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Intrinsic mechanisms for axon regeneration: insights from injured axons in *Drosophila*

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Abstract

Axonal damage and loss are common and negative consequences of neuronal injuries, and also occur in some neurodegenerative diseases. For neurons to have a chance to repair their connections, they need to survive the damage, initiate new axonal growth, and ultimately establish new synaptic connections. This review discusses how recent work in *Drosophila* models have informed our understanding of the cellular pathways used by neurons to respond to axonal injuries. Similarly to mammalian neurons, *Drosophila* neurons appear to be more limited in their capacity regrow (regenerate) damaged axons in the central nervous system, but can undergo axonal regeneration to varying extents in the peripheral nervous system. Conserved cellular pathways are activated by axonal injury via mechanisms that are specific to axons but not dendrites, and new unanticipated inhibitors of axon regeneration can be identified via genetic screening. These findings, made predominantly via genetic and live imaging methods in *Drosophila*, emphasize the utility of this model organism for the identification and study of basic cellular mechanisms used for neuronal repair.

Introduction

As the fundamental conduit for communication with other neurons, a neuron's axon is one of its most vulnerable features. An injury at any position in an axon's length leads to a silencing of its function. How do nervous systems cope with axonal damage? Neurons are expected to last for an animal's lifetime, so re-development and replacement of the damaged neuron is not an option in the adult nervous system. Instead, neuronal repair, when it occurs, requires that the neuron maintain survival through the damage and then initiate new axonal growth (termed 'axon regeneration') to re-form its lost connection. A neuron's ability to do this varies widely depending on what type of neuron it is, where the damage occurs, the presence of both extrinsic and intrinsic inhibitors of regeneration, and the cell's ability to organize its cytoskeleton to initiate new axonal growth. Towards the most idealistic goal of stimulating repair after nervous system damage, there is much interest in understanding what these factors are. Over the past decade, studies in invertebrate model organisms such as

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Drosophila and *C. elegans* have made increasingly important contributions to this goal, with discoveries made through genetic screens and live imaging techniques in these model organisms that take advantage of their simplified nervous systems and powerful genetic tools. In cases where it has been examined thus far, mechanisms identified in the invertebrate models have later been confirmed to be important in mammalian neurons.

In this review, we highlight recent and provocative discoveries made in *Drosophila* around the topic of responses to axonal and dendritic injuries. A number of different injury assays have been developed in the adult and larval nervous system and are reviewed in more detail in [1-3]. Studies of axon degeneration in *Drosophila* have made groundbreaking discoveries and are also reviewed in more detail in [3,4,55]. Here we focus on axon regeneration, and also some of the interesting comparisons that can be made between injuries to axons versus injuries to dendrites.

***Drosophila* as a model to study intrinsic mechanisms for repair**

A central feature of the mammalian nervous system is that axon regeneration occurs readily in the peripheral nervous system (PNS), yet fails to occur in the central nervous system (CNS). There has been great interest in understanding this dichotomy, since regeneration failure in the adult mammalian CNS is a major debilitating aspect of many neuronal injuries. One known reason for the dichotomy is the presence of proteins in CNS myelin that inhibit axonal growth, termed ‘extrinsic’ inhibitors (reviewed by [5]). Another is an ‘intrinsic’ incapability of neurons to initiate new axonal growth after damage in the CNS. Several landmark studies have shown that the intrinsic incapacity to regenerate can, at least for some neurons, be overcome through manipulations to cAMP or mTOR intracellular signaling pathways [6-9]. How this capacity is either locked or unlocked, including how these pathways are regulated and utilized for this gating, is a topic of great interest in the field.

