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Abstract

Neurite degeneration is a hallmark feature of nearly all neurodegenerative diseases, occurs after most brain trauma, and is thought to be the underlying cause of functional loss in patients. Understanding the genetic basis of neurite degeneration represents a major challenge in the neuroscience field. If it is possible to define key signaling pathways that promote neurite destruction, their blockade represents an exciting new potential therapeutic approach to suppressing neurological loss in patients. This review highlights recently developed models that can be used to study fundamental aspects of neuronal injury using the fruit fly *Drosophila*. The speed, precision, and powerful molecular-genetic tools available in the fruit fly make for an attractive system in which to dissect neuronal signaling after injury. Their use has led to the identification of some of the first molecules whose endogenous function includes promoting axonal degeneration after axotomy, and these signaling pathways appear functionally well conserved in mammals.

Key Words: axon; dSarm; Hiw; Phr1; Sarm1; Wallerian degeneration; Wld^s

Introduction

Neuronal injury, whether by toxic insult, genetic disease, or trauma, can lead to neurite degeneration, synapse loss, or even neuronal death. Neurite degeneration, which functionally disconnects the neuron from its circuit, is widely believed to underlie much of the functional loss in patients suffering from nervous system injuries or neurodegenerative disease. The molecular mechanisms controlling the degeneration of axons, dendrites, or synapses remain poorly defined, despite their pervasive and central roles in acquired neurological deficit. That apoptotic cell death is a genetically programmed event is now widely appreciated, and the molecular cascades leading to cell body death are well defined in many contexts. The notion that neurites might also drive their own destruction by a neurite-specific death program was an intriguing concept, but it lacked molecular support. A revolution in our thinking regarding neurite biology came from the discovery of the slow Wallerian degeneration (Wld^s) mutant mouse, in which the distal fragments of severed axons survived for weeks after injury (rather than hours) in the absence of support from the cell body. Wld^s taught us that neurites can indeed survive (and function) for long periods of time autonomously and inspired a deeper investigation of what happens to severed neurites as they degenerate.

How does Wld^s block axon death? More fundamentally, is there in fact an active program of auto-destruction in injured neurites that drives their destruction? This is a critically important question to answer because blockade of neurite degeneration signaling events has a high potential for suppressing neurological loss in a broad spectrum of patients. In the past decade, *Drosophila* has emerged as an extremely useful system in which to explore fundamental aspects of neuronal biology after neural injury (Fang and Bonini 2012). This review is meant to briefly describe the utility of specific preparations that can be used in the fly to explore injury-induced degeneration and functional loss of axons, dendrites, and synapses. We also highlight how some of these models have led to a much deeper understanding of changes in neurite biology after injury. For example, how does Wld^s protect axons? Finally, we describe how forward genetic approaches in *Drosophila* led to the identification of the first signaling pathways that, when blocked, potently suppress axon degeneration and that are conserved in mammals.

Olfactory Receptor Neuron Axotomy Assay

The olfactory receptor neuron (ORN) model was the first system developed to study axotomy in *Drosophila* (MacDonald et al. 2006). ORNs are the chemosensory neurons found in the adult third antennal segment or maxillary palps, which send axonal projections through the antennal and maxillary nerves, respectively, to synapse in the antennal lobe of the fly brain. Axotomy is induced by surgical removal of third antennal segments or maxillary palps, which ablates ORN cell bodies and fully transects the antennal or

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maxillary nerves in the process. The remaining distal axon segments and synapses in the central nervous system (CNS) undergo stereotyped degeneration over the course of approximately 6 to 12 hours. The antenna and maxillary palp are nonessential tissues, so their surgical ablation is nonlethal, and based on its morphology (i.e., all ORNs studied are in the antenna or maxillary palp), surgical removal of the entire tissue leaves very little room for error—in nearly all cases 100% of ORN axons can be considered severed. Previous work to map the projections of fly ORN into the antennal lobe has provided a wealth of tools useful for the investigation of neuronal injury in specific subsets of neurons in the adult fly. These include different Gal4 drivers based on odorant receptor (OR) gene promoters that label highly reproducible subsets of ORNs. Some OR-Gal4 drivers label small numbers of neurons that fan out within the antennal lobe, whereas others label tight bundles containing many ORN axons. Thus, one has the ability to visualize degenerative events or axon biology in either single axons or whole ORN populations, depending upon the particular OR-Gal4 driver used.

