation (Huntwork-Rodriguez et al., 2013; Yan and Jin, 2012), and may be relevant to the role of DLK in axon regeneration.

4. Larval laser axotomy models

The dendritic arborization (da) neurons are sensory neurons that have their cell bodies and dendrites in the larval body wall and project axons to the VNC (Grueber et al., 2003). The location of the cell bodies in the larval body wall makes them easily accessible for laser axotomy. da neurons were first studied to examine how diverse dendrite morphologies and dendritic territories are established. There are 15 da neurons per abdominal hemisegment that can be divided into four distinct morphological classes based on their dendritic branching complexity (Class I: least complex; Class IV: most complex). Selective *GAL4* lines express in distinct classes of da neurons allowing subtype-specific genetic control and single axon resolution. Injury is induced by laser axotomy, allowing for transection of individual axons or dendrites. Work in this system has demonstrated that the da neuron injury model recapitulates several interesting aspects of mammalian axon regeneration.

Since da neurons were originally studied in the context of dendrite development, the earliest injury work in this system focused on dendrite regeneration. Uemura and colleagues tested the ability of the dendritic arbors to efficiently tile the larval body wall in response to ablation of a single dendritic branch, finding that 8 of 18 cells extended (“regenerated”) branches from the injury site (Sugimura et al., 2003). Recently, the Rolls and Jan labs have adapted this system to focus on axonal and dendritic regenerative responses in da neurons using a laser injury (Song et al., 2012; Stone et al., 2010; Sugimura et al., 2003).

In brief, 2nd instar larva (40–48 h after egg laying) are mounted on a slide and a UV laser severs the axonal or dendritic connection (Fig. 1B) triggering distal degeneration within 24 h. Subtype specific *GAL4* lines drive GFP expression in a single class of da neurons, allowing for easy visualization as well as genetic manipulation of the target population. Since the anatomy is stereotyped, it is possible to image the same neuron at 24-h intervals over the course of 3 days post injury in order to score regenerative capacity. Although

potentially more challenging, it should be possible to injure axons of motor neurons with lasers since axons in the ventral nerve cord were also targeted in these studies (Song et al., 2012).

Early studies focusing on axon regeneration in this system demonstrated that when the axon is severed very close to the cell body ($<50\ \mu\text{M}$) it is not competent to regenerate. Instead a new axonal process is respecified from a neighboring dendrite (Stone et al., 2010). During this conversion there is a 10-fold up-regulation of the number of growing microtubules and the microtubule polarity is reversed via a JNK dependent mechanism (Stone et al., 2010). When the axon is severed further from the cell body, the neuron regenerates using the remaining axonal stump (Song et al., 2012, 2015; Stone et al., 2012). Reversal of microtubule polarity in dendrites and axons occurs in this injury paradigm as well as in an *in vitro* motor neuron axon regeneration assay, suggesting this may be a general injury response (Lu et al., 2015; Song et al., 2012; Stone et al., 2010).

In mammals different classes of sensory neurons have varying capacities to regenerate after injury (Kalous and Keast, 2010; Leclerc et al., 2007). Similarly, in flies, injury to class IV da axons (ddaC and v'ada) leads to robust axon regeneration as measured both by the percentage of axons that regenerate and the length of axonal growth after injury. However, class I and class III da neurons demonstrate little regenerative capacity (Song et al., 2012). This dichotomy creates a powerful system to identify genes that can block regeneration in class IV neurons or restore regenerative potential to class I or III da neurons. One example is the RNA-processing enzyme Rtca. Increased expression of Rtca reduced axon regeneration of class IV neurons whereas loss of function enhanced not only the regeneration of class IV neurons, but of class III neurons as well (Song et al., 2015).