The *Drosophila* nervous system lacks myelin and many of the known extrinsic inhibitors of regeneration that are expressed by oligodendrocytes (eg. Nogo, Ogm, and MAG). However, despite this absence, the PNS/CNS dichotomy for axon regeneration may potentially also exist in *Drosophila*: new axonal growth can be observed in several different injury methods to motor and sensory neurons in the larval and adult PNS (Figure 1 and reviewed in [1]). However, in two injury assays described thus far in the larval and adult CNS, limited axonal sprouting was observed after injury [10,11], and this lack of regeneration can be at least partially bypassed by manipulations to the cAMP or the mTOR pathways [10,11], echoing observations in mammalian neurons [6,7,9,12,13]. Injuries in the CNS are inherently more likely to disrupt multiple neurons and synapses, and it is difficult to make direct comparisons across different injury methods and locations, so further studies are needed. However the similarities noted thus far suggest that neurons across the animal kingdom use similar intrinsic mechanisms to promote or inhibit their capacity to regenerate. *Drosophila* is therefore a reasonable model system to study these intrinsic mechanisms, and it benefits from the vast number of existing genetic tools to manipulate cellular signaling pathways on a single cell level in the *Drosophila* nervous system.

Intrinsic mechanisms of axon regeneration: discoveries from *Drosophila*

a) Cellular rearrangements in microtubules and organelles

An important technique for studying cellular responses to axonal damage is live imaging, which allows one to track changes in the structure of damaged neurons over time, and changes in the localization and abundance of their organelles and cytoskeletal components. For such studies, the dendritic arborization (da) neurons that line the larval body wall have been an excellent model, since these cells can be imaged in their entirety (cell bodies, dendrites, and axons) through the relatively transparent larval cuticle. Based on dendritic branching complexity, these sensory neurons are divided into four classes (Class I: most simple; Class IV: most complex) [14]. With specific GAL4 lines expressing in each type of these neurons, they can be genetically labeled and manipulated with single cell resolution. In these neurons, cellular responses to axonal injury, such as changes in the microtubule cytoskeletal structure and endoplasmic reticulum (ER) distribution have been characterized, and may be coupled to the neuron's ability to initiate axon regeneration (Figure 2). Here we briefly review some of these changes that take place in neurons as they respond to axonal injury.

A useful tool to study the organization of microtubules in these neurons is to follow growth of individual microtubules via live imaging, using the microtubule plus-end binding protein EB1-GFP. In axons the microtubules are uniformly oriented with their growing ends ('plus ends') pointed away from the cell body, while microtubules in mature dendrites are oriented with an opposite polarity [15,16] (Figure 2). Axonal injury (but not dendritic injury) induces a dramatic and global increase in the number of growing microtubules throughout the cell body, dendrites, and proximal axon [17] (Figure 2).

What is the function of this massive induction in new microtubule growth? It appears rapidly, within minutes, in neurons that are able to regenerate their axon [17], so it may reflect or occur alongside important cellular changes that establish this capacity. This capacity in the da sensory neurons is truly remarkable: if the axon is completely removed (leaving no axonal stump attached to the cell body) then a dendrite becomes restructured and transformed into an axon, demonstrating a strong homeostatic drive to have an axon. This transformation entails a re-organization of microtubules within the dendrite to form a new process with the plus-end out polarity appropriate and specific to axons (Figure 2). Manipulations that inhibit microtubule growth, including knock-down of the microtubule polymerase *msps*, prevent the dendrite-to-axon transformation, however knock-down of gamma-tubulin, which inhibits the formation of new microtubules, does not inhibit the transformation [17,18]. So the new microtubules may not be primary effectors of axon regeneration, but may instead reflect broad and global effects of an axonal injury response. In fact, current data favors the idea that the induced microtubule growth is a neuroprotective response: once the dynamics is induced, neurites that are damaged during subsequent injuries become resistant to the process of Wallerian degeneration [18,19]. Intriguingly, overexpression of expanded polyglutamine proteins (a form of proteotoxic stress) seems to engage the same protective response pathway, which involves microtubule nucleation via gamma-tubulin and is controlled by activation of Jun N-terminal kinase (JNK) signaling

[18]. After time (72 hours after axonal injury), the microtubule dynamics ultimately quiet down, and manipulations that prolong the response can actually inhibit axon regeneration [17,18,20]. This builds a model that major aspects of the axonal injury response, including the global changes in microtubule dynamics, may serve as an initial protective response but must ultimately subside before the neuron can initiate substantial new growth from its axon.