ORNs in the olfactory system are highly amenable to sophisticated genetic mosaic analysis. This has the distinct advantage of allowing one to make genetic mosaic animals in which genes are knocked out in small clones of homozygous mutant ORNs. Forward genetic screens using such approaches will be essential to define the molecular pathways that promote Wallerian degeneration, and this is especially true if key Wallerian degeneration genes are otherwise organismal lethal. Indeed, forward genetic screening in mosaic animals has led to the identification of dSarm. A number of efficient tools are available for clone induction. For instance, one can induce clones using flippase expression driven by an *hsp* promoter, which allows for temporal control of induction, or by using the *eyeless-flp* genetic tool, which leads to the production of clones in the antenna beginning at early stages of antennal development. When combined with the mosaic analysis with a repressible cell marker technique, one can even specifically label homozygous loss-of-function mutant clones with markers such as green fluorescent protein (GFP).

To visualize distal axon segments, fly heads must be removed and fixed, and the fly brain must be dissected from the head. This process, although not particularly difficult, can be time consuming and is the limiting factor if large-scale screens are being considered. Another limitation is that the cell bodies and dendrites are removed during the injury process, so the effects of different manipulations on these cellular compartments cannot be evaluated. The same is not true of the larval chemotherapy or nerve crush models.

One major advantage of the ORN model is that the injured flies can be aged to normal lifespans, meaning several weeks, because antennal or maxillary palp ablation is nonlethal. Aging flies is particularly useful when working with Wallerian degeneration mutants, such as WLD^s, as was shown by initial studies that characterized this system. Notably, WLD^s was found to protect axonal and synaptic integrity for up to 50 days (MacDonald et al. 2006) after axotomy, and key differences between molecular variants of WLD^s were only clearly discernible by comparing axon degeneration at different time points over weeks after axotomy. Another advantage with using the ORN model is that the dissected brains can be antibody stained. This allows the visualization of not only axonal and synaptic components but also the glial responses to nerve injury. Importantly, the initial characterization studies by MacDonald and colleagues (2006) showed that both the neuronal and glial responses to axotomy in flies are comparable with their respective mammalian equivalents: axons underwent Wld^s-modulated degeneration, and glial cells rapidly became reactive and engulfed degenerating axons and synapses. These observations indicated that Wallerian degeneration is a conserved molecular event in even distantly related species.

The ORN model was used to dissect molecular pathways governing glial responses to axonal injury. MacDonald and colleagues (2006) discovered the cell corpse engulfment receptor Draper was a key mediator of glial activation after injury and engulfment of neuronal debris. Ziegenfuss and colleagues (2008) then showed that Shark/Syc and Src42A are required for signaling events downstream of Draper, which argued that Draper signaling was molecularly similar to mammalian immune signaling during engulfment events. Subsequent studies identified the specific subtypes of fly glia that engulfed axonal debris (Doherty et al. 2009), the molecular complex required for activation of phagolysosomal activity and internalization of axonal debris (Ziegenfuss et al. 2012), and the mechanism by which Draper also acts to terminate glial responses to injury (Logan et al. 2012).

At the same time, a number of studies explored the molecular basis of axon degeneration. Structure–function analysis of Wld^s in fly ORNs helped clarify precisely which domains of Wld^s were required for axonal protection (Avery et al. 2009), and these were remarkably consistent with similar analyses in mammals. More recently Osterloh and colleagues (2012) carried out a large-scale forward genetic screen using mosaic analysis with a repressible cell marker in the ORNs to identify recessive mutations that block axon degeneration after axotomy. The gene identified, dSarm/Sarm1, a Ca^{2+}-sensitive kinase scaffolding molecule, was shown to be essential for axon degeneration in vivo in both *Drosophila* and mouse. This key study identified the first so-called axon death gene, which, when knocked out, prevented severed axons from degenerating with a potency similar to that of Wld^s.