Regeneration of da neurons recapitulates several aspects of mammalian CNS regeneration. Mammalian dorsal root ganglion (DRG) neurons have cell bodies located outside of the CNS and have a bifurcated axon that projects both a central and a peripheral branch. The axon branch in the PNS can regenerate robustly while the CNS branch has limited regeneration potential. Da neurons project a single axon to the ventral nerve cord with portions of this axon inside and outside of this CNS structure (Fig. 1B). When the axon of the class IV da neuron is injured in the periphery it can regenerate robustly. However, when these same axons are injured in the VNC there is limited regeneration, with most axons stalling at the lesion site (Song et al., 2012). This inability to regenerate axons after CNS injury may be due to the local CNS environment or because the lesion is more distal from the cell body in the CNS lesion. The commonalities between da and DRG neuron axonal regeneration extend to molecular mechanisms. In mammals, enhancement of the Akt pathway can lead to increased regeneration after CNS injury. This is conserved in *Drosophila*. Both loss of function mutants in PTEN, a negative regulator of Akt, or Akt overexpression leads to an increase in sprouting of axons from da neurons upon VNC injury (Song et al., 2012). Hence da neurons recapitulate some functional and molecular aspects of mammalian CNS injury.

No large scale unbiased genetic screens for genes controlling axon regeneration have been conducted in *Drosophila*. However, the RNA processing enzyme Rtca was identified using a

candidate based genetic screen that focused on genes regulated after axotomy (Song et al., 2015). Loss of function of *Rtca* enhances CNS axon regeneration after injury in the VNC. *Rtca*, along with *Rtcb* and *Archease*, are components of an RNA repair and splicing pathway. *Rtca* likely functions in the pathway upstream of *Rtcb* and acts as a brake on enzyme activity, while also inhibiting axon regeneration. Both *Rtcb* and *Archease* are essential for the RNA ligation process. Consistent with the above results, mutants in *Archease* have impaired regeneration suggesting that the RNA repair and splicing pathway is important for regulating axon regeneration. *Rtcb* was also identified in *C. elegans* as an important regulator of axon regeneration, however in worms *Rtcb* mutants exhibited increased regeneration (Kosmaczewski et al., 2015; Nix et al., 2014). These differences may reflect unique functions of *Rtcb* vs. *Archease* or differences in the mechanism of regeneration in worms and flies.

What might be the substrate and mechanism for *Rtca/Rtcb/Archease* function in *Drosophila*? One hint is that the mRNA of *Xbp1*, a central component of the Unfolded Protein Response (UPR), is ligated by the *Rtca/Rtcb/Archease* ligase complex. Indeed, mutants in *xbp1* have a mild defect in axon regeneration in the periphery (Song et al., 2015). Moreover, overexpression of the spliced (activated) form, *Xbp1s*, in class IV da neurons significantly enhanced axon regeneration in the VNC, and promoted regeneration in the periphery when overexpressed in the usually refractory class III da neurons (Song et al., 2015). Of note, neither gain nor loss-of-function mutants in *Xbp1* affected axon regeneration in the worm (Kosmaczewski et al., 2015). However, this pathway may play a role in mammalian axon regeneration since retinal ganglion cells from *Rtca* mutant mice have a substantial increase in the number of regenerating axons (Song et al., 2015). Additionally, the UPR is activated by sciatic nerve crush in adult rat sensory neurons with enhanced expression of the core components CHOP and XBP1 (Ying et al., 2015). Finally, addition of tunicamycin, which induces the UPR, can rescue the decrease in axon regeneration seen in mutants of the ER-resident transcription factor Luman/Creb3 (Ying et al., 2015). Hence, the RNA ligase complex may promote axon regeneration by facilitating the capacity of the UPR to promote axonal regeneration.

5. Dendrite regeneration in sensory neurons

While most studies focus on axon regeneration, dendrites are also damaged by disease or injury and must undergo a regenerative response to regain connections or reestablish a dendritic receptive field. Since da neurons were first studied for their role in dendritic tiling, several studies focused on their response to dendrite injury. While studying the diversity of the dendritic trees of da neurons, Uemura's lab was the first to investigate how these neurons respond to dendritic injury (Sugimura et al., 2003). Subsequent studies revealed robust dendrite regeneration whether a single dendrite was injured or all dendrites were removed from a neuron (Song et al., 2012; Stone et al., 2014).