In addition to the global changes in microtubule structure induced by axonal injury, injury within any neurite (either axon or dendrite) also induces local changes in microtubule polarity [10,21]. This likely occurs via calcium-stimulated depolymerization of microtubules near the injury site, and treatments that block this polarity change in cultured neurons (eg., low concentrations of vinblastine applied immediately following axonal injury) completely inhibit axon regeneration [21]. These local changes allow for microtubules of opposing polarities to orient at the new 'tips' of the injured neurite. This creates a scenario for microtubule sliding, driven by the kinesin-1 motor protein, which can link to oppositely polarized microtubules and power them further apart [21]. The microtubule sliding ability of kinesin-1 plays an important role in promoting axonal outgrowth during development [22,23], and genetic knockdown of kinesin-1 leads to failed axon regeneration in *in vitro* assays [21].

Another important factor in axon regeneration is the microtubule severing protein Spastin: deletion of a single copy of Spastin can dominantly impair regeneration, and over-expression of Spastin also impairs regeneration, suggesting that the process is very sensitive to gene dose of Spastin [24]. Manipulations of Spastin have no obvious effects upon the global increase in microtubule dynamics induced by axotomy [24]. Instead, a recent study suggests an important role for Spastin in its ability to associate with the ER as well as microtubules. Spastin and Atlastin, another protein with similar ER-microtubule association roles, mediate an enhanced localization of ER and microtubules into the growing tips of regenerating axons ([25] and Figure 2). Since the calcium-releasing function of ER in the growth cone has been shown to play an important role in growth cone guidance and axon regeneration [26,27], the localization of ER to a regenerating axon tip could promote regeneration by providing intracellular calcium locally.

b) Axonal damage signaling via the DLK kinase

Work in multiple model organisms from *C. elegans* and *Drosophila*, followed by later studies in mice has identified the dual leucine zipper kinase (DLK, also known as Wallenda (Wnd) in *Drosophila*) as an essential mediator of a neuron's ability to initiate new axonal growth (reviewed by [28]). This kinase is not required for axonal outgrowth during development, but its function is essential for neurons to initiate new growth after injury. Upon axonal injury, Wnd/DLK mediates the retrograde transport of the 'injury signals' in axons. The JNK MAP Kinase and transcription factor Fos are required for Wnd/DLK mediated axonal regeneration, suggesting a transcriptional response is involved [29]. Moreover, studies in *C. elegans* found that the DLK pathway also regulates microtubule dynamics after injury via tubulin posttranscriptional modifications [30]. This is consistent with the findings in *Drosophila* that JNK and Fos mediate acute changes in microtubule dynamics after injury [17,20]. Therefore, Wnd/DLK appears to function as an upstream

regulator of multiple cellular responses to axonal damage. Interestingly, Wnd/DLK is actively transported in axons and becomes acutely activated upon axonal injury [29,31], hence may be triggered by local signals in damaged axons.

What determines DLK's activation after axonal damage? It is interesting that multiple studies in *Drosophila*, *C. elegans* and mammalian neurons have suggested that Wnd/DLK signaling is sensitive to manipulations that alter cytoskeletal structure in neurons, including mutations in spectraplakins, TCP1 α or TCP1 β , and treatment with nocodazole or cytochalasin D [32-37], hence axonal damage *per se* is not the only way to activate DLK. These observations suggest a central role for Wnd/DLK as both a 'sensor' and regulator of cytoskeletal structure in neurons. However, it is not yet clear how this kinase actually senses cytoskeletal changes on a mechanistic level. This is an interesting area of future work, particularly since several recent studies in mammalian neurons have implicated DLK in neuronal degeneration and death, including in models of glaucoma and excitotoxicity [38-41]. Therefore, while DLK activation is important for axonal regeneration, it may be of greater interest to inhibit (rather than promote) in order to minimize pathology in neurodegenerative diseases and injury.