Larval Nerve Crush

The larval nerve model was established to investigate injury responses in neuronal cell bodies residing in the larval ventral nerve cord, distal axonal and synaptic degeneration, and axon regeneration in the surviving proximal axon stumps (Xiong et al. 2010). The segmental nerves in *Drosophila* larvae carry motor and sensory axons between the CNS ventral nerve cord (the equivalent of the spinal cord) and each

segment of the larval body wall. Fluorescently labeled nerves can be easily visualized through the transparent larval body wall cuticle. To injure these nerves, larvae are anesthetized with carbon dioxide, and forceps are used to crush the nerves through the ventral cuticle posterior to the ventral nerve cord. Many, if not all, of the axons in the segmental nerves are injured depending upon the site of crush. The crush typically results in paralysis of the segments distal to the injury but is usually nonlethal because the larvae are still able to crawl and feed. After injury, one can examine axon degeneration for as many as 2 to 3 days after axotomy, although this is a much shorter period of time compared with experiments in the adult. *Drosophila* motor neurons, which are generally severed during crush injury, have a beautifully defined neuromuscular junction, for which there is a plethora of molecular markers, and one can even record electrophysiologic output of severed motor neurons over time by recording from the larval body wall muscles.

Crush injures nearly all of the axons (both sensory and motor) and nearby glia within the nerve. As in mammals, this approach results in some level of unpredictability in the precise number of neurons severed in each experiment, which can lead to variability in levels of axon degeneration or regeneration. However, alterations in the number of severed axons can be used to examine nerve responses at different degrees of injury. It is important to note this approach also causes collateral damage to the larvae, including perturbation of muscle and epidermis; however, one might consider this to be more like a true nerve injury experienced by a patient. One important biological limitation of the larval model is that larvae are still developing and continuously grow before entering the pupal stage—how different a developing neuron will be in its responses to injury compared with a terminally differentiated neuron remains an open question. The responses could be quite different, although based on work done thus far, larval motor neurons appear to respond quite similarly to adult ORN axons.

A major advantage with using larvae versus adult animals is that mutations in many genes are lethal during pupal or adult stages but are compatible with larval life and can be studied in the larval nerve injury system. Another advantage is that the segmental nerves can be visualized through the cuticle in an intact larva. Xiong and Collins (2012) took advantage of the ability to see the nerves in the intact larvae to show that a preconditioning lesion can delay axon degeneration caused by a second crush injury in the proximal stump by 16 hours. Because larvae are easily dissected, the brains and nerves can be stained with antibodies, and the cell bodies and dendrites within the CNS ventral nerve cord can also be visualized. Using a transcriptional reporter, Xiong and colleagues (2010) showed that the JNK pathway was activated in motor neuron nuclei after crush injury. Activation of JNK was found to require the kinase Wallenda but was inhibited by the E3 ubiquitin ligase Highwire, which negatively regulates Wallenda. Accordingly, injury was found to increase Wallenda levels and decrease Highwire levels in the injured neurons.

This same group later showed that mutations in *highwire* strongly inhibit axon degeneration at 24 and 48 hours in larval sensory and motor neurons and up to 20 days in olfactory receptor neurons (Xiong et al. 2012). Larval neuromuscular junctions were also preserved and shown to be largely functional 2 days later. However, in light of these findings, it remains unclear why Highwire levels would decrease after injury in the context of normal axon degeneration. Perhaps there are differences in Highwire signaling in proximal versus distal segments of severed axons. Studies distinguishing the difference between proximal and distal axon stumps may clarify this discrepancy. Nevertheless, this work in *Drosophila* inspired a second group to examine the role of mammalian Highwire (Phr1), which was also shown to be strongly protective of severed mouse axons (Babetto et al. 2013).