With dendrite regeneration assays in hand, it is possible to probe the molecular machinery involved and compare the process with axonal regeneration. Dendrite regeneration does share similarities with axon regeneration in that the PTEN-Akt pathway is important for both axon and dendrite regeneration (Song et al., 2012). While this pathway functions in

both compartments, there are selective regulatory mechanisms at play in dendrites. The microRNA Bantam regulates dendrite regeneration by dampening Akt activity (Song et al., 2012). Interestingly, Bantam functions non-cell-autonomously in epithelial cells to regulate dendrite regeneration, demonstrating that both intrinsic factors such as PTEN and extrinsic factors such as Bantam impinge on this pathway. While the Akt pathway controls both axon and dendrite regeneration, the requirement for the DLK-JNK signaling pathway differentiates these two forms of regeneration. As described earlier, the DLK/JNK pathway is an essential component of axon regeneration in flies, worms, and mice. In *da* neurons, however, JNK signaling is not activated by dendrite injury, and neither Wnd/DLK nor downstream components JNK or *fos* are required for dendrite degeneration (Stone et al., 2014). Interestingly, in these very same cells, axon injury does trigger a JNK signal and Wnd/DLK, JNK, and *fos* are all required for axonal regeneration (Stone et al., 2014). Hence, dendritic and axonal compartments of *da* neurons must utilize independent injury surveillance systems and pro-regenerative programs.

6. Adult models of regeneration

Larval assays for regeneration are informative and recapitulate many of the early stages of regeneration. However, larval models have some limitations. In larvae, regeneration occurs in a developing animal and hence the response may be different or more pronounced than in the adult fly. Indeed, this is the case in mammals where embryonic DRG neurons have increased regenerative capacity compared to adult DRG neurons. A second limitation is the short time frame of development, there are only 1–3 days to assess the regenerative response after injury before the start of metamorphosis. As such, there is no larval system in which axons regenerate to their target fields and form connections. These issues highlight the need for adult models of axonal regeneration in the fly. The first effort to develop an adult system was a whole-brain explant model (Ayaz et al., 2008), though recently, new assays more amenable to large-scale genetic screens have been developed in the adult wing. Derived from assays used to study axon degeneration (Fang et al., 2012; Neukomm et al., 2014), these new approaches allow for a comprehensive analysis of the genetic programs controlling injury-induced axon regeneration in the adult.

7. Axon regeneration in the adult wing

The nerve tracts of the wing are physically and genetically accessible allowing for easy manipulation of these neurons (Fig. 2). The nerve running along the L1 longitudinal wing vein consists of approximately 225 mechanosensory and chemosensory neurons (Fang et al., 2012). Cell bodies are spaced along the length of the L1 vein and axons project into the CNS. The *appl-GAL4* driver expresses in these neurons, allowing for selective labeling and genetic control. The wing model was originally developed as a method to study axonal degeneration following a cut to the wing. To study axonal regeneration, the Bonini lab developed a laser-induced injury that maintains cell bodies and proximal axons allowing for studies of regeneration (Soares et al., 2014).

Using this system, the Bonini lab followed the regenerative response for 14 days after laser ablation. Although a majority of axons showed some regenerative response, none of the

axons grew into or through the injury site in wild type animals, suggesting a limited regenerative potential of adult neurons (Soares et al., 2014). Of interest, a noticeable scar forms at the injury site. The scar may act as a physical barrier since fluorescently labeled dextran (3 kDa) injected into the wing does not penetrate the injury site (Soares et al., 2014).

