● **PERSPECTIVE**

Hope on the (fruit) fly: the *Drosophila* wing paradigm of axon injury

Axon degeneration and regeneration are processes that are central to neural insults including spinal cord injury, brain trauma, ischemia, infection, inflammation, neurodegenerative diseases, and aging (Conforti et al., 2014). Injured axons undergo progressive self-destruction, termed "Wallerian degeneration", which is named after August Volney Waller who was the first to describe degeneration of severed nerves in 1850 – the injured nerve fibers become fragmented like threaded beads and are gradually cleared away.

Since Dr. Waller first severed the glossopharyngeal and hypoglossal nerves of his favorite animal model the "frog" more than 160 years ago, neuroscientists have been trying to model axon injury in many different organisms, such as rodents, fish, nematodes and fruit flies, using various means to damage nerves, such as severing, crushing, and laser ablation. Studies in mammals and other vertebrate models have laid the groundwork of characterizing the pathology and cellular responses to neural injury. More recently, experimental models in invertebrates have provided novel insights and identified new pathways that regulate axon degeneration and regeneration. For comprehensive reviews on invertebrate models of axon injury, readers are referred to Bejjani and Hammarlund (2012) and Fang and Bonini (2012). Here, we highlight a newly developed *in vivo* model of axon injury of the adult *Drosophila* wing (Fang et al., 2012, 2013; Neukomm et al., 2014; Soares et al., 2014).

The seemingly thin and simple structure of the *Drosophila* wing contains exquisitely precise neural components. As illustrated in **Figure 1**, there are 225–250 neurons in the L1 and coastal veins along the anterior wing margin and in the L3 vein. These chemosensory and mechanosensory neurons dispatch dendrites into the bristles to sense environmental cues, such as flavors of a banana and pressure changes of the airflow when the animals move. Each wing neuron extends a long axonal projection; these merge to form a thick nerve track (**Figure 1B**) and the wing nerve connects to the central nervous system (CNS) by projecting into the thoracic ganglion (the "spinal cord" of the fly, **Figure 1A**). Similar to the mammalian nervous system, the fly wing nerve is wrapped by glia and surrounded in circulating hemolymph (the "blood" of the animal) that perform the phagocytic and immune functions. Thanks to the vast genetic approaches in *Drosophila*, individual components of the wing nerve can be independently labeled and manipulated. Moreover, the semitransparent nature of the *Drosophila* wing allows an unmatchable optical advantage to visualize fluorescent protein-highlighted neurons in live flies. Thus, the fly wing paradigm provides a unique and powerful *in vivo* system that allows identification of not only intrinsic neuronal factors regulating axon degeneration and regeneration, but also mechanisms involved in the interactions between axons and other cell types in response to neural insults.

The initial wing paradigm was developed with a pair of handheld Vannas spring scissors to cut off the wing at the point where the L1 vein meets with the costal vein (Fang et al., 2012, 2013). This is where the axon fibers of the last few L1 neurons join the wing nerve bundle. As such, a cut at this site transects the entire L1 nerve, leaving the costal nerve intentionally intact so it can be used as an internal control to distinguish injury-induced degeneration from axon deterioration caused by other factors, such as aging. Other injury sites have been since developed, as in Neukomm et al. (2014). Axotomy at the L1-costal junction site, however, unifies the number of axons transected, minimizes variations in the extent of damage, simplifies the assessment of the subsequent degeneration severity, as well as facilitates the comparison between different samples and across different groups. In the most recent study using the wing paradigm, this axotomy site has been adapted to laser ablation, in order to study the potential of axons within the wing to undergo regeneration following severing (Soares et al., 2014).

Upon injury, the severed wing axons exhibit key features of Wallerian degeneration including progressive axonal fragmentation. This is seen as early as 1 day post injury, with the axonal fluorescent signals largely undetectable by 7 days post injury (Fang et al., 2012; Neukomm et al., 2014). Such neural injury induces a robust glial response (Fang et al., 2013), and the glial gene *draper* is required for the clearance of axonal debris in the injured wing (Neukomm et al., 2014). In addition, laser axotomy of the wing nerve stimulates rapid hemocyte migration toward the injury site, with a visible scar forming near the injury site in the L1 vein (Soares et al., 2014).

