The Pennsylvania State University

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INVESTIGATING THE RELATIONSHIP BETWEEN THE ORGANIZATION OF THE MICROTUBULE CYTOSKELETON AND THE REGENERATIVE PROPERTIES OF NEURONS IN ATYPICAL CASES OF COMPOSITION AND DAMAGE.

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by

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ABSTRACT

Sensory neurons are irreplaceable cells that suffer damage that is both frequent and unavoidable. As such the ability of sensory neurons to regenerate is inseparable from maintenance of the sensory system that is necessary for normal function. The goal of this study is to gain additional insight into the characteristics of sensory neurons so as to be able to better anticipate their responses to damage, with the long-term goal of potentially improving regenerative outcomes.

While previous work has shown that axon and dendrites rely upon different pathways to regenerate after injury, I have taken this a step further to demonstrate that not only are these pathways separate, but that they are seemingly independent can operate concurrently without interference with each other. After laser -based removal of all neurites, DA (dendritic arborization) neurons were able to regenerate distinct axonal and dendritic compartments, with proper morphology, microtubule polarity, and localization of a known neurite specific marker. This shows that data showing several types of neurons demonstrate axon injury is toxic to the dendritic arbor, that nothing is inherently compatible about the pathways governing their regeneration.

I have answered questions left by previous work about the microtubule polarity of the sensory arbor of DRG sensory neurites. I have performed an optical in-vivo characterization of the microtubule polarity of intact zebrafish DRG sensory neurites and found them to have completely uniform axonal polarity throughout their entire length. I have also demonstrated the possibility that neurons with uniform plus-end-out polarity in all neurites can maintain a centralized MTOC at a developmental timepoint long after neurons with mixed polarity dendrites would be expected to have eliminated theirs, and that this is the case across multiple cell types and distant species relations. I have also shown evidence for the use of polymerizing microtubule

minus ends as being relevant in the organization of the cytoskeleton of mature DRG sensory neurites.

Axons regenerate via a molecular pathway that is conserved across both species and cell type. I have established that despite an unambiguous axonal microtubule polarity, that DRG sensory neurites in fish regenerate by a pathway that functions even with homozygous loss of function mutations to the DLK and LZK genes, which in the same species has been shown to be sufficient to abrogate motor neuron axon regeneration.

In summary, I have established that the pathways by which axons and dendrites regenerate are orthogonal to each other, as well as answered decades old questions about the cytoskeletal composition of DRG sensory neurites, and shown a potential exception to the accepted model by which neurons organize their microtubule cytoskeletons and further shown that axonal microtubule polarity is not necessarily indicative of dependance on the classic axon regeneration pathway for neurites to recover after injury.

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Traditionally, this is the part where the author, being at the end of a long and difficult process euphorically describes the positive nature of their experience and the traits of people they have worked with. As such, readers would be well served to remember the principles of the $\chi 2$ test.

This is my thesis, but no person is an island, and any degree of success I have had has been in great part due to the people around me, whether by getting me to reexamine my assumptions (this possibly more than anything else), offering encouragement, direct assistance, or even in some cases demonstrating with incredible enthusiasm what not to do.

Objectively speaking, a lot of work went into these projects, and not all of it was mine. Jenna Mandel's unflinching perseverance in proving me wrong is what made that project work. Greg Kothe singlehandedly enables projects to move forward by ensuring that the necessary tools not existing is just a minor inconvenience. Without Michelle Stone's assistance that allowed me to split my time between projects, some would have unfortunately gotten shelved and never seen the light of day (there is a reason at the time of this writing she has 798 citations). Though their work is not featured here, I was also fortunate enough to mentor a number of promising undergrads, among them Aabha Vora and Mila Tamminga, who made great discoveries in the field of "the effects of explosions in live nonanesthetized animals", which will hopefully be published at some point. Additional thanks are owed to everyone else who has in their own way made this lab a great place to be over the last 6 years, even if that has involved a truly surprising number of times moving furniture, or driving someone to the ER at 2AM because they tried to swan dive into less than three feet of water.

Lastly, people are complex entities with mixes of traits that cannot be properly summarized in a succinct statement, and I strongly believe the reflex to shoehorn people into

archetypes is a form of cognitive laziness to be avoided. It is the context of having expressed an understanding of this I would hope to convince any reader that Dr. Rolls is a truly excellent scientist whose commitment to ethics is a very large part of what drew me to her lab at a time when I was thinking of quitting grad school, seriously enough that I had already lined up a job teaching high school math in Arizona. She has built an environment where students who want to, can do science to get results they can believe in, and in doing so know they will see a return on their efforts. She is also the only boss I've had to date where I haven't thought things would be better off if we switched positions. It's taken almost 13 years to go from soldier to scientist but wherever I end up next, I am absolutely sure I will miss this phase of my life (except for feeding the fish, which I will never miss. Ever.)

Chapter 1

Introduction

Neurons are described as polarized cells. This term is applied to a variety of aspects of neurons, from the literal polarization of their membrane that results in it having an electrical potential that is central to their core function, to the polarization of their morphology that facilitates the polarization of the function of different subcellular compartments, to the polarization of their microtubule networks, which enables polarized trafficking of different materials to different parts of the cell so they can behave differently. The initial descriptions of axon and dendrites go back to purely morphological classifications made from silver staining in the 1800s. Since then, we have made a great deal of progress investigating their respective functions, and the differences in the cell biology of each that facilitate that function. Definitions have progressed from possibly coincidental descriptions based solely on morphology to biological ones based on the cellular activities taking place in each neurite. Unfortunately, much like how the complexity of biology renders the traditional definition of a neuron as a cell that fires action potentials and releases neurotransmitter useless by then including a variety of unintended targets from muscle cells to prokaryotes, the complexity of neuronal subtypes across different species makes perfect definitions for axons and dendrite difficult.

In this work I attempt to further probe the distinctions between axons and dendrites in nontraditional scenarios where existing definitions create ambiguity, by using multiple metrics to examine the cell biology of the neurites in question, as well as their response to perturbations which have previously been described to produce differential responses in axons and dendrites.

Because this work will cover aspects of neuronal biology where interpretation of the results relies heavily on the specific definitions used in the field, I have included a series of sections quickly covering basic material relevant to the experiments performed. A great deal of it is at the textbook level, but it should serve to make the experiments performed and the interpretations drawn from their results more accessible to anyone outside the field of neurobiology.

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Membrane polarity

Neurons form complex networks with a variety of connections, and in order to engage in the signaling necessary to participate in these networks, neurons modulate their membrane polarity to initiate and process signaling events. This is nearly an entire field in and of itself, and one which is still very much an active area of research. Dating back to experiments conducted on the giant axon of a squid in the mid 1900s which earned Alen Hodgkin and Andrew Huxley a Nobel Prize, electrophysiologists have given us a significant, but still incomplete understanding of this most fundamental aspect of neurons. A neuron described as at rest (not currently receiving or reacting to input) has an electrically charged membrane with a potential in the vicinity of -65 to -70mV. This is accomplished by the use of a series of ion pumps, such as the sodium-potassium pump that by importing potassium ions and hydrolyzing ATP drives sodium out of the cell into the extracellular environment, against both the electrical and chemical gradients. In conjunction with a membrane that is at rest, largely impermeable to passive sodium transport, and potassium leak channels, which together allow the cell to establish and maintain a negative electrical charge at the expense of ATP production. In a simplified model, signaling events to the neuron alter its membrane polarization by locally modifying membrane permeability. When ion channels are

opened, ions will move across the membrane as determined by the sum of the electrical and chemical gradients. If the opening results in a net decrease in positive charge within the cell, the negative charge of the membrane increases. If the opening results in a net increase in the positive charge of the cell, the negative charge of the membrane decreases. If this happens to a sufficient degree, and with sufficient speed, it can activate sodium channels gated not by ligands but by the alteration in voltage, resulting in a cascade of sodium influx called an action potential that can switch the membrane potential of the cell from negative to positive. It is this action potential that is the unit of signaling in neuronal networks as it results in a variety of outcomes, most notably the fusion of intracellular vesicles with the membrane of the synaptic terminal of the axon, which results in release of neurotransmitter into the synaptic cleft between the neuron in question, and its downstream partner (Kandel, Schwartz, & Jessell, 1991). This alteration in polarity is how neurons signal, and will be useful to keep in mind as the activity behind the function of the neurites I will describe.

Classic axon definition

While work had previously been performed in nerves, axons themselves were originally described in the 1800s as the "axis cylinder" of a neuron by Otto Friedrich Karl Deiters (Muzio & Cascella, 2021). At the time, sparse labeling necessary to resolve individual neurons was achieved via silver staining, so the distinction was based purely upon morphology. It would not be until the next century when electrophysiological characterization of the giant squid axon would give us mechanistic information about how the axon functions (Clay, 1998). Today, via a combination of injected tracers, electron microscopy, electrophysiology, immunohistochemistry, cell culture, and creative applications of fluorescent protein fusion, we have a somewhat more comprehensive understanding of this portion of neurons.

Though the following will sound familiar to anyone with a basic knowledge of neurobiology, it is in large part these characteristics upon which current definitions of neurites are based, and because the experiments I will perform will call these definitions into question, it seems prudent to first examine them. Axons are generally understood to be the portion of the neuron with the specialized role of conducting signaling events outward, to another neuron where they engage in chemical synapse. In order to accomplish this, they have a multitude of characteristics which optimize their axo-plasmic vs membrane resistance for the propagation of signals via voltage gated sodium channels positioned at varying density along their length, which initiate a depolarization cascade as a result of the cell reaching a depolarization threshold from a range of potential inputs. This depolarization, upon reaching the axon terminal, induces fusion of internal vesicles with the plasma membrane, releasing their contents into the synaptic cleft and inducing some response in the postsynaptic cell, which can vary depending on which of many neurotransmitters is released, what receptors the downstream cell express (both at and outside the synapse) and the current state of the postsynaptic cell. The importance of this context within the postsynaptic cell cannot be overstated, as the context can vary to the extent where effects that are completely opposite from standard scenarios have been observed. To illustrate the complexity of this, I offer two examples: first, excitatory glutamatergic input has been demonstrated to, depending on the circumstance, be able to induce either long term increase (Long Term Potentiation, LTP) or long term decrease (Long Term Depression, LDP) in the excitability of a neuron, altering how it participates in its network (Malenka & Bear, 2004). Second, in an even clearer scenario, we can examine the heteropentameric chloride channel formed by GABAa receptors (Sigel & Steinmann, 2012). Under normal conditions, a presynaptic neuron releasing GABA onto a GABAergic synapse results in the opening of this chloride channel, allowing additional negatively charged chloride into the neuron, which inhibits firing. In the atypical but documented case of a neuron where chloride levels are higher on the inside of the cell than the

outside, this same signal from the presynaptic cell results in chloride efflux from the ion channel at the core of the GABAa receptor, which is excitatory (Ben-Ari, 2002; Ben-Ari et al., 2012; Bregestovski & Bernard, 2012). Even in a two-neuron model, the results of a specific signaling event are as complex as the variety, activity, and level of receptors and pumps the neurons express.

Simplified models have the axon as an unbranching linear segment that extends from the cell body of the presynaptic cell, to make synaptic contact with the postsynaptic cell, but axons are also known to form "en passant" synapses with a number of targets along their length, or even branch at their terminal in order to contact multiple targets. Trafficking to the axon is specialized, with a number of cargos present elsewhere in the cell normally being excluded from it (Gumy & Hoogenraad, 2018).This is partly enabled by a microtubule cytoskeleton that is configured differently than other parts of the cell by having uniform plus-end-out microtubule polarity (see "the polarity of the neuronal microtubule network" below) , complete with different microtubule associated proteins (MAPS) (Peng, Binder, & Black, 1986), but also by a complex diffusion barrier present at the Axon Initial Segment (AIS) (Hedstrom, Ogawa, & Rasband, 2008; Sobotzik et al., 2009). A great deal of work has gone into describing the AIS (that is adjacent to, but not to be confused with the axon hillock, which is part of the cell body), which besides serving as a diffusion barrier that prevents dendritic receptors from diffusing into the axon, also hosts a high density of voltage gated sodium channels and has been implicated in the initiation of firing an action potential (Kole, Letzkus, & Stuart, 2007). While myelination by oligodendrocytes is described as a feature of axons that reduces membrane capacitance and in doing so facilitates the firing of an action potential, it is by no means universal (Windle, 1923). During outgrowth, axons are capable of complex pathfinding based on local chemical gradients they traverse in order to properly innervate their synaptic target, which can be either local in the case of interneurons, or distant in the case of projection neurons. Despite this great distance, immunohistochemical and

fluorescent protein fusion data suggest that mature axons at baseline largely lack assembled ribosomes and thus at do not for the most part engage in protein synthesis, requiring a great many proteins to be trafficked in (as relying on linear diffusion over a meter length would make adapting to stimuli prohibitively slow). Recent evidence has revealed however that this situation is somewhat more complicated than previously thought. (Dalla Costa et al., 2021). Axons have been described to lack golgi apparatus, which further emphasizes their dependence on active trafficking from the cell body of components which are synthesized and post translationally modified, though recent evidence suggests that like protein translation, this may also be more complicated than previously thought (Merianda et al., 2009). We have established a great many characteristics of axons which are --usually present, but as the number of these traits increases, it raises the question of what happens to our definition when one of them is absent.

Classic dendrite definition

Though the word dendrite had previously existed in other contexts, it was applied to neuronal morphology to describe the relatively short, branched “protoplasmic processes" of the neuron (Muzio & Cascella, 2021). Like axons, they were initially visualized by silver staining, and so their designation was also purely morphological in nature. The same technologies that have facilitated the characterization of axons have been applied to dendrites, and from them we have developed the understanding that, primarily by participating in the postsynaptic side of a synaptic partnership with axons, they facilitate information input into the cell. While dendrites do not fire action potentials, they integrate excitatory and inhibitory input from a variety of sources at different positions, and it is the sum of these local graded potentials, processed by the membrane properties of the dendritic arbor, which is thought to inform whether the cell reaches the necessary membrane voltage to fire an action potential and initiate a signaling event to its

postsynaptic partner (Kandel et al., 1991). Dendrite arbors develop receptive fields where they monitor input, and these are dynamic, capable of growing and shrinking in response to different stimuli, and even being able to differentiate extra synaptic vs synaptic stimulation from the same neurotransmitter (Karpova et al., 2013). Unlike axons, dendrites clearly have the machinery for local protein translation and processing (Craig & Banker, 1994), and even receive biased trafficking of some mRNAs to be translated locally (An et al., 2008). While axons have both cytosolic and membrane diffusion barriers (Hedstrom et al., 2008; Sobotzik et al., 2009), no comparable structure has been described in dendrites, making them seem to be to some degree an extension of the cell body. As such, a traditional neuron seems to divide into two regions: the axon, and a somewhat unified section consisting of the cell body and dendrites as an extension of the cell body.

The polarity of the neuronal microtubule network

Like all eukaryotic cells, neurons have a cytoskeleton. In neurons the microtubule portion of the cytoskeleton plays an important role in neuron specific functions, such as anchoring postsynaptic densities, stabilizing dendritic spines in response to activity, and controlling axon guidance. Like in other cell types, the neuronal cytoskeleton also facilitates active and directed transport of cargo to achieve speed and specificity greater than would be enabled by relying on random diffusion. This role takes on a much greater importance in neurons; if simple diffusion is insufficient for transporting cargoes in a small spherical cell, then the increased need for active and directed transport for a neuron needing to control the distribution of specific cargos to different neurites up to a meter away should be quite evident. While other cytoskeletal components like actin and spectrin are also present and important for neuronal function, here I would like to focus on the

microtubule portion of the neuronal cytoskeleton, as this has clear and immediate applications to the questions we asked in the experiments we performed.

Microtubules are composed of repeating alpha-beta tubulin dimers. The result of this is that each microtubule can be seen to have directionality, with an exposed alpha tubulin on one end (the minus end or -tip), and an exposed beta tubulin on the other (the plus end, plus tip, or +tip). Early EM data has shown that in neurons, axons have a microtubule polarity where the plus end is pointed out away from the cell body (plus end out), whereas dendrites are characterized by the presence of microtubules where the minus end is pointed out away from the cell body (minus end out) (Baas, Deitch, Black, & Banker, 1988). Microtubules are thought to initiate nucleation at their minus end at gamma tubulin ring complexes (YTURC) complexes, and to grow outward from the exposed beta tubulin plus end. Microtubules are known to grow from active polymerization at the plus end which is enabled by a host of proteins, whereas the gamma tubulin capped minus end is thought to be stationary. However, severing events can separate the AB dimers composing the microtubule and as a result convert a single microtubule into two shorter microtubules, each with its own plus and minus ends. This new minus end that is generated as the result of a severing event is not associated with a gamma tubulin nucleation complex. CAMSAPs, a species of MAP (microtubule associated protein), have been found to bind to and increase the stability of the minus end (Jiang et al., 2014), though not always to the extent of preventing minus end growth (Feng et al., 2019). Minus end growth and stability have been implicated in the initial establishment of minus end out microtubules in dendrites, as unlike the plus end out configurations of axons, this cannot be achieved by simply growing in a plus end out manner from a nucleation site in the cell body. Because of how motor proteins interact with microtubules, with kinesins walking out towards the plus end, and dynein walking out towards the minus end (Vale, 2003), the different polarity of axonal and dendritic microtubule networks (Baas et al., 1988) are thought to contribute to a neuron's ability to direct actively trafficked

cargos to different subcellular compartments (Kapitein et al., 2010; Rolls, 2011), and thus facilitate the specialized functions of axons and dendrites. Given that specialized trafficking allows the cell to do different things in different places, I would like to draw the reader's attention to this difference in microtubule polarity between the axon and dendrite as part of what enables the neuron to give different neurites axonal or dendritic characteristics.