The role of the JNK pathway was tested in this system. Surprisingly, down regulation of the pathway via expression of a dominant negative form of *jnk* led to enhancement of the regenerative response, with axons able to cross the injury site in 22% of mutant animals (Soares et al., 2014). Consistent with this result, over activating the pathway with a constitutively activated hemipterous (MAPKK) resulted in a decrease in axonal regeneration (Soares et al., 2014). The finding that this pathway inhibits regeneration is unexpected because there is a clear requirement for this pathway to promote axon regeneration in worms, mammals, and other neurons in developing flies (Hammarlund et al., 2009; Shin et al., 2012; Xiong et al., 2010). Whether this is due to differences in the developmental state, neuronal cell types, or other factors is unknown. Since the JNK pathway modulates the size of the scar and the size of the scar correlates with the amount of regeneration (Soares et al., 2014), this surprising result may reflect a non-cell autonomous role of JNK in scar formation rather than an intrinsic role for JNK within the neuron.

Two improvements to the wing regeneration model should emerge from recent advances to the wing axon degeneration system. The Freeman lab developed an elegant series of transgenic lines that use genetically encoded FLP-FRT chromosomes to achieve reliable MARCM (Mosaic Analysis with a Repressible Cell Marker) clone induction in L1 wing vein neurons. MARCM is a genetic technique for creating individually labeled homozygous cells in an otherwise heterozygous fly (Lee and Luo, 2001). This allows for expression of GFP from homozygous mutant neurons and provides single axon resolution, making feasible large-scale mutagenesis studies (Neukomm et al., 2014). In addition, these same transgenic lines allow for genetic control of motor neurons in the *Drosophila* leg, providing visualization of both the axon and its synaptic connections (Sreedharan et al., 2015). Further development of this model will allow for the genetic analysis of motor neuron axon regeneration and may provide a system for the analysis of regeneration to the target field and the reestablishment of functional connectivity in *Drosophila*.

8. Additional models of regeneration

8.1. Axon growth in the mushroom bodies

In *Drosophila*, the mushroom body (MB) is a brain structure required for olfactory learning and memory that is comprised of three classes of neurons organized into distinct mushroom body lobes. During the early larval stages MB γ -neurons extend a bifurcated axon that projects to the medial and dorsal lobes (Fig. 3). At the onset of metamorphosis, γ -neurons prune their axons back to the branch point. Later in development, γ -neurons re-grow to form an adult-specific, medial lobe (Lee et al., 1999). The developmental pruning of γ -neurons is a well-studied model of axon degeneration, however analysis of the re-extension of the γ -neurons during development as a model of axon growth and regeneration is relatively new (Yaniv et al., 2012). This system potentially allows for the dissection of three distinct axonal growth programs within the same neurons: initial axon outgrowth during circuit formation,

re-extension of axon growth during developmental remodeling, and finally axon regeneration after injury.

A genetic screen to identify novel genes involved in mushroom body γ -neuron remodeling identified genes involved in developmental pruning and axon re-extension (Yaniv et al., 2012). Mutants in the nuclear receptor Unfulfilled (UNF) (also known as Nr2e3) do not re-extend MB γ -axons into the adult specific lobe after pruning. UNF is required for the developmental axon regrowth, but not for initial axon outgrowth since larval γ -neurons in the mutant develop normally. This result suggests that axonal re-growth following pruning is mechanistically distinct from initial axon growth. In support of this view, mutants in the serine/threonine kinase *mTOR* also have defects in developmental re-extension, but do not have defects in initial axon growth. mTOR is also important for axon regeneration after injury in many systems (Abe et al., 2010; Park et al., 2008, 2010). Might other genes important in axon regeneration play a role in developmental re-extension? Several genes with well-established roles in axon regeneration were tested in this system to assess overlap in the two growth programs. Mutants in *akt1*, *InR*, *pten*, and *wnd/dlk* all display normal developmental re-growth, suggesting that developmental axonal re-growth does not use the core axonal regeneration program (Marmor-Kollet and Schuldiner, 2015).