Chemotoxicity in the Larva

Cancer chemotherapeutics, such as platinum drugs and taxol, cause treatment-limiting neuropathy. Axons and dendrites of sensory neurons are injured by the drugs, which leads to neuronal dysfunction and degeneration of these structures. *Drosophila* larvae have also been used in a chemotoxicity model of neural injury (Bhattacharya et al. 2012). In this model, larvae are placed on food that contains a drug such as cisplatin or taxol. As the larvae live in and crawl through the food, the drug is absorbed and/or ingested, effectively treating the whole animal. It is assumed that dosage is determined by rates of ingestion or absorption, but this is challenging to measure. As such, dosages of any drug to be used to study neuropathology are determined empirically. Like other systems using *Drosophila* larvae, advantages include speed and simplicity, especially because there is no surgical manipulation of the animals. However, an important consideration that limits this model is timing; larvae are continuously growing and only remain larvae for approximately 4 days before pupating. Thus, only acute effects of the drugs can be assayed; longer term effects might be examined more readily in the adult. Additionally, some drugs may not be readily absorbed by the larvae.

As with the larval nerve injury approach, larval chemotoxicity assays can be used to study responses of cell bodies, dendrites, and axons using the wealth of markers for *Drosophila* neurons and neuromuscular junctions. This may be especially important when using chemotherapeutic drugs because both the axons and dendrites degenerate. Interestingly, Bhattacharya and colleagues (2012) showed that, as in mammals, *Drosophila* larval sensory neurons are selectively sensitive to taxol, whereas motor neurons are largely unaffected. They also found that expression of the axon and dendrite protective NMNAT can effectively suppress the degeneration caused by the chemotherapeutic drug, consistent with previous findings in mammalian dorsal root ganglion sensory neurons. Further, they showed that loss-of-function mutations in *wallenda* are able to suppress the loss of sensory axons after treatment with taxol, as does knock-down of *retinophilin*, identified through

an RNAi -based screen. These studies show the utility in using larvae to screen in a treatment-relevant setting, which will likely be exploited in the future to understand the cellular basis of the effects of a number of drugs on the nervous system. Bhattacharya et al. (2012) also identified potential drug targets towards preventing the debilitating neuropathy seen in patients taking chemotherapeutic drugs.

Laser Axotomy in Peripheral Nerves or Sensory Neurons

The ability to image neuronal axons and dendrites in *Drosophila* larvae, coupled with the relative transparency of the larval cuticle, allows for live imaging of neuronal processes as well as photomanipulation of the neurons. Live imaging with markers for key cellular compartments, organelles, single molecules, or cellular indicators (e.g., GCaMP), combined with photoactivation or fluorescence recovery after photobleaching (FRAP) experimentation, opens the door to examining almost any aspect of cell biology during and after neurite injury. Very fine processes, or even single axonal or dendritic branches, can be severed with ultraviolet-pulsed lasers, similar to mechanical transection, but with great precision and significantly reduced collateral damage. This targeted injury allows for very specific ablations of axons and dendrites and for noninjured control neurites nearby or even within the same neuron.

Although laser ablation of neuronal processes can be very precise, this precision decreases with increasing depth of the target. Fortunately, the larval nervous system is located ventrally, and even some neurons in the brain are accessible for ablation. If necessary, one can perform an open filet preparation of the larva, whereby the entire nervous system is made accessible surgically, but survival in this preparation is only robust for upwards of 6 hours. A limitation of laser axotomy in the intact animals is that the larvae must be held motionless while the ablation is being performed. This is achieved mechanically by mounting the larvae between coverslips or by incapacitating the larvae with anesthetic. As with all of the larval models, the timeframe with which to use the system is limited due to the growth and development of the larvae into pupal stages—within 2 to 3 days most larvae will pupate. Therefore, only relatively acute processes can be examined with this model.

The primary advantage of this approach is the ability to look at acute changes in cell biology after neuronal injury. Avery and colleagues (2012) used laser ablation of larval axons to investigate the nature of injury-induced calcium bursts within severed axons, as well as mitochondrial motility after injury. They found that severed larval axons exhibited a dramatic increase in axonal calcium levels after axotomy and that mitochondrial motility was dramatically impeded. Remarkably, both laser-induced calcium spikes and blockade of mitochondrial motility were suppressed in severed axons if they expressed the neuroprotective Wld^s molecule. These findings argue that Wld^s can act very early after an injury event and were the first cell-biological roles ascribed to WLD^s beyond it ability to suppress fragmentation. It has been proposed based on this work that Wld^s localizes to mitochondria and, by enhancing their buffering capacity, blocks the induction of an axon degeneration-inducing Ca^{2+} spike.