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	axon	dendrite
synaptic position	post	pre
presence of minus end out microtubules	no	yes
diffusion barrier	yes	no
role in signaling	outgoing	incoming
golgi	no	yes
ribosomes	no	yes
MAPS	Tau	MAP2
fires action potential	yes	no
myelinated	yes	no
forms receptive field	no	yes

Table 1: Comparison of Classic axon and dendrite features

The simplified model neuron

Combining these expectations for axons and dendrites, one can construct the expectations for a model neuron. It would have a single largely linear axon containing presynaptic hardware that innervates a single target expressing matching postsynaptic hardware with which it forms a synapse, upon which, when sufficiently excited it releases excitatory neurotransmitter. It has a dendritic arbor that forms a receptive field that expresses post synaptic machinery to form synapses with and integrate signaling from the axons of neurons upstream in the motor signaling process (See Figure 1A). Its axon would have a plus end out microtubule polarity, and its dendrites would have a mix of plus and minus out microtubules which would in conjunction with the diffusion barriers at the AIS, assist in sorting protein and RNA cargos to their destinations. As this description seems to greatly resemble motor neurons (Kandel et al., 1991), neurons like this do exist, and serve as a useful model both for educational purposes and for forming general expectations.

Non-classical neurons and examples across species

While the simplified model neuron is in some circumstances useful, it is just a simplified model. The complex biology of the nervous systems shows us many examples where reality deviates from this. Rather than edge cases, or pedantic technicalities, some of these are neuron types that are both abundant and critical for survival in humans as well as other animals. It is these exceptions we wish to probe, both to increase our understanding of the diversity of the nervous system, and also to better understand the functions of these important but atypical cells, and

hopefully prevent us from losing potential insight by trying (metaphorically) to force a square peg in to a round hole with regard to defining their composition.

One of the first oversimplifications to expose is the ubiquity of the axo-dendritic chemical synapse. The location of synapses varies greatly across the nervous system, as does the effect of its placement between two neurons. Many axons synapse on the cell body of the downstream cell, and some even synapse on the axons of other neurons.(Cover & Mathur, 2020)

Another oversimplification is that signaling is necessarily directional. While this is true of transmission within chemical synapses, retrograde chemical signaling has been described between synaptic partners. (Arancio et al., 1996). Additionally, neurons can form gap junctions with other neurons called electrical synapses, which allow ions from the cytosol of one cell to pass through the gap junction into the cytosol of the electrically synapsed cell. This has the effect of shunting the results of signaling directly from one cell to another in a bidirectional fashion. While this shunting would seem to dampen the effects of signaling to one of the cells, because of the potential for sodium influx cascades in signaling, it also allows for the synchronization of firing between electrically synapsed neurons in a network.

The last oversimplification I will address is that dendrites necessarily receive synaptic input from a presynaptic cell. Separate even from the case of electrical synapses which enable bidirectional dendro-dendro synapses (Nagy, Pereda, & Rash, 2018), invertebrate dendrites in the periphery form a receptive field that integrates input from non-synaptic sources (Robertson, Tsubouchi, & Tracey, 2013). In both C. elegans and D. melanogaster highly branched arbors of peripheral sensory neurons with minus end out microtubule polarity (Harterink et al., 2018; Stone, Roegiers, & Rolls, 2008) form receptive fields that instead monitor things like heat, diffuse chemical content, or stretch(He et al., 2019; Hwang, Stearns, & Tracey, 2012; Tsubouchi, Caldwell, & Tracey, 2012; Zhong, Hwang, & Tracey, 2010). These neurites have dendritic microtubule polarity(Rolls, 2011), and a dendritic morphology that facilitates the coverage of areas to monitor

for sensory input, and a traditional dendritic role in signaling (Grueber, Jan, & Jan, 2002). They differ from CNS dendrites primarily in what receptors are expressed and their distribution across the membrane. The periphery is unique in that it gives a perfectly clear example of something that is difficult to find in the CNS: a circuit with an unambiguous starting point. In this scenario, it is in no way surprising that the input to this circuit is not synaptic in nature (Figure 1).

While the dendrites of invertebrate sensory neurons deviate from the simplified model, their primary difference is the synaptic vs non-synaptic nature of input, which given their role as being the neuron to initiate signaling directly as the result of environmental stimuli is currently accepted without controversy, However, vertebrates such as mice, fish and humans, have an abundant and critical cell type which moves yet another step away from classic definitions: the sensory neurons of the dorsal root ganglion (DRG neurons). Both in their role and morphology, these neurons greatly resemble invertebrate sensory neurons. They have a single unbranched neurite which extends towards a post synaptic target in the CNS, and a large branched neurite that forms a receptive field mediating sensory input in the periphery. It is through these cells and this atypical neurite that we receive the majority of our touch sensation. This neurite bears a striking difference from invertebrate sensory neurites in that the trigeminal neuron sensory neurite (a very close relative of the DRG neuron sensory neurite) has been shown to have axonal microtubule polarity (Topp, Meade, & LaVail, 1994) along the linear section before the arbor. This implies a scenario that requires the acceptance of a further degree of deviation from traditional models: an axon with a receptive field whose purpose is to integrate signaling and convey this information back towards the cell body. While this would be heretical from a signaling standpoint, nothing about the biology of an axon requires directionality of the propagation of an action potential. That said, a plus end out portion before the branched arbor does not indicate the arbor will be plus end out as interneurons in D. melanogaster show us an example of a dendrite with a minus end out arbor extending from a linear plus end out base (Stone et al., 2008), so exploring the as-yet-unexamined

microtubule polarity of the branched arbor would be necessary before committing to this receptive axon classification.

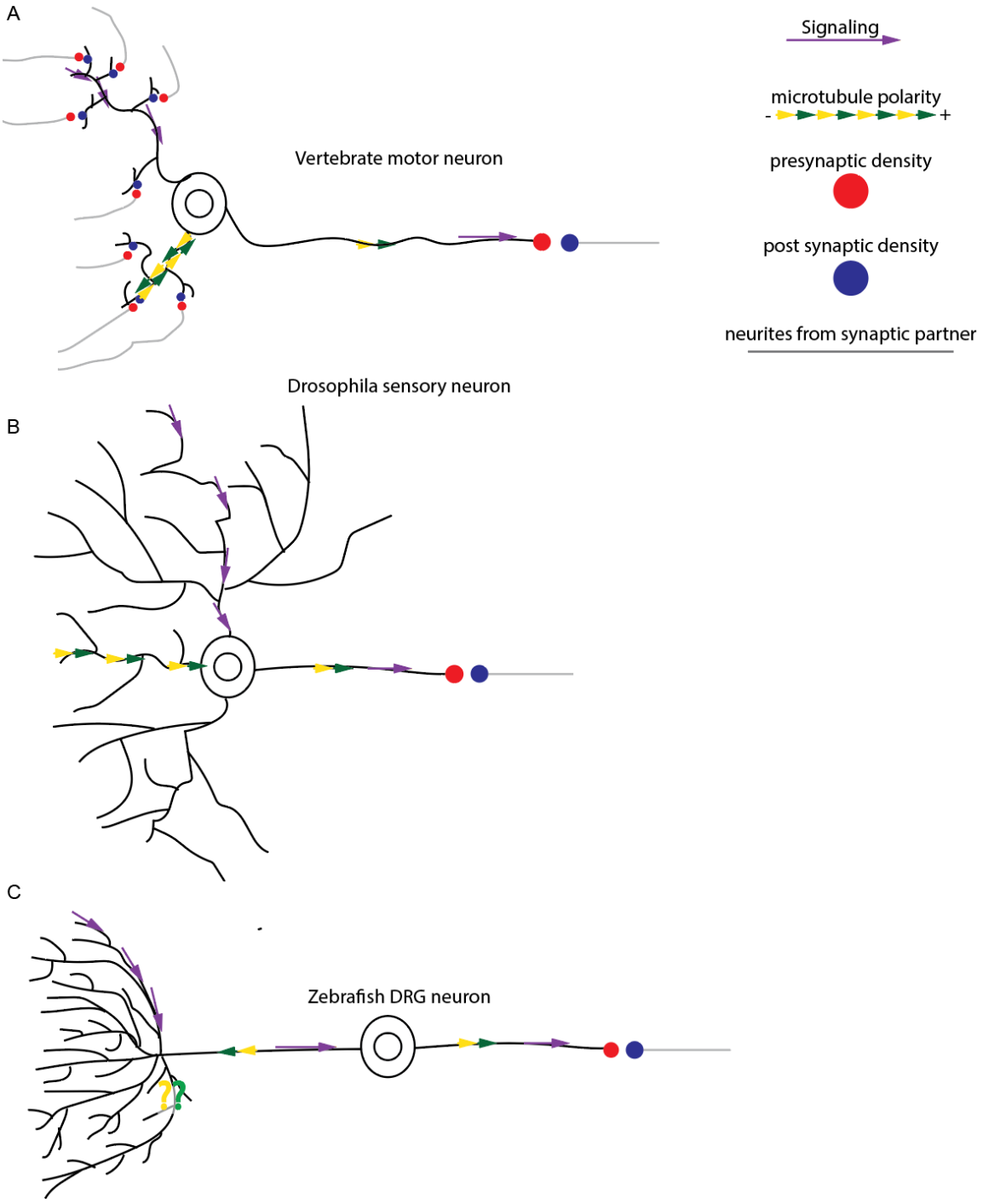

Figure 1: Microtubule and signaling orientation in representative neuron types. **A**. stereotyped vertebrate CNS neurons with synaptic input into mixed microtubule polarity dendrites, and signal output via the plus end out axon to a downstream synaptic partner. **B**. Drosophila peripheral sensory neuron receiving non-synaptic environmental input through minus end out dendrites, and out putting signal through a plus end out axon to a downstream synaptic partner. **C**. Zebrafish DRG sensory neuron receiving non-synaptic input environmental input from an arbor of

unknown polarity attached to a plus end out shaft, and outputting signal through a plus end out axon to a downstream synaptic partner.
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Axon damage and regeneration

Intact neural circuits are critical for normal function in organisms, and vertebrates do not grow new neurons to replace damaged ones, so when damage does occur, the ability of the neurons in a circuit (such as those mediating motor or sensory function) to regenerate is what determines whether function is recovered after injury. Speaking historically, in a very real sense the field of axon regeneration --is the field of neuronal regeneration. The emphasis the field places on the axons of neurons can be seen in the descriptions of neurons as nerve cells, and the same length and proximity to the surface that makes axons so vulnerable to damage also facilitates their study. While the earliest experiments predated the fields of genetics and molecular biology as we currently understand them, work demonstrating the role of nerves in conducting function from the brain have been documented as performed by Aelius Galenus (129-210ad) who is described as having written about the function of motor nerves, and held public demonstrations of severing the motor nerve to the vocal cord to in live pigs. By 1823 experimenters were already investigating "nerve dependent regeneration", how damage to the sciatic nerve affected the regeneration of leg tissue it would otherwise have innervated in salamanders (Kumar, Godwin, Gates, Garza-Garcia, & Brockes, 2007), and by the late 1800s experimenters were already attempting to use substrates to facilitate nerve repair by bridging the site of injury (Battiston, Papalia, Tos, & Geuna, 2009). It may not be surprising then that with almost two thousand years of work on the topic, that the challenge is not the absence of publications purporting to elucidate an aspect of the process, but rather sorting them by relevance, and identifying which ones to consider when constructing a model. To facilitate this, both in interpreting existing work as well as designing new experiments, it is necessary to take into

consideration a theme that I will be going over repeatedly in this work: that experimental conditions can produce results by manipulating several distinct parts of the regenerative process. First, the ability of the neuron to sense damage to the axon, and transduce this signal into one for outgrowth to replace the damaged portion. Second, the ability of the neuron to respond to that outgrowth signal. Third, non-cell autonomous factors affecting regeneration, such as physical obstruction, secretion of inhibitory factors by surrounding tissue, and that for projection neurons, regeneration often requires pathfinding though tissue that may have significantly changed since the developmental period during which the initial extension occurred (Giger, Hollis, & Tuszynski, 2010).

Sensing axon damage: discovery of the role of DLK-1 in axon injury

While the tendency of the axons of projection neurons to fasciculate into bundles has enabled experiments in nerve regeneration that predate the field of molecular biology as we know it, arguably the largest advance towards understanding axon regeneration at a signaling level was made by a forward genetic screen using C. elegans where the worms, due to a mutation in unc-70 (beta spectrin) possessed fragile axons, that while having no defect in initial outgrowth, spontaneously broke as a result of the body movements of the worm. This allowed the screen to identify defects in axon regeneration simply by looking for broken axons that had not undergone the re-fusion they otherwise would have in C elegans. Sequencing a worm line deficient in axon regeneration identified the mutation responsible to have occurred in the gene DLK-1 (Dual Leucine zipper bearing Kinase 1). In follow up experiments with the DLK1- mutant, after axons were injured with a 440nm pulse laser, damaged axons did not initiate outgrowth or form a growth cone, regardless of how much time passed since the injury, indicating that it was not simply an alteration

to the rate of regeneration, but rather the damage sensing function which had been impaired (Hammarlund, Nix, Hauth, Jorgensen, & Bastiani, 2009). This discovery was interesting in that, rather than the screen identifying a previously uncharacterized gene, or finding an existing gene engaging in signaling with partners that had not previously been observed to generate a novel effect, it was a previously characterized gene interacting with its previously characterized signaling pathway. At the time DLK-1 was already known to be a MAP3K antagonized by RPM-1 (Figure 2) and to signal through the mixed lineage kinases MKK-4 (a MAP2K) and PMK-3 (a MAPK homologous to p38) in the non-injury context of synaptic regulation (Nakata et al., 2005). These relationships were confirmed to also be relevant to regeneration as RPM-1 overexpression strongly reduced axon regeneration, and mutation of either the downstream MKK-4 or PMK-3 recapitulated the DLK-1 mutant defect in axon regeneration (Hammarlund et al., 2009). While this signaling cascade may have other effects, in terms of regeneration the relevant output has been shown to be alterations to the transcriptional profile of the cell, with an early roll for Jun and/or Fos depending on the organism and what specific downstream responses are being investigated. (Chen et al., 2016; Dragunow, 1992; Ghosh-Roy, Wu, Goncharov, Jin, & Chisholm, 2010; Itoh, Horiuchi, Bannerman, Pleasure, & Itoh, 2009). Further experiments have brought to light a number of details about how this sensor is regulated, and how its activation is transduced into a signal for outgrowth.

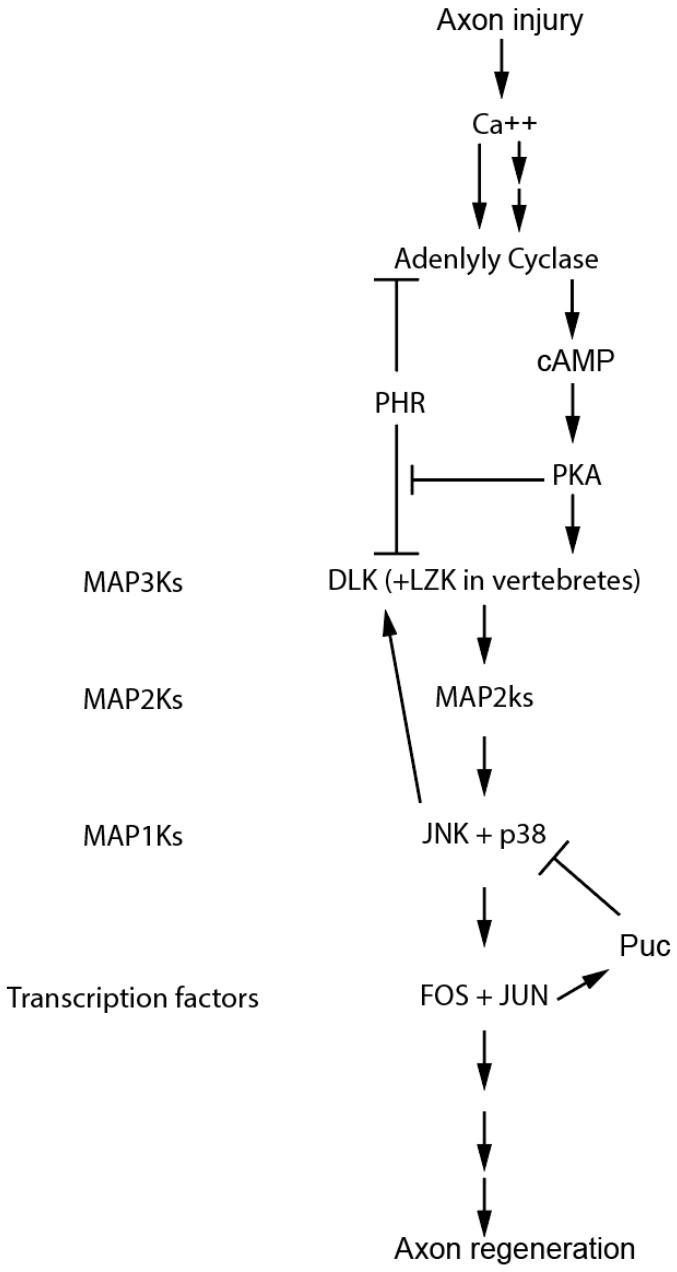


Figure 2: A functional model of DLK activation from injury and immediate downstream effects.

DLK activation and the initiation of the signal cascade

While the role of DLK as an early factor in the signaling cascade initiated by axon injury is clear, less information is available about how DLK is activated as a result of processes that result from axon injury. In C. elegans, DLK-1 has been shown to be directly activated by elevated levels of Ca^{++}(Yan & Jin, 2012), such as occurs after axon injury (Ghosh-Roy et al., 2010), but while Ca++ levels have been shown to increase in the neuron after axon injury in other species (Williams et al., 2014) not all species share this Ca^{++} binding domain on DLK, nor express an inactive isoform like c elegans DLK-1 that binds as a heterodimer in the absence of calcium. For several years the process by which DLK was activated would remain unclear, until a 2016 publication provided a critical link between phenotypes immediately following injury and the direct activation of DLK by PKA (Protein Kinase A) as a result of elevated cAMP (cyclic adenosine mono phosphate) levels (Hao et al., 2016), which I will proceed to describe (with some extrapolation) below.