Recently, nitric oxide was identified as a signaling factor that acts to both promote developmental pruning and inhibit axon re-growth in this system (Rabinovich et al., 2016). Nitric oxide represses developmental re-growth of γ -axons by inhibiting the physical interaction of the nuclear receptors UNF and E75. Nitric oxide synthase activity is downregulated developmentally at the onset of axonal re-extension, suggesting a mechanism for regulating the switch between developmental pruning and axon re-extension during MB development. Whether nitric oxide only plays a role in developmental contexts or plays additional roles in injury-induced axon regeneration is still an open and intriguing question.

While there is no *in vivo* model of axon injury in this system, the Schuldiner lab developed an assay to examine neurite sprouting following dissociation of MB neurons that recapitulates features of axon regeneration (Marmor-Kollet and Schuldiner, 2015). This assay is conceptually similar to assays of mammalian neurite outgrowth following dissociation of DRG neurons (Frey et al., 2015; Saijilafu et al., 2013). In brief, brains are dissected and neurons dissociated from flies expressing GFP in MB neurons. The dissociation of the neurons is a mechanical injury that leads to loss of neurites. Sparse cultures allow for single axon resolution as new MB neurites sprout in a process that may mimic axon regeneration after injury. Indeed, neurons from *wnd/dlk* mutants grow shorter neurites in this assay than do wild type neurons (Marmor-Kollet and Schuldiner, 2015). Perturbation of other genes important for axon regeneration, such as Akt and mTOR, also sprout shorter neurites in this system, supporting the validity of the model for studies of injury-induced axonal regeneration. This culture assay is amenable to drug studies, and was used to show that the mTOR inhibitor rapamycin inhibits neurite outgrowth in this assay (Marmor-Kollet and Schuldiner, 2015). This proof-of-concept study validates this assay as a potential platform for large-scale drug screens to identify novel modulators of axon regeneration. A similar primary neuronal culture assay has been developed for *Drosophila* motor neurons, in which neurons from dissected larval brains are dissociated and plated to

model axonal injury (Egger et al., 2013; Lu et al., 2015). One advantage of the motor neuron system is that the function of identified genes and drugs can be validated *in vivo* using the nerve crush assay described above.

8.2. The role of glia in the axonal regeneration response

The *Drosophila* system is not only good for exploring neuron intrinsic mechanisms of axonal regeneration, but as an *in vivo* model it is also useful for assessing the role of non-neuronal cells in the regenerative process. In mammalian systems glia play a central role in axon regeneration. *Drosophila* axons are not myelinated, however, the larval peripheral nerves are surrounded by 3 glia layers (perineural, subperineural, and wrapping glia) (Stork et al., 2008). In the CNS, the perineural and subperineural glia are paired with populations of cortex glia, ensheathing glia, and astrocytes (Freeman, 2015; Peraanu et al., 2005). Regenerating ddaC and v'ada axons grow along glial processes (Song et al., 2012). If these glial processes are injured along with the axon, this dramatically inhibits axonal regeneration, demonstrating the vital role of glia in the injury response (Song et al., 2012). Following peripheral nerve injury in mammals, Schwann cells undergo a c-Jun dependent program of dedifferentiation to generate repair cells essential for axon regeneration (Arthur-Farraj et al., 2012). Might *Drosophila* wrapping glia or other glial subtypes provide a similar function for axon regeneration? *Drosophila* may also provide a model of extrinsic factors that inhibit axonal regeneration. In a *Drosophila* CNS injury model, there is accumulation of glia at the site of injury reminiscent of the inhibitory glial scars in injured mammalian CNS (Cregg et al., 2014; Song et al., 2012) although myelin-associated inhibitors are not present in flies. The role that glia play in regeneration has largely been unexplored in *Drosophila*, and additional studies are required to determine whether glia at the injury site play a functional role in inhibiting axon regeneration. If so, this system will allow for the targeting of genes specifically in glia using selective *GAL4* lines while assessing axonal regeneration after injury.