In the initial study carried out using the wing paradigm, we demonstrated that the endogenous *nicotinamide mononucleotide adenylyltransferase* (*Nmnat*) gene is required for maintaining axonal integrity. Expression of the chimeric protein Wallerian Degeneration Slow (Wld^S) that contains the *Nmnat* coding sequence or upregulation of *Nmnat* can delay degeneration of injured axons, which has been known for decades and repeated in different experimental models (Conforti et al., 2014). Although it was hypothesized that the endogenous *Nmnat* gene might play a role in axonal maintenance, *in vivo* evidence was absent because loss of *Nmnat* function has a profound impact on cell and animal viability due to its function in NAD$^+$ biosynthesis. Using the *Drosophila* wing model, we expressed RNAi-Nmnat selectively in the wing nerve to downregulate the endogenous *Nmnat* level. This caused remarkable spontaneous axon degeneration that mimicked fragmentation induced by cutting the nerve, coupled with rapid depletion of axonal mitochondria (Fang et al., 2012). The axonal degeneration occurred earlier than any noticeable morphological changes in the neuronal soma, with a retrograde directionality reminiscent of
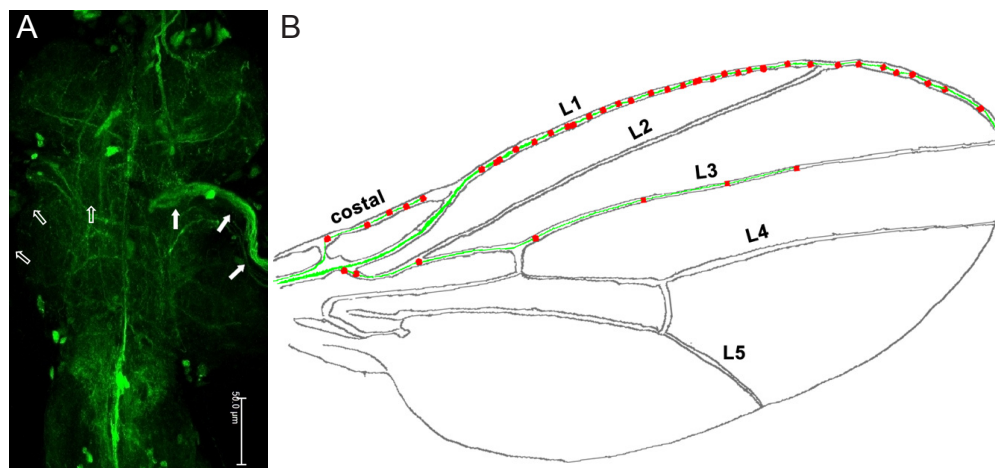
Figure 1 The adult *Drosophila* wing nerve and its CNS projections.
(A) A dissected thoracic ganglion showing the wing nerve projection labeled by mCD8-GFP, using the dpr-GAL4 driver which highlights a subset of the sensory neurons (Fang et al., 2012, 2013). Only one wing projection is present in the image (solid arrows); the projection of the other wing is absent (open arrows) due to injury-induced axon degeneration. Scale bar: 50 μm.
(B) An illustration of the peripheral nervous system in the *Drosophila* wing. The nerve projections (schematically illustrated in green) can be directly visualized when highlighted with fluorescent protein markers such as GFP and RFP. The wing nerve is comprised of the ~225 axons of the sensory neurons (positions of the neural cells bodies schematically illustrated in red) in the L1 vein, the coastal vein and the L3 vein.
CNS: central nervous system; GFP: Green fluorescent protein; RFP: red fluorescent protein.

dying-back diseases, suggesting that healthy axons require the function of the endogenous *Nmnat* gene. Indeed, shortly after our publication, four independent research groups reported that mutations in the human *NMNAT1* gene cause Leber's congenital amaurosis, an inherited eye disease of optic atrophy (Chiang et al., 2012; Falk et al., 2012; Koenekoop et al., 2012; Perrault et al., 2012). Thus, this study exemplifies the use and potential of the *Drosophila* wing paradigm to cast mechanistic insight into the study of axon degeneration.