In the model I propose, the elevated levels of Ca++ that have been observed to follow axon injury (Williams et al., 2014) drive increases in the levels of cAMP, which has been shown to occur after axon injury and been demonstrated to improve regenerative outcomes (Qiu et al., 2002). This is supported by the multiple lines of evidence that adenylyl cyclase can be modulated by Ca^{++}. Soluble adenylyl cyclase (sAC), has been shown in in-vitro experiments to be directly activated by Ca++ (Jaiswal & Conti, 2003), and knockdown of sAC has altered survival and regeneration in retinal ganglion cells after axotomy (Corredor et al., 2012) . Additionally, adenylyl cyclases 1 and 8 (AC1, AC8), which are also expressed in neurons, have been associated with neuronal phenotypes (Bernabucci & Zhuo, 2016; Yamanaka, Matsuura, Pan, & Zhuo, 2017) and have been shown to be activated by binding calcium-bound-calmodulin (Masada, Schaks, Jackson, Sinz, & Cooper, 2012) . While D. melanogaster and C. elegans do not express sAC (Roelofs & Van Haastert, 2002), AC8 has a homolog in drosophila by the name rutabaga (Levin et al., 1992). This seem to support a

model where at baseline cAMP levels are kept in check by PHR (Hiw, RPM-1) which negatively regulates both adenylyl cyclase (Pierre, Hausler, Birod, Geisslinger, & Scholich, 2004), and DLK (Hao et al., 2016), until post injury Ca++ levels increase enough to overcome this suppression, resulting in increased levels of cAMP which have been shown to be capable of inducing PKA to phosphorylate DLK, both leading to its activation, and increasing its abundance by rendering it resistant to ubiquitination by PHR. (Hao et al., 2016) (Figure2). The discovery that DLK is activated by PKA as a result of elevated cAMP levels is a critically important one, as it explains previous experiments showing that circumstances which elevate cAMP levels improve axon regeneration following injury (Ghosh-Roy et al., 2010; Pierre et al., 2004), and while it is possible that levels of this 2nd messenger also affect other pathways which may have effects on the neuron's ability to enable outgrowth, this shows a clear signaling pathway to connect elevated cAMP levels to the known phenotype of improved axon regeneration via the mechanism of increased DLK activation.

Regulation and downstream effects of DLK signaling

While the signaling pathway from DLK to it's MAPK was established before the discovery of it's role in axon injury signaling, a great number of fine details about how this signal cascade and the players in it are regulated has been discovered since. Though there is some disagreement on aspects of DLK signaling (see below) some aspects of how the pathway is regulated are quite clear, with no disagreement that at baseline the levels of DLK kept are in check by the balance between its negative regulator, the E3 ubiquitin ligase PHR1 (mammalian homolog of drosophila Hiw and C. elegans RPM-1 see (Grill, Murphey, & Borgen, 2016) for comparison of the gene homolog name cross species) and the de-ubiquitinating USP9X (Huntwork-Rodriguez et al., 2013). Wnd, the

drosophila homolog of DLK-1, has been shown to be trafficked into the axon, where after injury its levels increase in conjunction with a decrease in the levels of its negative regulator highwire, the drosophila homolog of RPM-1. (Xiong et al., 2010). This axonal trafficking appears to be mediated by combination of JIP3 and motor proteins and in part assisted by palmitoylation, which is required for the interaction of DLK with the JIP scaffolding protein, which also binds the MAP2K and MAP3K targets of DLK (Holland et al., 2016; Xiong et al., 2010). The post injury increase of DLK in the axon and its activation have been shown to result from phosphorylation by PKA that renders it resistant to the degradation by highwire that keeps it in check under basal conditions, and be further reinforced by a positive feedback loop where DLK is phosphorylated by the downstream MAPK bsk (JNK), until the transcriptional output of this pathway result in expression of sufficient levels of the JNK phosphatase puc to break this loop and terminate signaling through this pathway ((Hao et al., 2016).

Given the positive feedback loop involved in DLK activation (Hao et al., 2016), and the evidence of caspase activation as a downstream result (Chen et al., 2016), which potentially explains an aspect of DLK dependent cell death for RGCs (Retinal Ganglion Cells) in the optic nerve crush assay (Fernandes, Harder, John, Shrager, & Libby, 2014); it can be tempting to think of DLK as an emergency response factor that the cell keeps repressed, with no role on basal function. However, this view is an oversimplification, as DLK has been shown to have roles in other processes such as synaptogenesis and microtubule regulation (Klinedinst, Wang, Xiong, Haenfler, & Collins, 2013). What separates injury signaling of DLK from its basal signaling is still an open question being researched.

Besides what I have described above, there exists a great deal of information about the minutiae of the DLK pathway, but using it to assemble a comprehensive model past what I have described is complicated by the fact the body of work has been generated via both *in vitro*, and *in vivo* approaches leveraging multiple organisms, without all findings being formally crossed

checked between them. Besides axon regeneration DLK also appears to have a role in general development (Borgen et al., 2017) , synaptic regulation (Klinedinst et al., 2013), and apoptosis (Welsbie et al., 2017), which in conjunction with data coming in parts from multiple species, may contribute to some of the findings regarding the DLK pathway being difficult to reconcile. For example, *in vitro* data suggests that JIP1 acts to suppress basal DLK signaling by binding DLK an unphosphorylated monomer, and in doing so prevent the homodimerization and autophosphorylation that would otherwise occur (Nihalani, Meyer, Pajni, & Holzman, 2001). While not fully contradictory, other *in vitro* and cell culture analysis shows that palmitoylation dependent binding of DLK to JIP3 is necessary for both its proper localization and transport, and that this complex is important for facilitating the interaction with its MAP2K and MAP1K targets and thus may assist in signal transduction after it's activation, and in cells palmitoylation was required for the kinase activity of DLK (Holland et al., 2016). This is of interest because in C. elegans, MLK-1 is both necessary for axon regeneration, and able to compensate for DLK knock out (Hammarlund et al., 2009; Nix, Hisamoto, Matsumoto, & Bastiani, 2011), but MLK was shown not to be palmitoylated (Holland et al., 2016). Organism to organism difference are important to keep in mind as in mice, full loss of DLK is lethal shortly after birth, (Itoh et al., 2009), whereas in fish (see chapter 4 for fish data) and C elegans it appears to be well tolerated (Hammarlund et al., 2009). In C. elegans DLK-1 overexpression is well tolerated and pro regenerative (Hammarlund et al., 2009), but in mice DLK or LZK overexpression is toxic (Y. Li et al., 2021). It is possible that the differences between studies in overexpression data is a matter of degree due to the methods used in different models, with dose dependent outcomes ranging from the PHR eliminating any difference in total levels of DLK, to saturation of scaffold protein binding sites that could provide modest enhancement to regeneration as seen in the C elegans model (Hammarlund et al., 2009), to the overexpressed gene not receiving palmitoylation necessary for its kinase activity (Holland et al., 2016), or at truly high levels, overwhelming the regulation machinery and expressing at

sufficient levels to be activated by otherwise subthreshold stimuli, or undergo the dimerization and autophosphorylation seen *in vitro* (Nihalani et al., 2001). While the data from across species is generally in agreement and shows a great deal of conservation in the pathway, the differences highlight that care should be taken in automatically assuming every specific interaction will be conserved across species.

DLK-type signaling: complements, variants, and their effect on axon regeneration

While DLK has received a great deal of attention in the context of axon injury signaling, and it's modulation demonstrates strong phenotypes in a number of injury and regeneration assays, it is worth noting that depending on the organism, not all of the downstream signaling from axon injury is necessarily a result of DLK activation. In C elegans, due to crosstalk shown at the MAP2K level MLK-1 was shown to be able to rescue DLK-1 mutant axon regeneration (Hammarlund et al., 2009). MLK-1 was later shown to be jointly necessary with DLK-1 for axon regeneration to activate PMK-3 (p38) and KGB-1(JNK) both of which were required for regeneration. In vertebrates, while MLK (MAP3K9) is not associated with axon regeneration phenotypes, there is a recent copy of DLK (MAP3K12) termed LZK (MAP3K13). This is of interest because a number of experiments examining mouse neuronal regeneration in a DLK knock out background have displayed only partial impairment of regenerative outcomes, raising the question of whether DLK is required for regeneration, or simply involved. Injury experiments have been performed in a DLK gene trap mouse examining the injury response of DRG neurons (Dorsal Root Ganglion neurons) following sciatic nerve transection and found that at 24 hours post injury C-Jun phosphorylation was greatly reduced compared to injured wild type mice, but still increased relative to uninjured neurons of either genetic background (Itoh et al., 2009). Regeneration of sensory and motor axons

following sciatic nerve crush was assayed in a conditional DLK knockout mouse, but while regeneration was reduced relative to wild type, only partial reductions in axon regeneration were observed in both motor and sensory axons (Shin et al., 2012). Given that LZK overexpression has been shown to have similar effects to DLK overexpression in Purkinje cells (Y. Li et al., 2021) and when inhibited, to dramatically augment the protective effects of DLK inhibition in RGC cell death assays following optic nerve crush. (Welsbie et al., 2017) it seems highly possible that the partial phenotypes can be explained by an intact LZK locus, though experiments will be necessary to determine this.

Outgrowth of axons: avoiding red herrings

Despite the fact that axons are easy to find and damage, productively studying axon regeneration is difficult. It is telling that the clearest data we have on axon regeneration is on the initiation of axon regeneration, and how the players for that process are regulated and interact. After the early steps of transcriptional change, things become much less clear, with alterations to the expression of over a hundred genes (Costigan et al., 2002); even before factoring in gene redundancy, and a need for multiple basal processes working properly, in a scenario that complex, properly interpreting mild or even moderate regeneration phenotypes resulting from gene knock down becomes quite difficult.

However, in a post-DLK, post-sequencing era, there are some approaches that can be used to at least somewhat clarify matters, and that publishing without radically reduces the impact of a finding: examining whether the cell displays noticeable phenotypes at baseline is important, as these are indicative of a non-regeneration-specific phenotype probably effecting basal processes. Following that, using puc (VHP-1 in C. elegans) reporters allows identification of factors involved

in initiating the axon regeneration response from factors acting further downstream in the process (Martin-Blanco et al., 1998). Further insight can also be derived by examining the specific regeneration phenotype: Latency until initial outgrowth, degree of degeneration on the proximal side of the cut, extension past the cut site, rate of linear extension once past the cut site, are all different metrics, potentially modulated by different processes, and necessary to examine even simply for the sake of proper quantification. Besides the sum of factors involved with extension, axons also require, and could have phenotypes relating to the ability to path-find to, and re-engage with their original target. Alterations to each of these could be achieved by different means, and sub-classifying the nature of any given regeneration deficit allows for greater insight into the nature of the effect from any given perturbation.

Non-cell autonomous factors affecting regeneration

While the DLK pathway which senses axon damage and initiates a transcriptional response is thought o be cell-autonomous, not all factors affecting axon regeneration are. The axon of a regenerating neuron, after having met all internal criteria to initiate, and then sustain a regenerative process, must navigate through tissue like it did during its initial outgrowth in early development. That environment however, may have changed greatly since that time. Unlike early development, the tissue through which it must pass is not specifically designed by the developmental process to facilitate this with a combination of positive factors like neurotrophins and lack of negative factors that collapse growth cones. Additionally, aside from simple developmental changes, regenerating axons have shown to have difficulty growing past a glial scar at the injury site which is enriched for inhibitory factors such as semaphorins, NOGO, CSPGs (chondroitin sulphate proteoglycans), and some of the greatest improvements in regeneration have come from grafting in a more

permissive substance for neurons to extend through. (Fawcett, Schwab, Montani, Brazda, & Muller, 2012). A number of studies have shown this can be overcome to a degree by elevating cAMP (Fawcett, 2020), potentially by enhancing DLK activation (see below). This shows that improvements to regenerative outcomes must take into account the interactions of the growing axon with its environment if successful regeneration is to be optimized.

The clinical impact of axon damage

Axons exist throughout the human body, whether in the periphery like the sciatic nerve, tracts in the spinal cord, or the white matter of the brain. Damage to these areas that severs the axon disrupts the circuit the axon was part of, and compromises whatever function it was involved with. Thousands of American each year are affected by paralysis and in 1995 there were over 50,000 peripheral nerve repair procedures performed (Evans, 2001). Spinal cord decompression surgery improves outcomes, but is more a matter of reducing the damaging downstream effects of the original injury to prevent it from getting worse (Fehlings et al., 2017). Axon regeneration functions better in the peripheral nervous system than the CNS (Fawcett et al., 2012), and even there with the assistance of surgical intervention in the case of ulnar nerve repair only about half of patients have good to excellent results, and up to one third have little to no recovery at all (Vastamaki, Kallio, & Solonen, 1993), showing that existing therapeutic options are limited in the scope of their effectiveness.

Potential therapeutic targets

Understanding and improving recovery from injury is one of the major drives in studying axon regeneration. As such it should not be surprising that discoveries in this process are often viewed

in terms of potential therapeutic application. I would like to highlight two approaches which have recently received a great deal of attention as potential therapeutics to facilitate recovery in a clinical setting that met with different results.

PARP inhibition

In the initial experiments characterizing the role of DLK-1 in axon regeneration, DLK-1 overexpression improved regenerative outcomes in E. elegans (Hammarlund et al., 2009). A follow up experiment using DLK-1 overexpression, cell sorting and RNA sequencing to examine the targets of DLK transcriptional regulation identified parg-1 and parg-2 (poly(ADP-ribose) glycohydrolases) as candidate to enhance axon regeneration phenotypes. As PARGs remove the post translational modification poly-(ADP-ribose) (PAR) from targets (N. Li & Chen, 2014), the authors attempted to improve regenerative outcomes by inhibiting the class of enzymes, PAR polymerases (PARPs), which antagonize this function by adding PAR to proteins, . Both genetic and pharmacological inhibition resulted in improved axon regeneration a C elegans model. (Byrne et al., 2016). While follow up experiments performed with shRNA against PARP1 improved regenerative outcomes in cortical neuron culture, neither genetic deletion of PARP1 nor administration of the PARP inhibitor veliparib altered outcomes in an optic nerve crush assay, or spinal cord hemisection (Wang et al., 2016). Given the pro-regenerative outcomes displayed in whole animal c elegans and cultured mammalian cortical neurons, this outcome is certainly disappointing. It is also fascinating in light work showing that PARPs are activated in response to contact with inhibitory factors and its inhibition was shown to be sufficient to allow cultured neurons to grow in the presence of MAG, CHO and NOGO (Brochier, Jones, Willis, & Langley, 2015). In context with the C. elegans findings this suggests a system where part of the process of DLK-1 activation is to increase PARG expression to antagonize parylation, and allow the axon to

extend through this hostile territory, which would seem to remove the simplest explanation for why PARP inhibition improved regenerative outcomes in both invertebrate, and mammalian neuron culture models, while failing to improve regeneration in an in-vivo mammalian model. This is especially confusing in light of the notable success in improving regenerative outcomes demonstrated by antibodies against NOGO-A (see below), which is just one of several inhibitory compounds to which PARP inhibition should render neurons insensitive.

Anti-NOGO antibodies

As discussed above in the context of axon guidance, the adult nervous system expresses levels of repulsive factors that reduce the plasticity of the system as a whole by increasing the difficulty of axons to grow through it. Injury modifies gene expression in the injured cell to reduce its sensitivity to some of these factors, but a marked difference between the regenerative capacity of axon in central vs peripheral contexts has been observed, with CNS glia being found to secrete a number of inhibitory factors which can interfere with growth cones via activation of Rho kinase to modify cytoskeletal properties (Brochier et al., 2015), the effects of which were solidly demonstrated by the improvement in regeneration of CNS axons induced by transplantation of peripheral nerve segments.(David & Aguayo, 1981) Of these inhibitory factors NOGO has received attention as a candidate for therapeutic approaches via antibodies to bind NOGO and prevent interaction with the NOGO receptor expressed on neurons (Mohammed, Opara, Lall, Ojha, & Xiang, 2020), and studies have shown increased regenerative response in the spinal cord of rats following injury (Schnell & Schwab, 1990), which was later shown to improve functional metrics of recovery (Bregman et al., 1995). However, related approaches shown that any improvement observed may not be exclusively due to effects on growth cone function (Sozmen et al., 2016) (Xu et al., 2020). Encouraging results have also been seen in a study examining recovery of motor control after spinal lesion in monkeys

(Freund et al., 2009). Nogo antibody injection has passed stage 1 clinical trials in humans for both applications in spinal injury (ClinicalTrials.gov Identifier: NCT03935321) and multiple sclerosis. Though the stage 2 trials for MS showed no effect, (Meininger et al., 2017) information is not yet available on the stage 2 clinical trial for spinal injury. While therapeutics for the improvement of regenerative outcomes are an exciting and much needed are of development, it can also be seen that is it quite difficult to anticipate the results of clinical trials from early *in vitro* data, and only time will tell if these particular approaches warrant approval for general use.