Other clues to DLK's activation mechanism may come from the biochemical identification of the upstream activators that can stimulate the activation loop of the Wnd/DLK kinase. Recent work has discovered such an activity for the cAMP effector kinase protein kinase A (PKA), which can induce Wnd's activation and function, and is required for its activation in injured axons [42]. Genetic interaction studies have also led to the suggestion that Wnd may also be regulated by TORC1 [43]. It is striking that, as discussed previously, both cAMP and mTOR signaling are known for abilities to unlock axonal regeneration potential. Importantly, DLK is also similarly regulated by cAMP and PKA in mammalian as well as *Drosophila* neurons [42]. This suggests an interesting possibility that DLK activation could be a key feature for the unlocking mechanism, and a potentially universal mechanism for stimulating axonal regeneration.

c) Regulation of RNA-processing pathways

Use of *Drosophila* injury models for forward genetic screens allows for the discovery of new unanticipated regulators of axon regeneration. A recent example is the discovery of an RNA processing enzyme Rtca (RNA 3'-terminal phosphate cyclase), as a potent inhibitor of axon regeneration: mutations in *rtca* allow for sensory neuron axons to grow in the CNS, and overexpression of *rtca* causes an inhibition to axon regeneration in the PNS [44]. Following on this discovery, Song *et al* then demonstrated that the mammalian ortholog of Rtca functions similarly as an inhibitor of axonal regeneration in adult DRG neurons in culture and in the adult optic nerve *in vivo*. Known substrates of Rtca include mediators in the tRNA splicing pathway, and the ER-stress response factor X-box protein 1 (Xbp-1), whose non-canonical intron shares features and regulation with the tRNA splicing pathway [45]. Song *et al* suggested that Xbp-1 may be the target of Rtca during axonal regeneration since the enhanced regeneration observed in *rtca* mutants was largely abolished in *Xbp-1*, *rtca* double mutant flies. Such a role for Xbp-1 in axon regeneration would be consistent with other recent studies that have noted roles for ER stress pathways in regeneration [46,47]. However,

the genetic data alone cannot exclude other potential (and yet unknown) targets of RtcA regulation. Interestingly, a recent study in *C. elegans* identified the tRNA ligase RtcB as an inhibitor of axon regeneration, but ruled out both Xbp-1 and tRNAs as functional targets for RtcB in this function [48]. These findings suggest that much remains to be learned about the 'RNA dimension' of responses to axonal injury: we may have glimpsed only the tip of an iceberg that remains to be charted.

How are responses to dendritic injury different from axonal injury?

Many neuronal injuries entail loss of dendrites as well as axons. The highly characterized anatomy of *Drosophila* da neurons has turned into an exciting model system for comparing and contrasting the differences between dendritic and axonal injury. After laser-induced removal, da neurons show a robust ability to regrow their dendritic arbors [10,49,50], even when carried out in adult flies [50]. While for some classes the regenerated dendrites cannot cover the entire lost territory and the architecture is not completely restored, the regenerated dendrites show a remarkable ability to re-grow the stereotyped pattern of primary branches according to their class type [49,50]. Interestingly, nearly all of the aspects of axon regeneration described above are not required for dendrite regeneration: dendrite regeneration does not require DLK or Spastin [24,50], and occurs independently of global microtubule rearrangements and ER relocation [17,25]. Dendrite regeneration and axon regeneration are therefore fundamentally different processes. This does not rule out the possibility of shared molecular pathways. For instance, AKT-PTEN signaling, which is classically associated with cellular growth, is required for both axon and dendrite regeneration [10]. Since mixed polarity microtubules form at the tips of injured dendrites [10], kinesin-driven microtubule sliding is another candidate mediator of renewed dendrite growth, particularly since microtubules also have mixed (opposing) polarity in dendrites during their original developmental outgrowth [16]. However, the molecular distinctions between mechanisms that promote dendrite growth versus axonal growth after injury are thus far poorly understood [51].