9. Conclusions

Understanding the neuronal response to axonal injury is a major challenge in neurobiology, with exciting implications for the treatment of neurological disease and injury. Over the past decade, studies in *Drosophila* have lead to major breakthroughs in our understanding of the molecular mechanism of axon degeneration. With the development of new models and tools described above, we believe that *Drosophila* is poised to deliver similar major insights into the mechanisms of axonal regeneration in both the peripheral and central nervous systems.

One highlight from the current studies of axonal regeneration in *Drosophila* is the strong evidence for evolutionary conservation of major regenerative pathways. In particular, the DLK/JNK and PTEN–Akt–mTor pathways appear to function similarly in both flies and mammals to promote axon regeneration. Hence, new pathways identified in the fly are good candidates to regulate mammalian axonal regeneration. Indeed, work in *Drosophila* demonstrated that cytoskeletal disruption activates the DLK/JNK pathway and promotes axonal regeneration, a finding later confirmed in mammalian neurons. On the other hand, not all pathways will be conserved across species, as seen with the divergent findings in

worms and flies for the RNA ligase RtcB/Archease and its substrate Xbp1. Such diversity underscores the need to study multiple models, neuronal subtypes, and organisms to gain a full understanding of axon regeneration.

With the development of new models of axonal regeneration in the fly, the stage is set for large-scale genetic screens that are a strength of the *Drosophila* system. Axon regeneration screens in *C. elegans* have been very successful, identifying many new genes involved in axon regeneration (Chen et al., 2011; Nix et al., 2014). Similar large-scale genetic screens have identified novel genes regulating axon degeneration in the fly, so studies of axonal injury are feasible (Bhattacharya et al., 2012; Osterloh et al., 2012). In the coming years we expect such screens to identify novel molecules and mechanisms that shape the axonal regeneration program in *Drosophila*, and for many of these insights to translate into a deeper understanding of evolutionarily conserved programs that can reestablish connectivity in the injured and diseased nervous system.

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Abbreviations

PNS	peripheral nervous system
CNS	central nervous system
DLK	Dual Leucine Zipper Kinase
Hw	Highwire
Wnd	Wallenda
VNC	ventral nerve cord
JNK	c-Jun N-terminal kinase
da	dendritic arborization
MB	mushroom body
DVGLUT	<i>Drosophila</i> Vesicular Glutamate Transporter

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Fig. 1.

Larval models of regeneration. (A) Schematic of the nervous system of 3rd instar larva depicting the ventral nerve cord (VNC) with motor neurons (blue) and sensory neurons (orange). Degeneration in the distal axon is marked by the dashed lines. Axons are injured with #5 forceps on the ventral side of larva leading to axonal injury (Xiong et al., 2010). Neurons are labeled by GFP expression under control of *GAL4* expression (*MI2-GAL4* for motor neurons; *ppk-GAL4* for sensory neurons). (B) The cell bodies of ddaC sensory neurons are located in the periphery and project to the VNC. In a second paradigm, injury is induced with a laser and axon regeneration of the PNS *versus* the CNS can be assessed based on the location of the injury (Song et al., 2012). This system is also amenable to studies of dendrite regeneration if the injury is targeted to a particular dendrite branch (Stone et al., 2010). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Fig. 2.

Axon regeneration in the adult wing. The adult wing is depicted with the nerve (green) running along the L1 wing vein expressing GFP under the control of the *appl-GAL4* driver. The nerve is comprised of approximately 225 mechanosensory and chemosensory neurons. Injury by laser induces both degeneration of the distal axon and regeneration from the proximal stump (Soares et al., 2014). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Fig. 3.

Developmental axon re-extension in the mushroom body (MB). Initial axonal growth as well as pruning and regrowth of γ -neurons is depicted in the MB's medial and dorsal lobes (gray structure). MB γ -neurons extend a bifurcated axon (blue) that projects to the medial and dorsal lobes. During metamorphosis, γ -neurons prune these axonal extensions and regrow an adult-specific medial extension (red). This model has been used to study both axon growth and re-extension in a developmental paradigm (Yaniv et al., 2012). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)