Dendrite damage response and regeneration

Unlike axon regeneration, where we have thousands of papers and the challenge is navigating the abundance of data to find the relevant points to connect, dendrite regeneration is only beginning to be studied, with only 16 papers (several of which are false positives, and one review) brought up by an NCBI search for "dendrite regeneration" at the time of this writing. This is primarily because of the difference in technical difficulty between experiments to investigate the regeneration of axons and dendrites has prohibited dendrite regeneration research until relatively recently. At the simplest, because of the tendency of projection neurons to bundle their axons into macroscopic nerve structures that travel far from the cell body, axon regeneration experiments can be performed by identifying a macroscopic nerve structure, damaging it with macroscopic methods, and monitoring the speed and degree to which function returns afterwards. This can be seen in the use of metrics such as the sciatic functional index (SCI) which measures walking ability of a mouse after sciatic nerve damage such as a crush injury. Dendrite regeneration is far more difficult, as dendrites project only a relatively short distance from the cell body, do not bundle, and generally exist in places densely packed with other neurons and their associated dendrites. This is especially true in vertebrate models where they seem to be restricted to the central nervous system (CNS),

which is deeper and harder to access. This means injury to the dendrites that reliably allows for survival of the associated neuron must occur at a proximity to the cell body which rules out macroscopic damage techniques such as crush or blades due to the collateral damage associated with their use. Similarly, the morphology of dendrites requires that the techniques used to label neurons for dendrite regeneration studies allow the investigator to be able to incontrovertibly resolve individual neurons. This can be achieved through sparse transfection in a cell culture scenario, but *in vivo* requires the additional difficulty of either using individually made mosaic animals, or generating a stable line that only labels well-spaced, resolvable neurons. The first whole organism used for this was D. melanogaster larva, where this was facilitated by a repeating body structure with largely planar sensory neurons in each segment that are either well-spaced or clearly tile, and preexisting transgenic lines that allow for labeling of neuronal populations where individual neurites close to the surface (Figure 3) can be resolved and damaged with UV lasers that cost a tenth that of two-photon source lasers necessary for ablation in deeper tissue. These factors have allowed for work in dendrite regeneration despite its inherent technical difficulty, and so it is in this system that most of the work on dendrite regeneration has been performed.

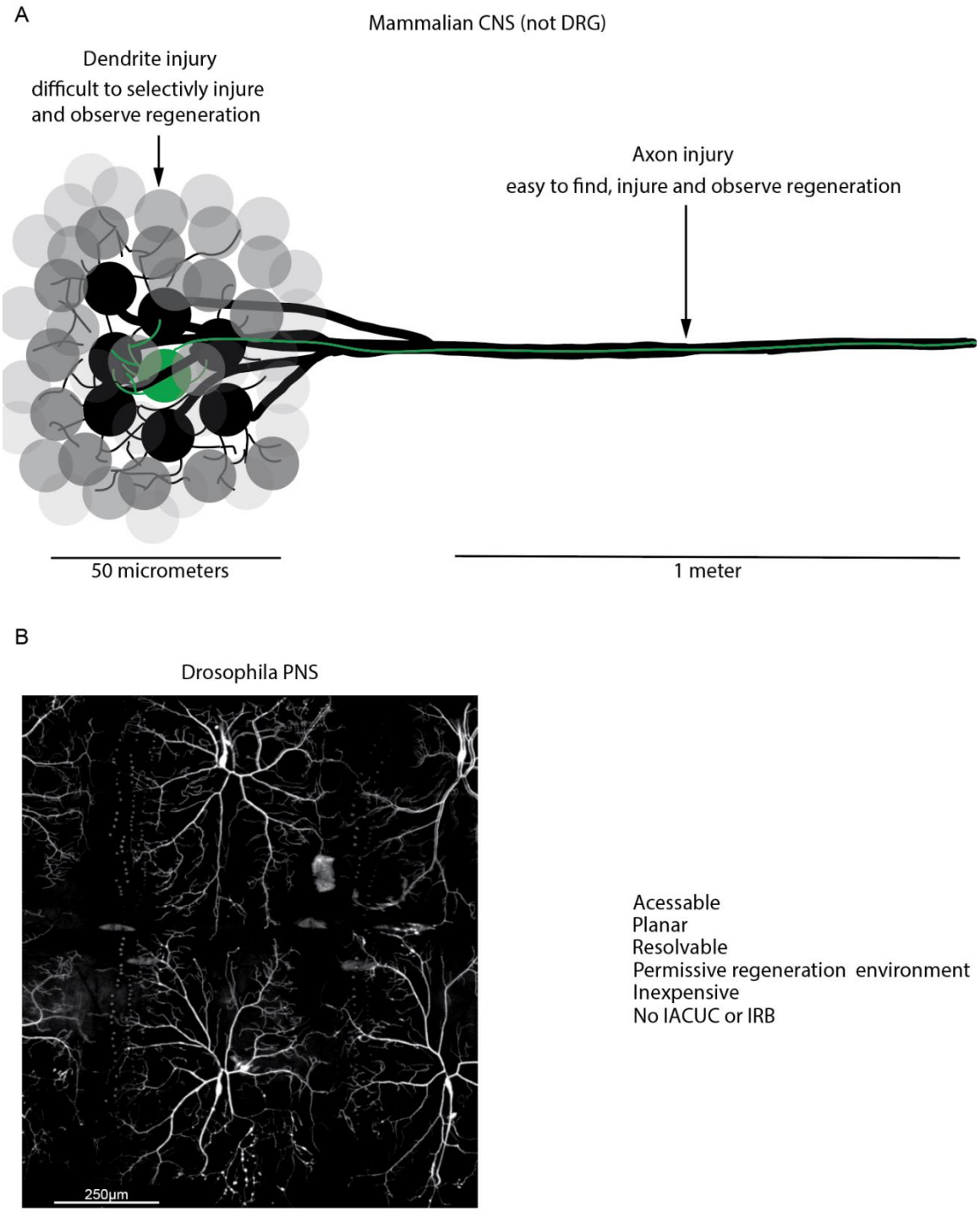


Figure 3: Labeling and accessibility for injury of neurites in different systems. A. Mammalian central nervous system demonstrating the ease of axotomy of projection neurons with no label and the impracticality of dendrite damage without a laser source and single cell labeling. **B.** Drosophila peripheral nervous system as seen by a TRP channel promoter driving GFP in a stable transgenic line labeling easily resolvable dendrites.

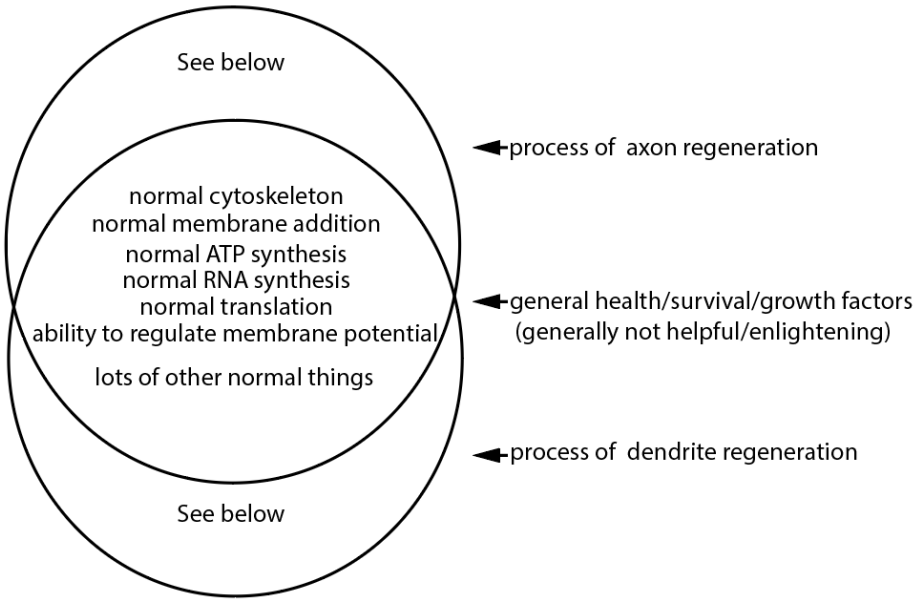
In order to understand the results of the handful of papers published on this topic, it is necessary to first define "dendrite regeneration" both because this would be necessary to properly evaluate experiments attempting to modulate it as well as because there has been some disagreement in the field about this definition. While it might initially seem tempting to define successful regeneration as a perfect return to a cell's exact pre-injury morphology, the complexity of the dendritic arbor seems to prohibit this. It is also not necessary to restore function, as given sufficient time ddaC neurons regrow an injured dendritic arbor to recover the entirety of their original territory, which in all probability restores their function, as for sensory neurons in the periphery, the idea of functional regeneration is somewhat different from the concept of functional axon regeneration, as there is no synaptic target to find and synapse with to restore function. Instead, as seen in the case of the ddaC neuron TRP channel PPK, receptors are evenly distributed throughout the membrane of dendrites (Zheng et al., 2008), implying their presence in any given area is sufficient for them to detect stimuli there. There has been debate however, over whether certain neuron types regenerate at all, even within the same lab (Song et al., 2012; Thompson-Peer, DeVault, Li, Jan, & Jan, 2016). Despite this other studies have found post injury collateral sprouting of ddaE dendrites returns them to their pre-injury branchpoint complexity (Stone, Albertson, Chen, & Rolls, 2014). Whether collateral sprouting constitutes regeneration may be clarified by looking back to the case of ddaC neurons and their functional recovery, which seems to offer the most relevant metric. Whereas ddaC neurons are nociceptive, and need to cover surface area in order to monitor it for exposure to localized noxious stimuli, ddaE neurons function by detecting the folding of the skin (He et al., 2019) and may not need to regrow to coverage to do so. That they consistently regrow instead to pre-injury complexity is strongly suggestive of this, but admittedly not definitive without a functional assay such as imaging of ddaE neurons expressing GCaMP during locomotion

and comparing the degree of activation relative to their contralateral counterpart, as this would probably produce a definitive answer.

Meaningful analysis of dendrite regeneration requires distinguishing between the same types of function as axon injury: First, the ability of the cell to sense the type of damage that occurs and initiate a program to restore the function lost due to the damage. Second, the cell-autonomous ability to respond to that signal and engage in the outgrowth necessary to restore function. Third, the degree to which the environment permits this recovery (Figure 4). While it may seem daunting to design experiments where the gene being modulated may produce a partial phenotype in the output of the assay that could be from modulating any of these three factors, especially in the absence of any known initiator like DLK that would give us starting point, we are fortunate that existing work in the field has greatly assisted in deconvoluting which factor is responsible. First, the PNS of drosophila appears to be very permissive to regeneration (K. S. Rao & Rolls, 2017; Stone et al., 2014; Stone, Nguyen, Tao, Allender, & Rolls, 2010) and while it is always possible that this could be altered by an experimental variable, when using genetic techniques to restrict perturbations to the neurons being examined this can be generally ruled out. Second, the single largest revelation to date in the field of dendrite regeneration was that the early steps of the DLK pathway by which axons sense damage are completely dispensable for dendrite regeneration (Stone et al., 2014). Besides being interesting in its own right as it strongly implies that neurons have separate pathways to detect damage to different subcellular compartments, it also critically useful as it allows us to control for perturbations that would generally alter outgrowth by ruling out factors which also alter axon regeneration as general-purpose cell health or growth factors such as the Pten-AKT pathway (Song et al., 2012), or ones which have pre injury outgrowth defects (Kitatani et al., 2020). Without this, one can easily imagine a long list of perfectly correct and equally unhelpful experiments demonstrating genes like Pol2, ATP synthase, and tubulin etc. to be necessary for dendrite regeneration.

Aside from the dispensability of the DLK pathway, the field is only beginning to identify factors specifically involved in dendrite regeneration, as there are to date two papers which have after controlling for pre-injury morphology and axon regeneration effects, identified factors specific to the regeneration of dendrites. One paper demonstrated interfering with Wnt signaling in drosophila peripheral neurons specifically impairs dendrite but not axon regeneration (Nye et al., 2020). The underlying mechanism was characterized in the jointly published paper, in which it was shown that Wnt signaling controls microtubule nucleation in dendrites of drosophila peripheral neurons (Weiner et al., 2020). The other publication demonstrated that knockdown of proteins in the kinetochore complex (primarily known for its role in cell division) in mature drosophila peripheral neurons produced the surprising effect of disrupting microtubule nucleation in the dendritic arbor. These same neurons displayed deficits in dendrite, but not axon regeneration, that were rescued when nucleation was restored to normal levels (Hertzler et al., 2020). While the fact that knockdown of kinetochore proteins and disruption of Wnt signaling produced dendrite specific regeneration deficits is encouraging in terms of beginning to shape an understanding of dendrite regeneration as a process, further work will need to be performed to distinguish whether this is part of process by which dendrites sense damage, or if growth and extension of dendrites may have somewhat different requirements or sensitivities than growth and extension of axons. This would not be completely unexpected as it has been shown that there are differences in what is involved in the extension of axons and dendrites during regeneration (K. Rao et al., 2016), and thus we may wish to at least consider the possibility of factors with a bias towards differentially influencing outgrowth capability of one or the other. At the very least it seems safe to conclude that dendrites are more sensitive to disruptions of microtubule nucleation than axons in terms of their regenerative process.

A


See below
normal cytoskeleton
normal membrane addition
normal ATP synthesis
normal RNA synthesis
normal translation
ability to regulate membrane potential
lots of other normal things
See below
process of axon regeneration
general health/survival/growth factors
(generally not helpful/enlightening)
process of dendrite regeneration

B

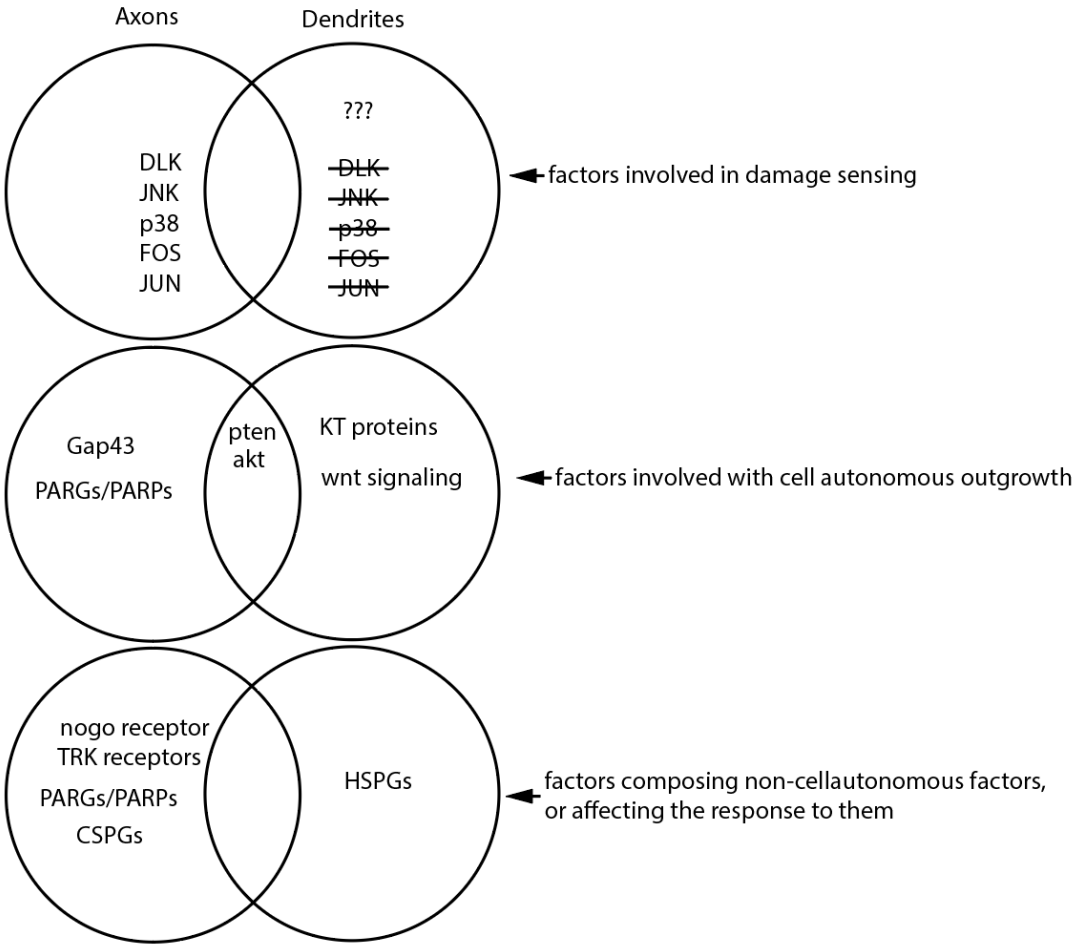

Axons
Dendrites
DLK
JNK
p38
FOS
JUN
???
DLK
JNK
p38
FOS
JUN
factors involved in damage sensing
Gap43
PARGs/PARPs
pten
akt
KT proteins
wnt signaling
factors involved with cell autonomous outgrowth
nogo receptor
TRK receptors
PARGs/PARPs
CSPGs
HSPGs
factors composing non-cellautonomous factors,
or affecting the response to them

Figure 4: Template for a model of analysis for regenerative phenotypes. A. Ven diagram of factors involved in dendrite and axon regeneration. B. An example of better separation of variables for analyzing regeneration phenotypes.

Given that the DLK pathway was not, as originally expected, involved with the regeneration of dendrites, and our general lack of knowledge in this new field from which to generate informed candidates for testing, an appealing strategy would be to leverage the invertebrate model's reproductive rate and to perform a forward genetic screen. Unfortunately, due to the reasons described above regarding the technical difficulty of dendrite damage, to date no one has designed a system by which dendrites can be reliably damaged in a high throughput manner. This technological barrier will first need to be overcome to enable a large-scale screen. In the meantime, the findings that dendrite regeneration is both ddaC and ddaE neurons is sensitive to the control of microtubule nucleation seems to be a promising direction to investigate.

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Preface to Chapter 2

Chapter 2 is a project that was published in the September 2020 of the journal Developmental Biology. The co first authors of this manuscript are Matthew Shorey, Michelle Stone, and Jenna Mandel. The PI for the project was Melissa Rolls. The manuscript was written by Matthew Shorey and Melissa Rolls. Experimental work represented in the manuscript was distributed evenly between Matthew Shorey and Michelle Stone, with Jenna Mandel performing a long series of supporting experiments that identified the conditions under which the assay could be performed. This project contributes to the thesis as an investigation of an as-yet-unexplored neuronal injury paradigm, and is a contribution to the field as it builds on previous work by Michelle Stone demonstrating the regenerative capacity of invertebrate peripheral dendrites and axons, and as a result broadens our understanding of conditions under which robust neuronal regeneration can occur.

A supplemental figure not present in the publication has been included after the references to show the response of ddaC neurons to the full removal of all neurites in order to show that the results from the publication are not restricted to a single cell type.