Comparison of dendritic injury with axonal injury has also revealed some differences in degeneration, defined here as the process by which the injured piece that is separated from the cell body becomes dismantled. This process appears to involve the initiation of a cellular pathway within the injured neurite that leads to specific destruction and degeneration of the axonal or dendritic pieces that are no longer connected to the cell body. Studies of axon degeneration in *Drosophila* have identified many key players in this process (reviewed by [4] [55]). Some of these players influence the degeneration of both axons and dendrites [52]. However, studies of dendrite degeneration in *Drosophila* suggest the existence of aspects that are distinct from axons, such as specific requirement for the microtubule-severing protein Fidgetin in dendrite but not axon degeneration [53]. Live imaging data suggest that Fidgetin promotes an increase in microtubule number, presumably via its severing activity, followed by microtubule disassembly in distal dendrites. This response contrasts with axonal injury as described above, which promotes a global increase throughout the cell body (including all dendrites and the proximal stump), but not in distal axon stump [17]. These differences further emphasize that axons and dendrites respond distinctly to injuries and distinct mechanisms are involved.

Can functional reconnection occur after axon injury in *Drosophila*?

Initiation of new axonal growth is only the first step towards repairing lost connections. True repair requires that regenerating axons find their targets and form functional synapses. In general this process of ‘synapse regeneration’ is poorly studied in the field. To date, it has not been noted in any of the *Drosophila* models of injury, however the *Drosophila* system may present future opportunities to study this process. Earlier studies in other invertebrate, including cockroaches, leech, *Aplysia*, snails, crayfishes and crickets, suggest functional regeneration can indeed happen (reviewed by [55]). By following the process in a system that is tractable to cell-specific genetic manipulations, live imaging and behavioral screening, one could determine whether synapse regeneration follows the same pathways as synapse development, whether axon regeneration pathways need to be turned off to initiate synapse formation, and what role remaining synapses within the injured circuit play in the process. We predict that exciting work lies ahead in this powerful model organism.

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Highlights

- Powerful genetics, a simplified nervous system, and amenability to live imaging make *Drosophila* an ideal model system to study mechanisms of axon and dendrite regeneration;
- Molecular pathways that gate the intrinsic ability to regenerate injured axons are highly conserved between *Drosophila* and mammalian neurons;
- New axon regeneration regulators, including RNA processing enzyme Rtca, have recently been identified via genetic screens in *Drosophila*;
- Recent studies in *Drosophila* demonstrate that dendrites and axons respond to injury and regenerate via distinct mechanisms.

Figure 1. Axons regenerate to varying extents in different *Drosophila* axon injury models

New axonal growth after injury, cartooned in pink, occurs to varying degrees after injuries in the adult and larval PNS. Some of the sensory neurons that line the larval body wall initiate remarkable regeneration along the original path of the lost axon [10]. Other injury models in the adult wing and larval peripheral nerves note extensive new axonal sprouting [29,54]. This undirected growth ('sprouting') may reflect an absence of salient cues to guide directed growth for the regenerating axon. In some cases, sensory neurons in the adult wing can initiate extensive directed growth, however this occurs along a new path that is distinct ('misrouted') from the original path [54]. This may be a side effect of massive tissue damage and scar formation at the injury site that prevents the axon from finding its correct path. In contrast to the PNS injuries, the two studies thus far that have injured axons in the CNS have noted very poor growth responses [10,11]. The contrast is particularly interesting for the Class IV da sensory neuron axons, since the axons grow robustly after injury in a PNS location but very poorly after injury in a CNS location [10,44].

Figure 2. Injury triggered microtubule dynamics in neurons

Microtubules are organized in axons and dendrites with distinct orientations of their growing (plus) ends: in axons microtubules orient with plus ends facing away from the cell body (plus-end-out), colored green, while in dendrites microtubules orient with minus-ends-out, colored red. Axonal injury, but not dendritic injury, triggers a global increase in the number of growing microtubules. In contrast, increased microtubule is observed in distal dendrite stump, but not distal axon stump. Mixed-polarity microtubules are observed in both proximal axon stump and dendrite stump. ER and microtubules are accumulated in growing axon tips, but not in growing dendrite tips, 96 hours after axotomy (for class I da neuron). In the case of complete axonal removal, one dendrite can switch microtubule polarity and become a growing axon.