The wing paradigm was designed and developed to meet the need for large-scale screening. It was proven successful in an initial P-element insertion screen (Fang et al., 2012), as well as in a recent ethyl methane sulphonate (EMS) mutation screen (Neukomm et al., 2014). The latter study adapted the mosaic analysis with a repressible cell marker (MARCM) approach to the wing paradigm and reached single axon resolution, which improved the scoring of degeneration of individual axons. The authors identified several mutant alleles of the *highwire* (*hiw*) gene, which delays degeneration of injured wing axons (Neukomm et al., 2014). *hiw* is a ubiquitin ligase that was known to regulate Nmnat protein degradation and promote axon degeneration (Xiong et al., 2012).

While the distal segment of the injured wing nerve degenerates, the proximal axons also respond to the insult. However, the regenerative process could not be assessed in the injury setup of the wing paradigm using the scissors cut: although the simple wing cut allows for rapid genetic screening, it removes the proximal neuronal cell bodies in the L1 vein (which were cut off with the wing blade), making it not feasible to examine the potential for regeneration. In the most recent development, Soares et al. (2014) used a pulsed laser to precisely sever the wing axons at the same site – after the last neural cell body

along the L1 nerve – while keeping the wing attached to the fly. In this way, the neural cell bodies are maintained. Upon injury, a rapid impact to the axonal cytoskeleton was observed by as early as 30 minutes, which propagated all the way back along the wing nerve to the neuronal cell bodies (Soares et al., 2014).

Notably, the *Drosophila* wing nerve showed a potent regenerative capacity. By 7 days post injury, about 50% of the injured animals show axon regrowth, some of which project close to the injury site. However, the scar that forms at the injury site seems to put a stop to the re-growing axons. Interestingly, some of the regenerated axons detour – avoiding the injury area and scar and misrouting into the nearby costal vein (Soares et al., 2014). This suggests that the scar in the injured wing nerve may be not only a physical barrier, but also secrete chemical cues that repel re-growing axons. In contrast, the uninjured costal vein appears to provide a permissive environment for the regenerating axons. How the re-growing L1 axons find this path and enter the coastal vein is an intriguing puzzle; further analyses of the hemolymph components in the vein before and after the formation of the injury scar may provide hints.

By directly testing for functional pathways that alter the regenerative response, downregulation of JNK signaling in injured neurons allows the regrowing wing nerve to project through the original injury site (Soares et al., 2014). Interestingly, the scar is also reduced, although features of the barrier (ability of dextran to flow through the region) remain unchanged; thus, downregulation of JNK signaling in the neurons appears to promote the ability of the axons to project through the injury site despite what may be an "injury" environment. These findings indicate that the adult fly nervous system has the capacity for regeneration. As JNK signaling pathway has been reported

to play an important role in regulating axon degeneration and regeneration in other animal models of axon injury (Miller et al., 2009), our work serves as a proof of principle that the wing paradigm can be applied to uncover evolutionarily conserved molecular mechanisms regulating axon regrowth (Soares et al., 2014).

Modeling axon injury in genetically amenable organisms is key for advancing understanding of the complex molecular organization and signaling pathways regulating axon survival and regeneration. *Drosophila* is a marvelous model organism that has been proven successful in elucidating genetic and molecular mechanisms that are evolutionarily conserved with humans. We initiated the approach of modeling axon injury in the adult *Drosophila* with the wing nervous system because of its accessibility for experimental manipulation, the minimal preparation for axotomy, the dispensability for animal survival, and the capability for visualization of injury responses directly in live flies (Fang et al., 2012, 2013). The exceptional suitability of the *Drosophila* wing paradigm for unbiased genetic screening is being continuously proven (Fang et al., 2012; Neukomm et al., 2014), while the latest development to combine laser ablation brings the paradigm to the research frontier of axon regeneration (Soares et al., 2014). The unique optical advantages of the *Drosophila* wing allow additional approaches as well, such as *in vivo* imaging of axonal transport and cytoskeletal dynamics in live animals (Fang et al., 2012; Soares et al., 2014); changes in these processes are frequently associated with neurodegenerative diseases. Moreover, although classically used to reveal unsuspected and novel pathways by genetic approaches, the fly may also prove amenable to discover small compounds with neuroprotective activity, some of which may impact pathways revealed by screens (Wang et al., 2014; Fang et al., 2012). With additional genome-wide, large-scale screens and continuous efforts to improve and broaden the use of the fly, the *Drosophila* wing paradigm holds the hope to systematically define the complex networks of genes and factors regulating axonal integrity, as well as reveal novel drug targets for treating neural injury and axon diseases.

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