Chapter 2

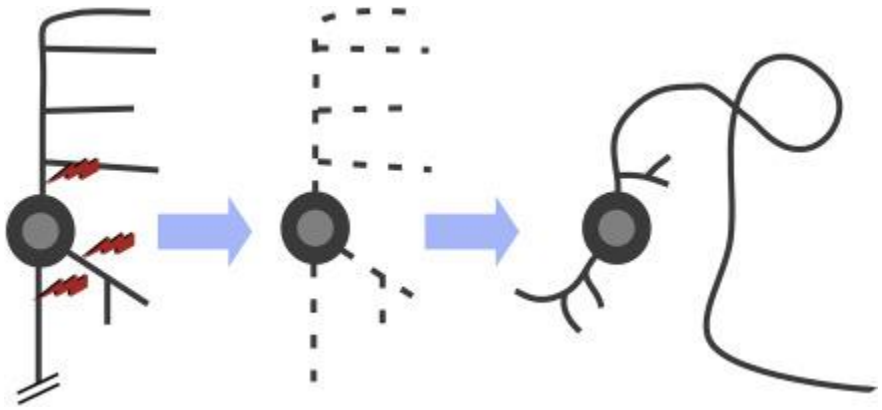
Neurons survive simultaneous injury to axons and dendrites and regrow both types of processes *in vivo*

Abstract

Neurons extend dendrites and axons to receive and send signals. If either type of process is removed, the cell cannot function. Rather than undergoing cell death, some neurons can regrow axons and dendrites. Axon and dendrite regeneration have been examined separately and require sensing the injury and reinitiating the correct growth program. Whether neurons *in vivo* can sense and respond to simultaneous axon and dendrite injury with polarized regeneration has not been explored. To investigate the outcome of simultaneous axon and dendrite damage, we used a Drosophila model system in which neuronal polarity, axon regeneration, and dendrite regeneration have been characterized. After removal of the axon and all but one dendrite, the remaining dendrite was converted to a process that had a long unbranched region that extended over long distances and a region where shorter branched processes were added. These observations suggested axons and dendrites could regrow at the same time. To further test the capacity of neurons to implement polarized regeneration after axon and dendrite damage, we removed all neurites from mature neurons. In this case a long unbranched neurite and short branched neurites were regrown from the stripped cell body. Moreover, the long neurite had axonal plus-end-out microtubule polarity and the shorter neurites had mixed polarity consistent with dendrite identity. The long process also accumulated endoplasmic reticulum at its tip like

regenerating axons. We conclude that neurons *in vivo* can respond to simultaneous axon and dendrite injury by initiating growth of a new axon and new dendrites.

Graphical abstract



Introduction

Most neurons use dendrites to receive signals and axons to send them, and function is lost if either type of process is damaged. As neurons typically cannot be replaced in mature nervous systems, repair pathways have evolved to allow neurons with damaged axons or dendrites to regain function. Axon injury and repair have been studied for over one hundred years, and some aspects of the axon injury response have been elucidated. After axons are severed, the portion of the axon distal to the cut site degenerates (Gerdts et al., 2016), and an injury signal is sent to the cell body (Abe and Cavalli, 2008; Rishal and Fainzilber, 2014). In many neuron types, including those in the vertebrate peripheral nervous system (PNS), and in invertebrates like Drosophila and C. elegans, this injury signal re-initiates axon growth (He and Jin, 2016; Mahar and Cavalli, 2018). In the vertebrate central nervous system (CNS), some neurons initiate growth in response to axon injury, either from the cut stump (Kerschensteiner et al., 2005) or from collateral

branches (Kerschensteiner et al., 2004). However, other CNS neurons fail to grow either because they do not mount the initial injury response or because their axons encounter an inhibitory environment (Curcio and Bradke, 2018; Liu et al., 2011). In vertebrate and invertebrate neurons that regenerate, the dual leucine zipper kinase (DLK) is a central component of the axon injury signal (Hammarlund et al., 2009; Shin et al., 2012; Xiong et al., 2010; Yan et al., 2009) that acts upstream of transcriptional changes that trigger regeneration in permissive cells (Mahar and Cavalli, 2018).

Although much less studied and much less understood, it has recently been demonstrated that- at least in Drosophila- neurons can initiate a program of dendrite regeneration after partial or complete removal of the dendrite arbor (DeVault et al., 2018; Song et al., 2012; Stone et al., 2014; Thompson-Peer et al., 2016). Although an injury-induced growth response, dendrite regeneration seems to use different molecular machinery than axon regeneration. It does not require DLK or its downstream transcription factors to initiate the injury response (Stone et al., 2014). Comparison of axon and dendrite regeneration in the same Drosophila cell type also revealed that axon regeneration requires endoplasmic reticulum (ER) localization to the growing axon tip, while dendrite regeneration does not (Rao et al., 2016). Further confirming this distinction between the two types of regeneration, dendrite regrowth requires the tyrosine kinase Ror, while axon regeneration does not (Nye et al., 2020). Thus, neurons seem to possess two distinct regeneration programs: one for axons and one for dendrites.

Some types of injury or stress are expected to damage axons and dendrites at the same time. For example, traumatic brain injury (TBI) can cause axon breakage followed by degeneration (Hill et al., 2016; Strich, 1956), and can also induce dendrite degeneration (Gao and Chen, 2011). Similarly, ischemic stroke can cause dendrite damage (Murphy et al., 2008; Risher et al., 2010) in addition to axon damage (Hinman, 2014). In the case of stroke, axon sprouting (Carmichael et al., 2017) and dendrite plasticity (Brown et al., 2007) are observed during

recovery, but it is unclear whether these derive from neurons that were damaged in the initial injury, or are part of a response by surrounding neurons.

If neurons survive the initial trauma to axons and dendrites, the next question is whether they can launch the axon and dendrite regeneration programs together. There are some hints that axon regeneration may antagonize dendrites. In some vertebrate neurons, axon injury causes retraction of dendrite arbors (Leung et al., 2011; Linda et al., 1992; Sumner and Watson, 1971; Wang et al., 2002) suggesting that axon injury responses including regeneration antagonize dendrite maintenance or growth. Similar retraction of dendrite arbors after axon injury has been observed in Drosophila neurons (Chen et al., 2012). From a more hypothetical perspective, both axon and dendrite regeneration require large-scale addition of membranes to the cell, and so regrowth of one type of neurite might well be expected to reduce the resources available for growth of the other type.

There are two scenarios where the outcome of simultaneous axon and dendrite damage has been established. One is in the generation of primary neuronal cultures. Preparation of cortical or hippocampal rodent cultures involves dissociation of embryonic neurons, which removes axons and dendrites. The remaining cell body is cultured with ample growth factors, and can successfully regrow axons and dendrites (Kaech and Banker, 2006). In this context of extra growth factors, neurons do survive and after an unpolarized phase axons are specified and grow, followed a few days later by dendrites (Bradke and Dotti, 2000; Dotti et al., 1988). The second example is amphid neurons in C. elegans, which have a sensory cilium rather than a branched dendrite arbor. These neurons are not capable of regenerating the sensory ending after it is removed, but simultaneous damage to this region and the axon stimulates extra outgrowth compared with axon injury alone (Chung et al., 2016). In this case, the extra outgrowth is not dependent on the standard DLK pathway.

Key unresolved issues include whether neurons with branched dendrites can survive simultaneous loss of axons and dendrites *in vivo*, and if they do survive, will they be able to regenerate both types of neurites? To address these issues we performed controlled injuries on the ddaE neuron in Drosophila larvae as this cell type has well-characterized responses to separate axon and dendrite injury. The ddaE neuron is a sensory neuron with a branched dendrite arbor that is positioned on the dorsal surface of Drosophila larvae and is a member of the simplest class of dendritic arborization neuron, Class I (Grueber et al., 2002). It is responsible for helping to coordinate movement (Hughes and Thomas, 2007) and signals in response to folding of the epidermis during larval crawling (He et al., 2019; Vaadia et al., 2019). Although it is a sensory neuron, ddaE has distinct axons and dendrites that are polarized in many of the same ways as mammalian central neurons. For example, axons have plus-end-out microtubule polarity, while branched dendrites contain minus-end-out microtubules (Hill et al., 2012; Stone et al., 2008), and dendrites contain protein synthetic machinery like ribosomes (Hill et al., 2012), while axons have little. Most importantly here, injury of ddaE axons triggers the conserved DLK/JNK signaling pathway (Rao and Rolls, 2017; Stone et al., 2014; Stone et al., 2010), which is used to initiate axon regeneration in many other neuron types from C. elegans (Hammarlund et al., 2009) to mice (Shin et al., 2012). Injury of dendrites does not initiate DLK signaling in these cells, and DLK, JNK and downstream transcription factor fos do not play a role in dendrite regeneration (Stone et al., 2014). In these neurons, laser-induced severing of the axon beyond approximately 50 microns from the cell body elicits classic axon regeneration from the severed stump (Rao and Rolls, 2017; Stone et al., 2012). In contrast, injury close to the cell body (within 20mm, see examples in Figure 1) converts a dendrite into a regenerating axon (Rao and Rolls, 2017; Stone et al., 2010), as described in mammalian neurons (Gomis-Ruth et al., 2008). In this study we severed axons close to the cell body as it allows length of the regenerating axon to be measured. ddaE neurons

also regenerate after removal of one or all dendrites, and recapitulate the same number of branch points that the arbor contained before injury (Stone et al., 2014; Thompson-Peer et al., 2016).

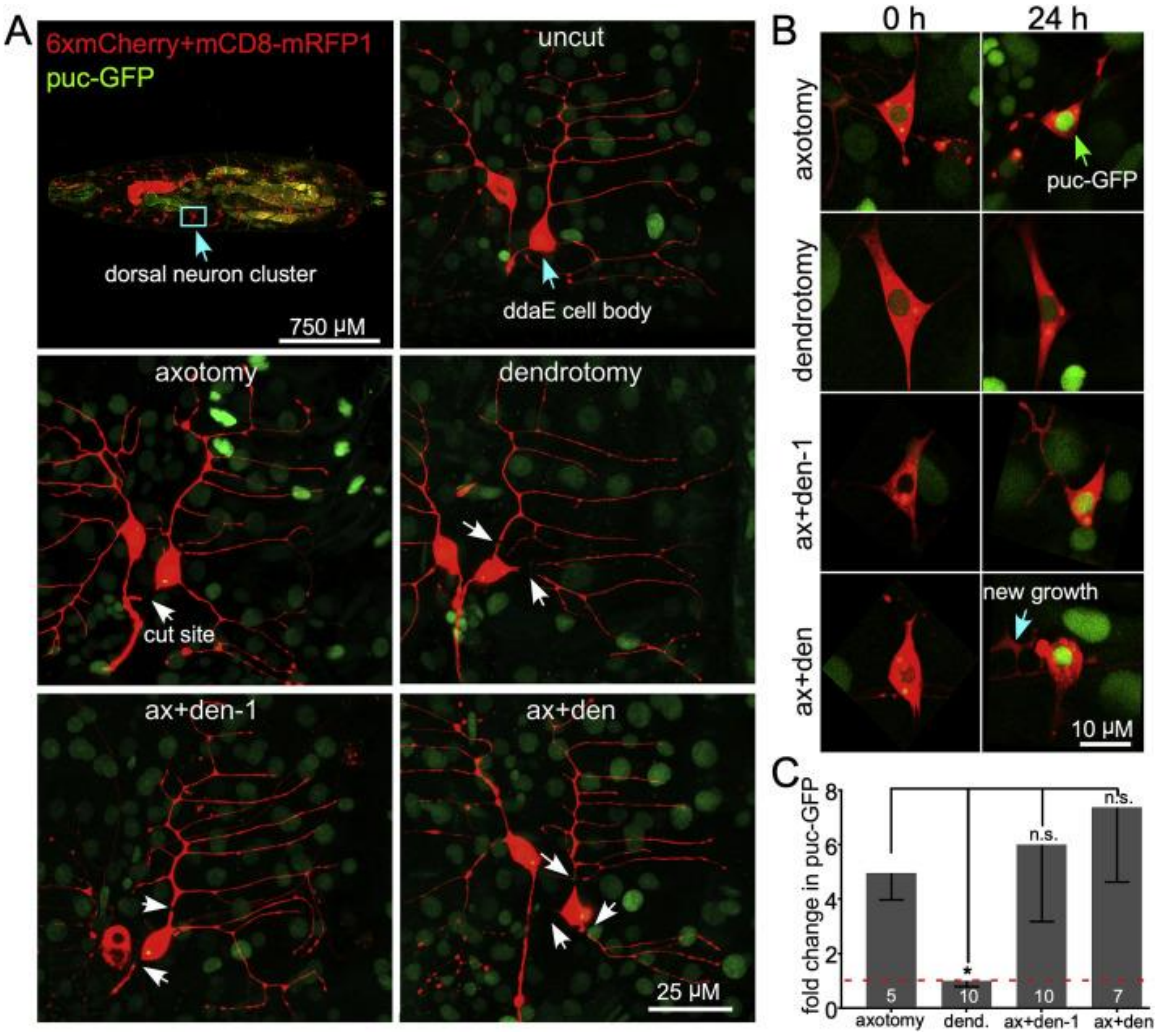


Fig. 1. **DLK pathway activation after axon, dendrite and combined injury**.
A. An overview of a Drosophila larva expressing mCD8-mRFP, and 6xmCherry under 221-Gal4, and GFP driven from the puc locus is shown in the top left panel. The box indicates a dorsal neuron cluster. The other panels show examples of ddaE neurons injured at different sites. White arrows indicate cut sites. **B.** Cell bodies of ddaE neurons expressing puc-GFP and red cell shape marker are shown immediately after injury and 24h later. Green arrow indicates the nuclear puc-GFP used to measure DLK pathway activation. The blue arrow points to new neurites growing out from the cell body. **C.** Quantification of puc-GFP change from 0 h post injury to 24h post injury in the indicated injury paradigm is shown. N for each condition is indicated within its column on the graph, error bars are graphed as the symmetric lower half of standard deviation. Asterisks denote statistical significance <0.001 as determined by unpaired two-tailed *t*-test.

Using this model system, we first investigated whether neurons of intact animals could survive simultaneous axon and dendrite injury. Surprisingly, we found that mature neurons survived removal of the axon and all dendrites and were able to initiate the conserved axon injury response pathway. We then tested whether neurons could regrow axons and dendrites over a longer timescale. We found that both types of neurites grew at the same time, and observed no reduction in growth compared to axon or dendrite regeneration alone.

Results

Neurons survive simultaneous axon and dendrite injury and initiate injury response signaling

To test whether ddaE neurons could survive injury to axons and dendrites at the same time, we performed different combinations of laser injuries in whole, intact Drosophila larvae. 221-Gal4 was used to express membrane-bound (UAS-mCD8-mRFP1) and soluble (UAS-6XmCherry) red fluorescent proteins (RFP) allowing for optimal visualization of cell shape and detection of severing. Incomplete cutting of an axon or dendrite could be detected by rapid recovery of mCherry into the region. Sites of axon and dendrite injury are indicated in Figure 1A. Images of neurons were acquired immediately after injury (0 h) and after 24h. Between time points animals were returned to normal growth conditions. As expected, ddaE neurons survived removal of the axon or all dendrites (Figure 1B). Survival was also observed in all cases when axon severing was paired with removal of all but one dendrite (ax+den-1). When the axon and all dendrites (ax+den), are removed all that remains is a free cell body beneath epidermal cells. Normally dendritic arborization neurons are attached to the body wall, in part by integrin-mediated

interactions between the dendrites and epidermal cells (Han et al., 2012; Kim et al., 2012; Yang and Chien, 2019). After stripping the neuron of all dendrites and the axon, the cell body tended to move. An example of this movement is evident in the 0 h cell body image (Figure 1B) from the double image of the axon stump captured in sequential frames. Some cell bodies drifted away into the body cavity, where we could no longer see them. By 24h after injury, denuded cell bodies that remained in place had processes emerging from the cell body (Figure 1B).

To determine whether axon injury signaling occurred normally when axon injury was paired with dendrite injury, we used a reporter of the DLK/JNK pathway. Puckered (puc) is a MAP kinase phosphatase that is transcriptionally upregulated after axon injury (Xiong et al., 2010). We used a GFP insertion in the *puc* gene (Morin et al., 2001) that results in GFP accumulation in the nucleus after axon injury (Stone et al., 2014) as a readout of DLK/JNK activation. As previously reported, axon- but not dendrite- injury resulted in increased puc-GFP signal in the nucleus (Figure 1B and 1C). Similar increases were observed when axon injury was paired with dendrite injury (Figure 1B and 1C) indicating that dendrite injury does not interfere with axon injury signaling. We conclude that ddaE neurons can survive injury to both axons and dendrites, and even removal of all neurites. Moreover, axon injury signaling is similarly initiated when axons are injured alone or in conjunction with dendrites.

Neurons can regrow long unbranched and short branched processes at the same time

The ability of neurons to survive simultaneous axon and dendrite injury prompted us to investigate whether they could regenerate both types of neurites over longer time frames. We began by monitoring growth after leaving a single dendrite (ax+den-1) as this condition allowed neurons to remain anchored to the epidermal cells and left a dendrite as a substrate for conversion to an axon. Control axotomy and dendrotomy experiments, where either the axon or all dendrites

were removed (middle panels of Figure 1A), were performed for comparison. In this study, unlike most of our previous studies, we killed the neighboring class IV ddaC neuron by aiming the pulsed UV laser at the cell body. The Gal4 driver used in this study results in some expression in the ddaC cell, whose dendrites overlap those of ddaE. In some cases, tracing regenerating ddaE neurites is confounded by overlapping ddaC dendrites, and this problem is eliminated by killing ddaC. After severing the ddaE axon close to the cell body in conjunction with ddaC ablation, a dendrite was converted to a regenerating axon as previously described (Figure 2A and (Stone et al., 2010)). Note one difference from our previous studies is the temperature at which animals were incubated between axon injury and monitoring outgrowth. In previous studies animals were incubated at 20C and imaged 96h after injury, while here animals were incubated at 25C and imaged after 72h. Development and growth occurs faster at 25C than 20C and so the two different time points are similar, though not identical. Under the conditions used in this study, new outgrowth from the tip of the original dendrite averaged 180 microns by 72h after injury (Figure 2D). Dendrite regrowth after removal of the entire dendrite arbor was also similar to that previously reported for these cells (Stone et al., 2014; Thompson-Peer et al., 2016), and the arbor regrew to the same complexity as before injury (Figure 2B and 2E). Uninjured ddaE neurons do not add new branches during the time window used (Stone et al., 2014; Sugimura et al., 2003), so branch addition represents a response to injury.