Dendrites can also be laser ablated, and work by Tao and Rolls (2011) has shown that dendrites, like axons, degenerate after being severed and that WLD^s can prevent this degeneration. They further showed that genes required for developmental pruning of dendrites (which exhibits fragmentation that looks very much like that occurring after neurites are severed) are not required for degeneration after injury. These findings suggest that dendrites and axons share an injury-induced degeneration pathway that is distinct from developmental pruning. Interestingly, Chen and colleagues (2012) found that dendrites could also be protected from injury-induced degeneration by a previous axonal injury in the same neuron. This is reminiscent of the delay in degeneration observed after a second crush injury to axons (Xiong and Collins 2012) and suggests that the protection afforded by a conditioning lesion may be through the same mechanism in both axons and dendrites.

Axotomy or Crush in the Adult Wing

The fruit fly wing provides an additional adult system to study neural injury. In the wing margin, hundreds of sensory neurons reside in the major anterior wing vein (the L1 vein), all of which project axons toward the CNS into the thoracic ganglion. They function to provide tactile and chemosensory information to the fly. At approximately 1.5 mm, axons of the most distal sensory neurons represent the longest axons in the fly. To injure L1 wing vein axons, the wing can be easily cut with scissors, which removes the cell bodies of the neurons distal to the cut site, similar to antennal ablations. The distal axon stumps, which remain on the intact part of the wing, can then be imaged in the remaining portion of the wing still attached to the fly. Because the sensory neurons are spread evenly throughout the wing margin, the location of the cut will determine precisely how many axons in the bundle are injured: the more distal toward the tip of the wing one cuts, the fewer axons will be transected. Because the wing is largely made from a waxy cuticle, it is not possible to use antibody stains. Therefore, any labeling of the neurons or glia in the vein must be by expression of markers in the cells of interest. The cell bodies and dendrites are also removed during the ablation, so it is not possible to investigate these structures after injury.

Major advantages to using the wing model are the ease and speed of the nonlethal injury and that no dissection is required for visualization of the neural components in the wing. To visualize the nerve, the wing is simply removed and put on a slide. Because dissection is not necessary, many wings can be processed in a short amount of time, and this rapidity lends itself to large-scale screens. Additionally, live imaging of the intact wing that is still attached to the fly can be performed. Fang and colleagues (2012) live-imaged GFP-labeled mitochondria in wings of flies embedded in agarose. They showed

that depletion of dNMNAT or Milton by RNAi in the wing neurons causes a progressive degeneration of the axons. Interestingly, expression of WLD^S or mammalian NMNAT could rescue the degeneration caused by RNAi targeting dNMNAT, but not Milton, consistent with previous results in mammals. Milton is required for anterograde transport of mitochondria in axons, and a depletion of axonal mitochondria was observed in the wing after injury, except in axons expressing WLD^S/NMNAT. The authors proposed that WLD^S/NMNAT functions to preserve axonal mitochondria and thereby delay degeneration, although it remains unclear whether the loss of mitochondria is a cause or consequence of axon degeneration or whether axon preservation by WLD^S/NMNAT might indirectly lead to preservation of mitochondria.

Conclusions

A number of excellent neural injury models have been developed in *Drosophila* that are proving extremely useful in defining the genetic and molecular bases of neurite degeneration. Key features include the speed of experimental approaches, the precision with which one can visualize degeneration (e.g., single axon, dendrite, or synaptic resolution), the ability to exploit powerful molecular-genetic tools, the opportunity to perform forward genetic screens, and the fact that all work is performed in vivo. To date, there has been a remarkable conservation in these processes at the molecular level, as demonstrated by *Wld^S*, *dSarm/Sarm1*, and *hiw/Phr1*. We propose that the detailed delineation of neurite death signaling will benefit significantly from model genetic organisms such as *Drosophila* in a way that parallels the central role invertebrate model organisms played in unraveling the genetic basis of apoptotic cell death.

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