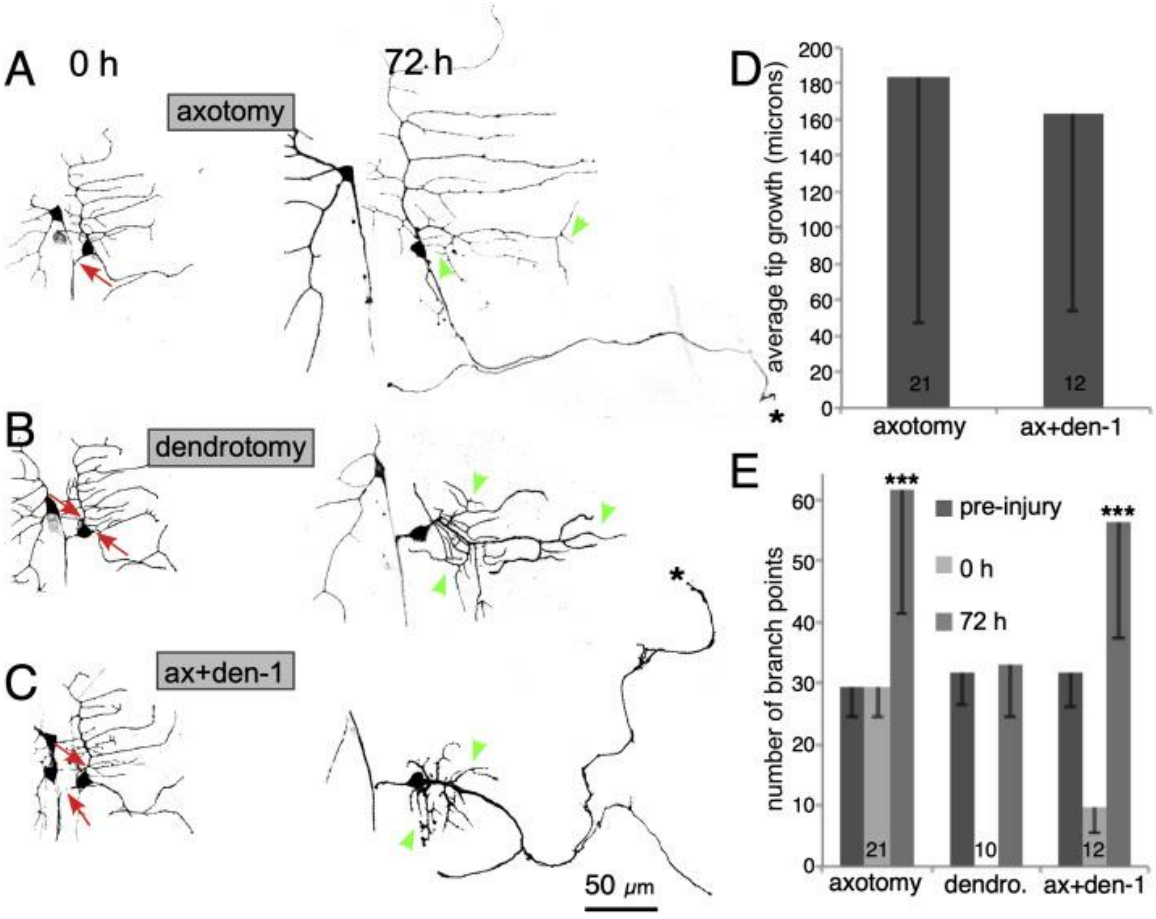


Fig. 2. **Analysis of neurite regrowth after different types of injury**.
A-C. 221-Gal4 was used to express mCD8-mCherry and EB1-GFP in ddaE neurons. Example images of mCD8-mCherry are shown at 0 h–72 h post injury. Red arrows indicate cut sites, green arrows point to new branches and the star indicates the tip of the growing axon. **D.** Quantification of tip growth in the axotomy and ax + den-1 injury paradigms is shown. See methods for a description of how measurements were made. **E.** Quantification of branchpoint addition in the axotomy, dendrotomy, and ax + den-1 paradigms. N for each condition is indicated within its column on the graph, error bars are graphed as the symmetric lower half of standard deviation. Asterisks denote statistical significance <0.001 as determined by unpaired two-tailed *t*-test.

Having recapitulated earlier studies that showed ddaE neurons could regenerate axons and dendrites separately, we injured axons and dendrites simultaneously, while leaving one dendrite intact (ax+den-1). The remaining dendrite initiated growth from its tip (Figure 2C) and was able to extend a similar length as neurons with only axon injury (Figure 2D). At the same time new branch points were added near the cell body (Figure 2C). The final number of branch

points was similar to that when the axon was injured alone. Although, many additional branch points had to be added to reach this point when the comb dendrite was removed with the axon (Figure 2E). We conclude that ddaE neurons can not only survive contemporaneous axon and dendrite injury, but can extend a long neurite as well as add short neurite branches at the same time, suggesting that both axons and dendrites can regenerate simultaneously.

One result that we had not anticipated was the increase in dendrite branching in response to axon injury (Figure 2E). New branches typically initiated from existing dendrites and were quite short (Figure 2A). Similar excess branching was observed when axon injury way paired with removal of one dendrite (Figure 2E). As the addition of new dendrite branches in response to axon injury was unexpected, we considered several explanations. One possibility was that induction of growth by axon injury could result in membrane addition to dendrites if the axon was unable to extend. Support for this idea was provided by a negative association between new axon length and branch number (Figure 3A-3D). We also considered the possibility that tissue damage caused by ddaC ablation could increase dendrite branching. Some support for this possibility was obtained from experiments in which ddaC neurons were ablated in the absence of ddaE axon injury. In this case a small increase in ddaE dendrite branch number was observed (Figure 3E-3G). When the percent increase was compared to that of ddaE neurons without ddaC ablation, there was a trend (p-value 0.06) towards having a slightly higher increase in branching with ddaC ablation. Thus, there may be a minor effect of adjacent injury on branching, and likely some relationship between low amount of axon outgrowth and higher levels of branching.

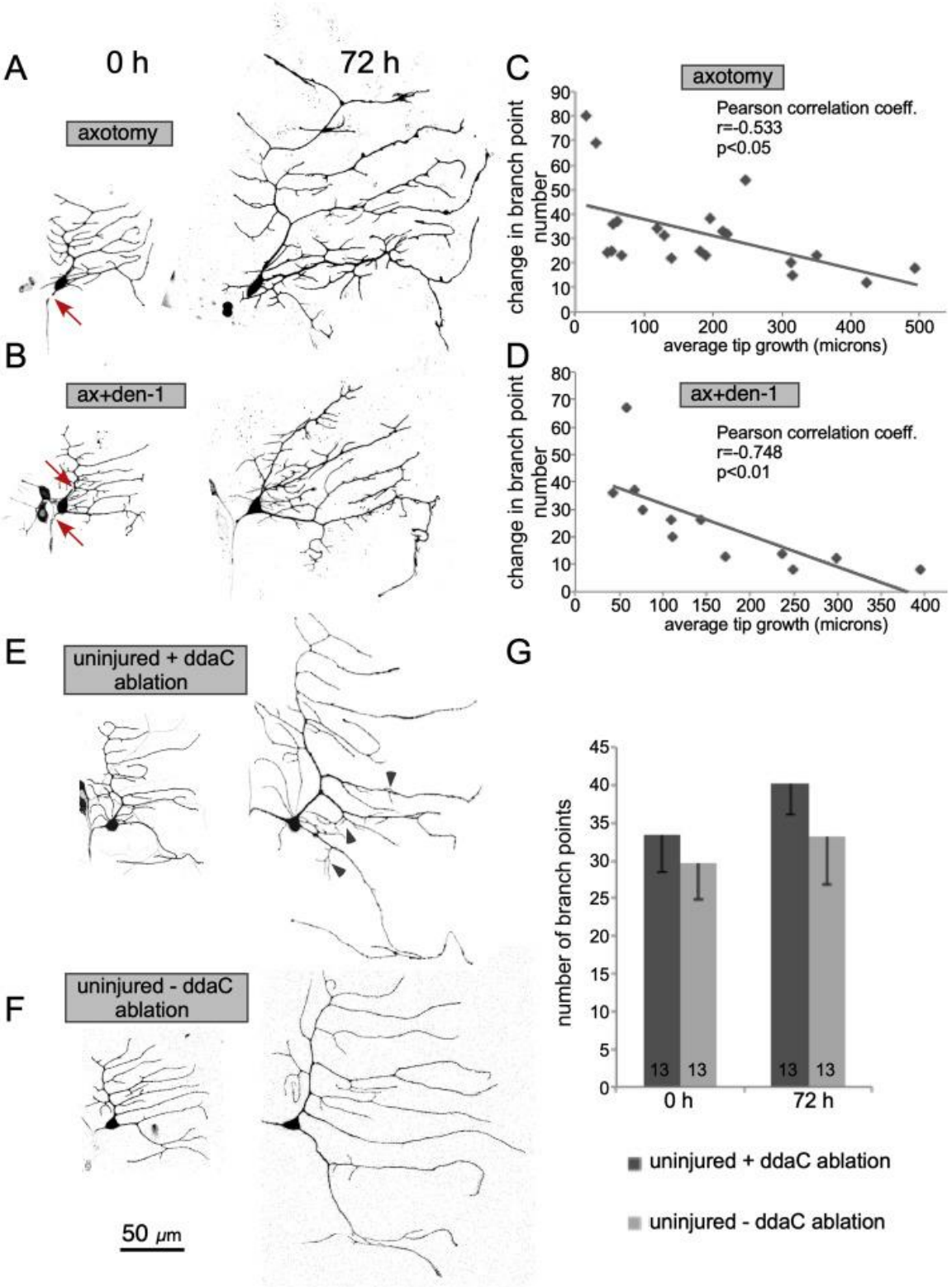

A
0 h
72 h
axotomy
B
ax+den-1
C
axotomy
Pearson correlation coeff.
r=-0.533
p<0.05
change in branch point number
average tip growth (microns)
D
ax+den-1
Pearson correlation coeff.
r=-0.748
p<0.01
change in branch point number
average tip growth (microns)
E
uninjured + ddaC ablation
F
uninjured - ddaC ablation
50 μm
G
number of branch points
13
13
13
13
0 h
72 h
uninjured + ddaC ablation
uninjured - ddaC ablation

Fig. 3. **The relationship between increases in ddaE branching and axon regrowth or ddaC ablation**.
A and B. Representative images of ddaE neurons 0 h and 72 h after the indicated injury type. Neurons expressed EB1-GFP and mCD8-mCherry, but only the mCherry channel is shown. Red arrows indicate cut sites. **C.D.** Graphs showing the relationship between branchpoint addition and axon outgrowth in the indicated injury type. **E.F.** Representative images showing the morphology of uninjured ddaE neurons at 0 and 72 h with and without ddaC ablation. **G.** Quantification of branchpoint addition in uninjured neurons from 0 to 72 h following with and without ddaC ablation. N for each condition is indicated within its column on the graph, error bars are graphed as the symmetrical lower half of standard deviation.

New processes with axonal and dendritic microtubule polarity are generated after complete neurite removal

We next wished to determine whether neurons could regrow neurites after complete removal of the axon and all dendrites, and if so, to probe identity of the regrowing processes. While we knew that axons could survive at least 24h after removal of all neurites (Figure 1), we hypothesized that regrowing all neurites from an isolated cell body would be more challenging than using a remaining dendrite as a platform for regeneration. To our surprise, a single long process as well as shorter branched processes emerged from denuded cell bodies (Figure 4A). The long processes grew, on average, over 300 microns (Figure 4B), which is comparable to the amount of regrowth following axon removal alone (Figure 2D). The absolute amounts of growth are difficult to compare across these two injury paradigms as conversion of a dendrite to a new axon results in growth of a pre-existing neurite from the tip. As the animal grows larger during larval development, the pre-existing part of the neurite will also expand as it does in uninjured animals. We therefore normalize growth from pre-existing neurites to account for body expansion and get a better estimate for outgrowth from the tip (see Methods). When all neurites are removed, the new process grows de novo and so the measurement is an absolute value of outgrowth from the cell body. Denuded neurons also regrew branched processes, and as with dendrite removal alone,

the pre-injury complexity was recapitulated by 72h (Figure 4A and 4C). Thus, even after removal of all neurites, cells were able to regrow a single long neurite as well as shorter branched ones.

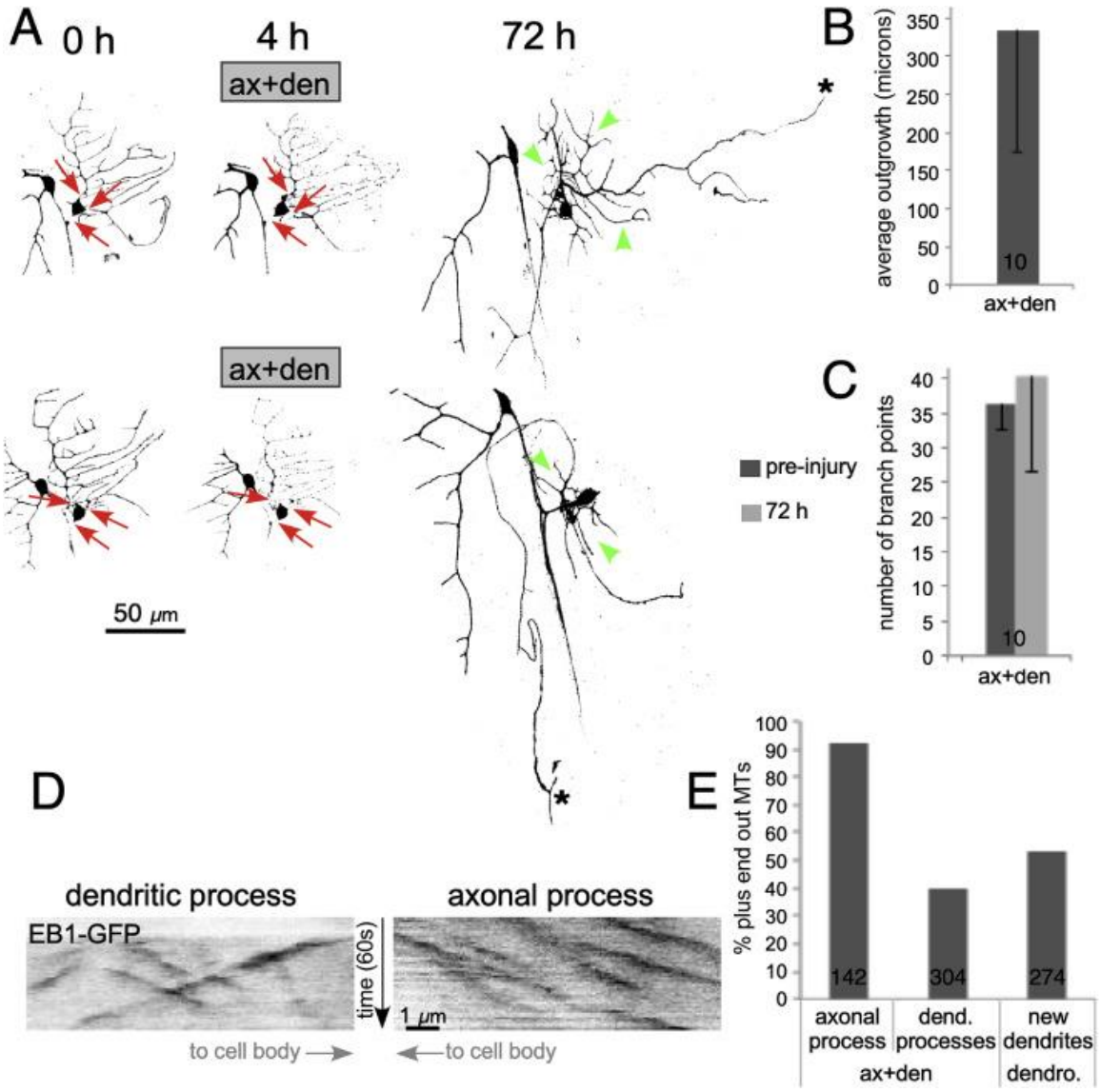


Fig. 4. **Analysis of ddaE neurons after removal of all neurites**.
A. ddaE neurons expressing EB1-GFP and mCD8-mCherry were subjected to complete neurite removal at 0h; images of mCD8-mCherry are shown. Cut sites are indicated with red arrows. Neurons were imaged 4h later (middle row) to confirm severing. Neurons were imaged again at

72h to monitor regeneration. Green arrows indicate new dendrite-like growth. Asterisks indicate the extending tip of the regenerating axon-like neurite. **B.** Quantification of absolute growth of the axon-like neurite during the 72 h post injury is shown. **C.** Quantification of branchpoint addition during the 72 h post injury is shown. **D.** Kymographs of EB1-GFP time series were generated from the new dendrite-like and axon-like processes 72h after injury. **E.** Quantification of microtubule polarity at 72h after injury in the new dendrite-like and axon-like processes after denuding, and in regenerating dendrites after dendrotomy is shown.

Based on morphology, we hypothesized that the long neurite was a regenerating axon and the shorter, branched ones were new dendrites. One of the most universal features that distinguishes axons and dendrites is microtubule polarity. In all species where it has been examined, the polarity of axonal microtubules is plus-end-out, meaning the more rapidly growing plus ends are oriented away from the cell body. In contrast, dendrites contain a significant population of minus-end-out microtubules (Baas and Lin, 2011; Rolls and Jegla, 2015). Mature ddaE neurons have axons with greater than 95% plus-end-out polarity and dendrites with greater than 90% minus-end-out polarity (Stone et al., 2008). To test whether polarity was reestablished after removal of all neurites, we assayed microtubule polarity by tracking movement of EB1-GFP. EB1 is recruited to microtubule plus ends as they grow (Akhmanova and Steinmetz, 2015; Jiang and Akhmanova, 2011), and direction of growth can be used to map microtubule polarity in neurons (Stepanova et al., 2003). Consistent with the long neurites having axonal identity, EB1-GFP comets moved almost exclusively away from the cell body in plus-end-out orientation (Figure 4D and 4E). The shorter branched neurites had comets moving in both directions (Figure 4D and 4E) and an overall polarity of about 60% minus-end-out microtubules, which is similar to new dendrites that are generated after removal of ddaE dendrites (Figure 4E). This difference in microtubule polarity supports the idea that both axons and dendrites can be correctly specified after removal of all neurites.

The endoplasmic reticulum concentrates at the regenerating axon tip in all injury paradigms

Thus far our data suggested that axon and dendrite regeneration could occur simultaneously after damage to both types of processes. To further test neurite identity, we assayed the distribution of an endoplasmic reticulum (ER) protein during different types of regeneration. Although the ER forms a continuous network throughout neurons, it becomes particularly concentrated at tips of regenerating axons, but not regenerating dendrites (Rao et al., 2016). One marker that can be used to track this distribution is Rtnl1-GFP (Rao et al., 2016). We expressed this tagged protein together with the plasma membrane marker mCD8-mCherry and analyzed its distribution after different injury combinations. As expected after axon removal, Rtnl1-GFP concentrated at tips of regenerating axons (Figure 5A). Compared to the base, regions near the tip of the growing neurite were about two-fold brighter. The remaining dendrites had roughly constant Rtnl1-GFP fluorescence along their length (Figure 5D). Similar fluorescence patterns were observed after axon injury was paired with removal of all, or all except one, dendrites (Figure 5B-D). The long neurite typically had a region of bright Rtnl1-GFP accumulation, while the only accumulations in the shorter, branched processes were slight increases at dendrite branch points, similar to those seen at branch points of dendrites that were not growing (Figure 5A). This difference in distribution of Rtnl1-GFP between the long neurite and short branched ones is consistent with them taking on axonal and dendritic identity respectively. We conclude that neurons respond to simultaneous axon and dendrite injury by initiating both regeneration pathways concurrently.

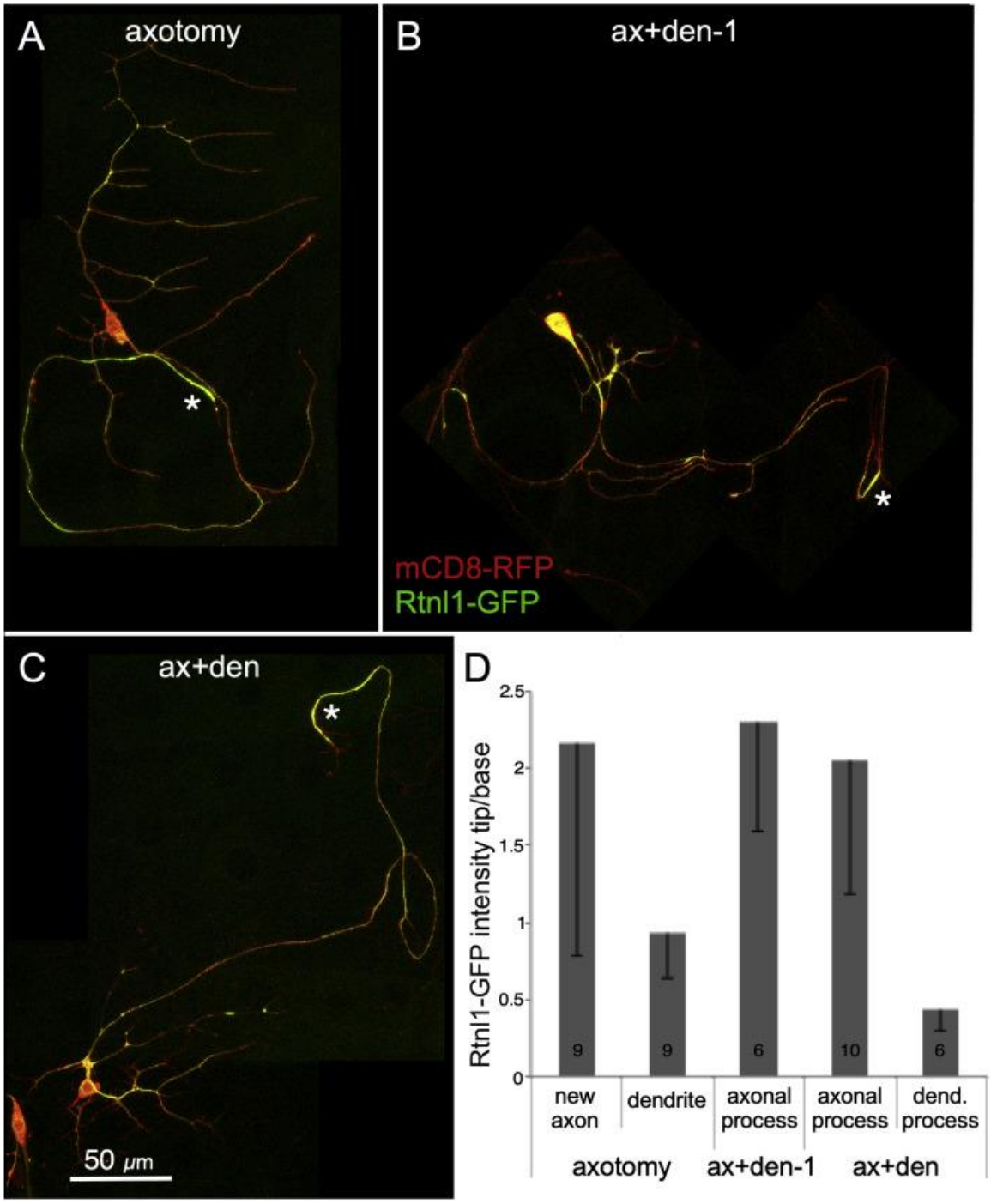


Fig. 5. **Rtnl1-GFP enriches at the tip of the growing axon-like neurite, but not the shorter dendrite-like neurites.A-C.** ddaE neurons expressing mCD8-mCherry and Rtnl1-GFP were injured at time 0 and imaged 72 h later. Images of neurons after different types of injury from the 72 h timepoint are shown. Asterisks denote the tip of the long axonal process. **D.** Quantification of the enrichment of Rtnl1-GFP in tips of regenerating neurites in the indicated injury paradigms. N for each condition is indicated within its column on the graph, error bars are graphed as the symmetrical lower half of the standard deviation.

Key resources table

Reagent or resource	Source	Identifier
Experimental Models: Organisms/Strains		
221-Gal4	Wesley Grueber	Gift
Puc-GFP	FlyTrap Project	G00426, Morin et al. 2001
UAS-mCD8-mRFP	Bloomington Drosophila Stock Center	BL27398
UAS-6xmCherry	Bloomington Drosophila Stock Center	BL52268
UAS-Rtnl1-GFP	Rolls Lab	Rao et al. (2016)
UAS-EB1-GFP	Tadashi Uemura	Gift
UAS-mCD8-mCherry	Bloomington Drosophila Stock Center	BL27392
Software and Algorithms		
ImageJ	https://imagej.net/Fiji	Schindelin et al. (2012)

Discussion

Using model neurons that have been shown to regenerate axons and dendrites individually, we determined that they could survive and regenerate after complete removal of all neurites. The neurons were able to reinitiate outgrowth of two different types of neurites at the same time. Moreover, coupling dendrite injury with axon injury did not reduce the amount of axon outgrowth by the cell or impair axon injury signaling. The two types of regenerating neurites were classified as axons and dendrites based on microtubule polarity and ER accumulation. We

conclude that mature, functional neurons can recover from complete axon and dendrite removal *in vivo* and reinitiate polarized outgrowth of axons and dendrites.

After complete neurite removal the regenerating axon was identical to regenerating axons converted from dendrites after proximal axotomy, with plus-end-out microtubule polarity and ER concentrated near the growing tip. However, regenerating dendrites did not attain minus-end-out polarity. During embryonic development ddaE neurons have a mixed polarity phase, and this resolves to about 90% minus-end-out in the large dorsal comb dendrite during larval stages (Feng et al., 2019; Hill et al., 2012). During dendrite regeneration in large ddaC neurons, microtubule polarity is initially mixed, but by 24h after dendrite removal is mostly minus-end-out (Feng et al., 2019; Stone et al., 2014). One explanation for the lack of resolution to minus-end-out in regenerating ddaE dendrites is the difference in branch organization in the regenerated dendrite arbors compared to the ddaE comb dendrite or ddaC dendrites where polarity was previously analyzed. In the comb dendrite and ddaC dendrites, many branch points are found along each dendrite. The regenerated ddaE dendrites tend to emerge in more of a star shape close to the cell body and are quite short. As dendrite branch points are key control points for uniform microtubule polarity (Mattie et al., 2010), it is possible the different branching pattern could impact the ability to reinforce polarity.

In other neuron types, including ddaC neurons, simplification of dendrites is observed after axon injury (Chen et al., 2012). This response was not observed in ddaE neurons (Figure 2), and instead short branches were added to dendrites. The lack of retraction could be due to the lack of plasticity of ddaE dendrites compared to ddaC. During larval life, the large, complex arbor of ddaC undergoes branch addition and retraction, with a net increase in branching (Sugimura et al., 2003). In contrast, the shape of ddaE is fixed very early in larval life (Stone et al., 2014; Sugimura et al., 2003). If axon injury shifts the balance of retraction and addition in ddaC, net simplification could occur. With no ongoing dynamics in ddaE, more active removal of branches

would need to take place. The branch addition we observed in ddaE seemed related to two different phenomena: first, an inverse relationship with the amount of axon outgrowth, and second, additional damage due to the ablation of a neighboring cell.

The ability of ddaE neurons to survive complete removal of all neurites is similar to that of embryonic cortical and hippocampal neurons dissociated from brains and recovered in culture. In culture, media is enriched to promote survival. Here the cell body has to rely on endogenous growth factors. ddaE neurons are normally closely associated with the epidermis and receive signals from epidermal cells during development (Li et al., 2016). It is possible that epidermal cells could increase growth factor production to support the cell after injury. Alternatively, ddaE neuron survival may not depend on trophic factors from surrounding cells.

The temporal pattern of ddaE outgrowth after complete denuding is slightly different from that of primary neuronal cultures or ddaE neurons during development. Cultured neurons first grow short unspecified processes, one of which becomes the axon at day two or three. The axon then grows, while the unspecified processes wait for several more days before initiating growth as dendrites (Craig and Banker, 1994; Dotti et al., 1988). During embryonic development, ddaE neurons also send out axons well before dendrite growth is initiated (Feng et al., 2019; Hill et al., 2012). After removal of all neurites, however, dendrite regrowth initiated rapidly, and all neurons had short branched processes like those seen in Figure 1B at 24h after injury. Thus, unlike primary neuronal cultures or embryonic ddaE neurons, ddaE neurite regrowth does not proceed through sequential polarization with a first axon-only growth phase followed by emergence of dendrites.

Overall, one striking aspect of the response to axon and dendrite removal was its robustness. Despite axon regeneration requiring reinitiation of large-scale outgrowth, it was not dampened by simultaneous regrowth of dendrites. This indicates that even though axon regeneration is itself impressive, it does not saturate the growth capacity of the neuron. Indeed,

the ability of ddaE neurons to regenerate at all is somewhat surprising. These neurons function primarily during larval life where they respond to epidermal folding as the larva crawls (He et al., 2019; Vaadia et al., 2019). In some specific segments ddaE neurons die during pupariation, while in others they survive, remodel and are observed in young adults; all are gone by the end of the first week of adult life (Shimono et al., 2009). Despite the relatively short utility of these neurons during the lifespan, they are able to survive and regenerate when they are injured.

If even transient ddaE neurons can survive complete neurite removal and initiate polarized regeneration, it seems likely that this capacity exists in other neuron types as well. It will be extremely interesting to probe other cell types in invertebrates, and also vertebrates. So far, however, it has not even been possible to determine whether vertebrate neurons can regenerate *in vivo* after dendrite removal. Vertebrate neuron cell bodies are typically protected under bone and so controlled injuries to dendrites will require overcoming technical hurdles. However, survival of neurons after simultaneous axon and dendrite damage could play a role in the plasticity that enables improvement after stroke, and perhaps also TBI.+

Acknowledgements

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Methods

Fly culture

All flies used for this study were maintained at 25C on cornmeal agar media with a per liter composition of: 4.5g agar, 15.5g yeast, 25.9g sucrose, 51.7g dextrose, 85.8g cornmeal, 4ml of propionic acid and 6ml tegosept. Flies used for experiments in this study were moved to fresh media every 24 hours to facilitate selecting appropriate age offspring, and the media with eggs was aged for 48h at 25C to generate larvae for imaging.

Fly lines

Two different tester lines were used in this study. For the puc-GFP assay, the tester line was UAS-mCD8-RFP; 221-Gal4, puc-GFP. All other assays used the 221-Gal4, UAS-mCD8-mCherry tester line. Virgin females were collected from each tester line and crossed to the appropriate males for each experiment. Males from the following lines were used: 20xUAS-6xmCherry, UAS-EB1-GFP, and UAS-Rtnl1-GFP. Information about each line is in the Key Resources Table.

puc-GFP assay

Virgin females from the UAS-mCD8-RFP; 221-Gal4, puc-GFP tester line were crossed to males from the 20xUAS-6xmCherry line. Whole, live two-day old larvae were selected and mounted for imaging on a plain glass slide. A 22x40 mm coverslip was placed over the larva and held down with tape to prevent larval movement. A Zeiss inverted LSM800 equipped with an Andor MicroPoint UV pulsed laser was used for neurite severing and imaging. All images were acquired

using GaAsP detectors. For GFP quantification, cells were imaged with a Zeiss 63x 1.4NA oil immersion objective set to a zoom of 3, a dwell time of 2.06 microseconds per pixel, 2x averaging, a pinhole of 43 microns and a 512x512 image size. The ddaE neurons were subjected to the indicated type of axon and/or dendrite damage, and a post injury z-stack image was acquired. Larvae were then incubated at 25°C on fly media for 24 hours before being re imaged. For quantification, the brightest 3 slices from each Z stack were manually aligned and combined using the maximum projection feature of ImageJ. Quantification shows fold change in nuclear GFP intensity from 0 hours post injury to 24 hours post injury.

Regeneration assays

Virgin females from the 221-Gal4, UAS-mCD8-mCherry tester line were crossed to males from the UAS-EB1-GFP line. Whole, live two-day old larvae were mounted on a glass slide with dried 3% agarose pad. A 22x40 mm coverslip was taped down on top of the animal with enough pressure to hold it still, but not damage it. Laser injury was performed on a Zeiss widefield AxioImager.M2 equipped with an Andor Micropoint UV pulsed laser. Sites of axon and dendrite injury in the ddaE neuron for each of the paradigms are shown in Figure 1A. Class IV ddaC neurons were killed by concentrating the pulsed UV laser at the nucleus to eliminate overlapping dendrites that might make it difficult to trace ddaE regrowth. After injury, mounted animals were moved to a Zeiss LSM 800 upright microscope and imaged using a 63x 1.4 NA oil objective and GaAsP detectors. After imaging, larvae were returned to fresh Drosophila food and kept at 25°C until they were remounted for follow up imaging at 72h. For the ax+den condition larvae were also imaged at 4h to ensure the cell was completely denuded.

Quantification of axon regeneration

Average tip growth for the regenerating axon in axotomy alone was calculated by measuring the length of the dendrite that initiated tip growth immediately after severing and again at 72hrs. Another dendrite that did not initiate tip growth was also measured to account for normal developmental growth. Tip growth beyond normal growth (regeneration) was calculated using the following formula:

$$\text{Regeneration}_{\text{tip growth}} = \text{Length}_{\text{new axon}} - [\text{Length}_{\text{0h}} / (\text{Length}_{\text{0h control dendrite}} / \text{Length}_{\text{72h control dendrite}})]$$

Average tip growth for the regenerating axon in ax+den-1 was quantitated using the same formula except the average ratio of $\text{Length}_{\text{0h control dendrite}} / \text{Length}_{\text{72h control dendrite}}$ from the axotomy alone experiments was used to account for normal developmental growth since the comb dendrite where a control dendrite is normally found has been removed in this condition.

Average tip growth for the regenerating axon in ax+den was quantitated by measuring total outgrowth from the cell body of the longest neurite+

Quantification of dendrite regeneration

Dendrite regeneration was quantitated by counting all visible branch points in the dendrite arbor at 0h and 72h. Average number of branch points at each time point was plotted.

Microtubule polarity assay and kymograph generation

Microtubule polarity was assayed in regenerating neurites after ax+den injury and regenerating dendrites after dendrotomy by tracking the movement of EB1-GFP comets either towards or away from the cell body. EB1-GFP videos to determine polarity were captured at 1 frame per

1.17, 0.93 or 1.86 seconds. Analysis was done using Image J software and only comets that were visible in three consecutive frames were scored. Kymographs were made using the Multi Kymograph Image J plugin, using a line width of 1.

Rtnl1 localization assay

Virgin females from the 221-Gal4, UAS-mCD8-mCherry tester line were crossed to males from the UAS-Rtnl1-GFP line and regeneration assays were performed as described above. Rtnl-GFP intensity in the regenerating axon/axonal processes from the axotomy only, ax+den-1, and ax+den conditions were measured using the previously described method (Rao et al., 2016). For regenerating axon/axonal processes, the brightest 10mM within 100mM regions at the tip and base were used for analysis. For the regenerating dendritic process, the brightest 10mM within 20mM regions at the tip and base of the longest regenerating dendritic process were used. Intensity measurements were performed using ImageJ (Schindelin et al., 2012).

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Chapter 2 supplemental information (unpublished)

Full removal of all neurites from ddaC neurons

Though it was not ready in time for the submission of the publication, we were able to observe survival and regeneration of ddaC neurons after full removal of all neurites. 72 hours after egg laying larva were underwent laser microsurgery with a UV pulse laser to remove all neurites from ddaC neurons, and were incubated at 25C for 72 hours, after which they were imaged. We

observed that the cells became very dim (figure s1A), which is congruent with published results showing the PPK promoter to be negatively regulated by DLK signaling (Wang et al., 2013). The ddaC neurons displayed a great deal of extension of a relatively simply axon like neurite, but growth was so often into areas of the larva with brighter ddaC innervation that it was not practical to track (Figure s1B). The ddaC neurons also regenerated dendrite like neurites, but these were relatively small compared to pure-dendrite injury where they would be expected to have recovered most to all of their territory by this time (Stone, Albertson, Chen, & Rolls, 2014) (Figure s1C). This was not entirely unexpected as ddaC neurons are known to have their dendritic arbor regulated by factors relating to axon injury, with studies showing that axotomy causes dendritic retraction in ddaC neurons (Chen, Stone, Tao, & Rolls, 2012), and that DLK signaling silences the expression of the transcription factor Knot (Wang et al., 2013), which is partially responsible for their morphology (Jinushi-Nakao et al., 2007). This suggests that the impaired dendrite regeneration in this injury paradigm may specific to cell relying on this or related factors for their pre-injury morphology.

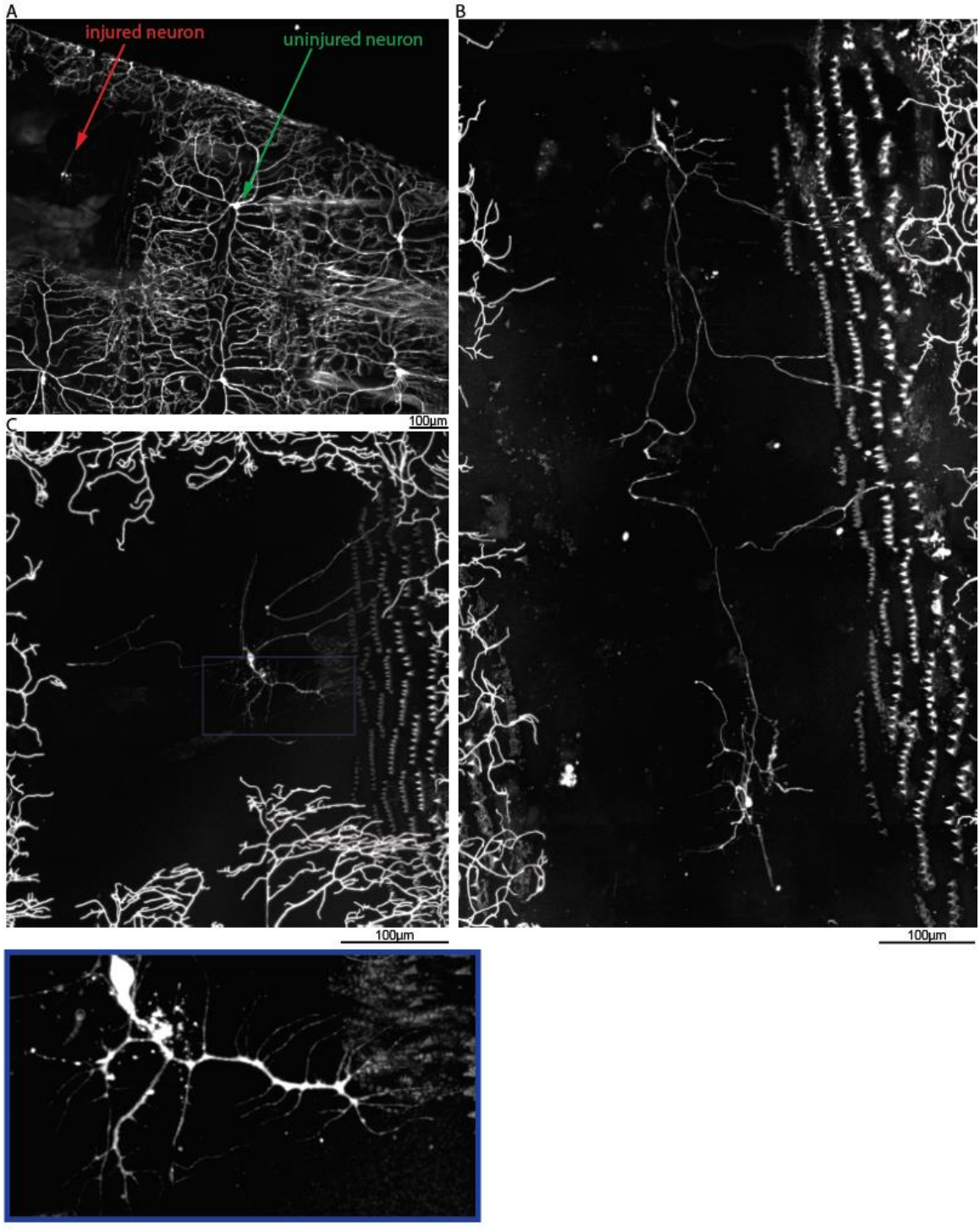
A
injured neuron
uninjured neuron
100µm
B
100µm
C
100µm

Figure s1: ddaC neurons survive and regenerate morphologically axon and dendrite like compartments after full removal of all neurites. A. 10x overview of day 6 Drosophila larva showing the effect of full removal of all neurites on the activity of the PPK promoter used to visualize cells. **B**. ddaC neurons engage in aggressive axon-like outgrowth by 72hpi at 25 Celsius**. C.** After full removal of all neurites ddaC neurons develop branched dendrite like neurites. Blue inset higher magnification to display branched morphology.

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Preface to chapter 3

Chapter 3 is a project that was published in the June 2021 of the journal Developmental Biology. The first author of this manuscript is Matthew Shorey, and contributing authors are Kavitha Rao, Michelle Stone and Floyd Mattie. PIs for the project were Alvaro Sagasti and Melissa Rolls. The manuscript was written by Matthew Shorey and Melissa Rolls. Kavitha Rao performed the mosaic characterization of RB neurons in day 1-4 zebrafish embryos, and Michelle Stone performed the imaging of the Nematostella vectensis neuron. All other work was performed by Matthew Shorey. This project contributes to the thesis by providing a more thorough characterization of the microtubule cytoskeleton of vertebrate sensory neurons than has previously been performed, by covering multiple cell types, developmental timepoints from embryo out to maturity, and examining the entirety of the peripheral portion of the neuron.

The microtubule polymerization videos from the paper have not been included, and I would strongly encourage anyone reading this with an interest in this chapter to view them online at https://www.sciencedirect.com/science/article/pii/S0012160621001494

Unpublished supplementary information:
I have also included at the end of the supplement an alternate graphic for displaying EB activity that was not included in the publication, but still may be informative as it contains more information than the simple profile plot.

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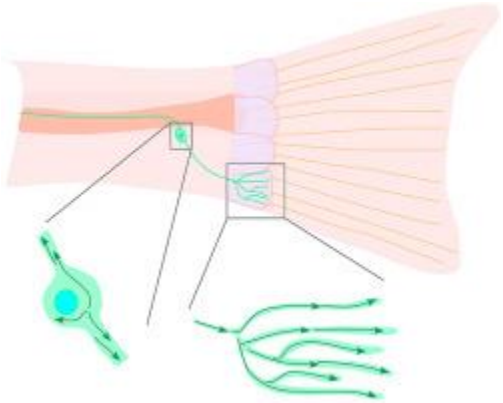
Chapter 3

Microtubule organization of vertebrate sensory neurons *in vivo*

Graphical abstract



Abstract

Dorsal root ganglion (DRG) neurons are the predominant cell type that innervates the vertebrate skin. They are typically described as pseudounipolar cells that have central and peripheral axons branching from a single root exiting the cell body. The peripheral axon travels within a nerve to the skin, where free sensory endings can emerge and branch into an arbor that receives and integrates information. In some immature vertebrates, DRG neurons are preceded by Rohon-Beard (RB)

neurons. While the sensory endings of RB and DRG neurons function like dendrites, we use live imaging in zebrafish to show that they have axonal plus-end-out microtubule polarity at all stages of maturity. Moreover, we show both cell types have central and peripheral axons with plus-end-out polarity. Surprisingly, in DRG neurons these emerge separately from the cell body, and most cells never acquire the signature pseudounipolar morphology. Like another recently characterized cell type that has multiple plus-end-out neurites, ganglion cells in Nematostella, RB and DRG neurons maintain a somatic microtubule organizing center even when mature. In summary, we characterize key cellular and subcellular features of vertebrate sensory neurons as a foundation for understanding their function and maintenance.

Introduction

Dorsal root ganglion (DRG) neurons mediate the majority of touch sensory input for vertebrates. The cell bodies of DRG neurons reside in ganglia adjacent to the spinal cord and extend neurites into the spinal cord and into the periphery where they innervate the skin (Lumpkin et al., 2010; Nascimento et al., 2018). Sensory endings embedded in the skin receive and integrate information and transmit it centrally to interneurons in the spinal cord. The sensory endings thus function like many dendrites as the input region of the neuron. Despite these neurons having been originally characterized in the 1800s, with thousands of papers published since, it is still unclear if DRG sensory endings possess any of the cellular features that distinguish dendrites from axons.

Whether DRG sensory endings have cellular features of axons or dendrites is more than an academic or semantic question. Cargo is transported into axons and dendrites using different machinery (Harterink et al., 2018; Kapitein et al., 2010; Zheng et al., 2008), and disruption of neuronal transport has been linked to several types of neurodegenerative disease (Guillaud et al., 2020; Prior et al., 2017). In addition, injured dendrites do not use the same machinery as axons to

sense damage and initiate regeneration (Stone et al., 2014), so determining whether DRG sensory endings have axonal or dendritic features could inform our understanding of how they regenerate. Efficient cargo transport and injury sensing are both particularly important for long-term DRG function. To sense the environment, their endings need to be positioned such that they are exposed and vulnerable. They are thus particularly vulnerable, and failure to maintain sensory endings leads to peripheral neuropathy. In 2016 alone 130,000 lower extremity amputations were performed due to downstream effects of sensory neuron impairment in diabetes. In order to understand sensory ending maintenance and regeneration, it is critical to know whether this compartment shares the basic features that underlie transport and injury responses with axons or dendrites.

The stereotyped neuron presented in biology classes resembles a motor neuron, and defines dendrites as being arborized and post-synaptic, and axons as singular and unbranched until reaching their terminus, where they are presynaptic and signal to the downstream cell (Alberts et al., 2007). Even 60 years ago this model was recognized as insufficient to describe common cell types including touch sensing neurons that have large arborized receptive fields that are not post-synaptic (Bodian, 1962). Bodian proposed that despite not being post-synaptic, sensory endings in the skin should be considered dendrites. In this framework, dendrites generate signals in the skin and the axon begins as sensory neurites converge and exit the skin to bundle into the nerve taking signals towards the central nervous system. At the time this model was proposed, few intracellular differences between axons and dendrites were known. With the ability to visualize intracellular features, additional differences between axons and dendrites emerged and can be brought to bear on the nature of sensory endings. For example, now we know that dendrites contain ribosomes, Golgi outposts and microtubules oriented with minus ends distal to the cell body (minus-end-out), all of which are rare in axons (Craig and Banker, 1994). Subcellular features have been examined in invertebrate branched sensory endings; Drosophila mechanosensory neurons with branched arbors possess dendritic cellular signatures including minus-end-out microtubules (Stone et al.,

2008), Golgi outposts (Ye et al., 2007) and ribosomes (Hill et al., 2012), while their axons projecting to the central nervous system do not. Sensory endings of vertebrate DRGs have not been examined in the same way.

To develop specialized neurites with distinct contents, cargoes need to be differentially distributed from the major site of synthesis in the cell body to their site of function. In many neurons, microtubule polarity plays a central role in the ability to direct cargoes to axons and dendrites. Axonal microtubules have microtubules with their dynamic plus ends pointed away from the cell body, while dendrites have mixed orientation in vertebrates or minus-end-out orientation in invertebrates (Baas and Lin, 2011; Rolls and Jegla, 2015). Thus, the presence of minus-end-out microtubules distinguishes dendrites from axons and allows the minus end-directed motor dynein to deliver cargo specifically to dendrites (Kapitein et al., 2010). While the role of microtubule polarity in dendrite identity is easiest to conceptualize in multipolar neurons in which axons and dendrites emerge separately from the cell body, loss of dynein also specifically affects dendrites in unipolar neurons in Drosophila (Liu et al., 2000). While microtubule layout has only been examined in a couple of unipolar neuron types in flies, the primary neurite that emerges from the cell body has plus-end-out microtubule polarity, as does the axon. The dendrites that branch from the primary neurite, in contrast, have minus-end-out polarity (Stone et al., 2008). Thus, dendrites with minus-end-out microtubules can emerge from plus-end-out processes, and dynein-mediated cargo transport is essential to their identity. We therefore hypothesized that the sensory endings of DRG neurons could have mixed or minus-end-out microtubule polarity despite originating from an axon. In primary cultures, adult DRG neurons typically generate one to four neurites that have plus-end-out axonal microtubule polarity and receive similar cargoes (Gumy et al., 2017; Moughamian et al., 2013). However, sensory endings do not differentiate *in vitro*, restricting assessment of their microtubule polarity to *in vivo* settings. *In vivo* assessment of microtubule polarity in peripheral nerves containing sensory neuron projections has been conducted. Trigeminal neurons, functional

equivalents of DRGs that innervate the head region, have plus-end-out microtubule polarity in peripheral nerves to the cornea (Topp et al., 1994). The branched endings that innervate the cornea were not, however, examined, and we are not aware of studies examining microtubule polarity in sensory endings of DRG neurons. To our knowledge, microtubule organization has only been described in sensory endings of one vertebrate cell type at a single timepoint early in development. Rohon-Beard (RB) neurons perform similar functions to DRG neurons in embryonic and larval fish and amphibians, including zebrafish (Ribera and Nusslein-Volhard, 1998; Spitzer, 1984). Their cell body sits in the spinal cord and elaborate branched sensory endings innervate the skin (Katz et al., 2021; Palanca et al., 2013). In zebrafish these cells die after several weeks (Williams and Ribera, 2020) as DRG sensory endings take over skin innervation (Rasmussen et al., 2018). Analysis of microtubule organization showed that peripheral RB arbors have plus-end-out microtubule polarity in embryos one day after fertilization (Lee et al., 2017), but later timepoints were not reported.

Two primary methods have been used to assess microtubule polarity in neurons. The first method was based on the observation that under specific buffer conditions free tubulin polymerizes onto the sides of microtubules in sections prepared for electron microscopy to form visible “hooks” whose curvature reflects microtubule orientation (Heidemann and McIntosh, 1980). This method was used to determine that axonal microtubules in a variety of neuron types have uniform plus-end-out polarity (Baas and Lin, 2011) and that dendrites in cultured hippocampal neurons have mixed polarity (Baas et al., 1988). However, this method is quite cumbersome and would be difficult to apply to sensory endings in the skin as they form a complex network in which it would be difficult to determine orientation of neurites in sections. The second method for polarity assessment relies on live imaging of proteins including end-binding proteins 1 and 3 (EB1 and EB3) that bind to polymerizing microtubule plus ends (+TIPs) (Baas and Lin, 2011). It was first used to confirm microtubule polarity in cultured hippocampal neurons (Stepanova et al., 2003) and has since been applied to a variety of vertebrate and invertebrate neurons *in vivo* (Goodwin et al.,

2012; Kleele et al., 2014; Maniar et al., 2012; Rolls et al., 2007; Stone et al., 2020; Stone et al., 2008; Yau et al., 2016). This +TIP-tracking method seemed most promising to use for probing polarity of sensory endings.

As +TIP tracking requires live imaging to assess microtubule polarity, optical accessibility was a key determinant for selection of a vertebrate in which to image sensory endings. While mice are a popular vertebrate model, their fur and high autofluorescence in the skin make them a poor choice for optical interrogation of fine sensory endings. In contrast, zebrafish are a vertebrate model organism with excellent transparency and low fluorescent background at early life stages, and quite amenable to imaging into adulthood if an appropriate mutant background is selected (Antinucci and Hindges, 2016; Moss et al., 2013; White et al., 2008). In this study we therefore used live imaging of +TIPs in zebrafish to evaluate microtubule polarity of vertebrate sensory endings.

Results

Rohon-Beard (RB) sensory endings maintain axonal microtubule polarity at maturity

RB neurons in embryonic and larval zebrafish have been used extensively for live imaging (Andersen et al., 2011; Andersen and Halloran, 2012; Lee et al., 2017; Liu and Halloran, 2005; Paulus et al., 2009; Wang et al., 2012). RB neurons begin central axon outgrowth 15-17h after fertilization (Andersen et al., 2011; Kuwada et al., 1990), and peripheral axon outgrowth initiates shortly afterwards (Andersen et al., 2011). To complement analysis of microtubule organization in the peripheral arbor at 24h post fertilization (Lee et al., 2017), we wished to observe the polarity of the endings all the way to their distal tip when the arbors are mature and stable.

To assess microtubule polarity in RB sensory endings, we expressed a fusion of GFP to mouse EB3, which has previously been used in zebrafish to track growing plus ends (Distel et al.,

2010). The *Isl1[ss]* promoter, which expresses in RB neurons (Higashijima et al., 2000; Palanca et al., 2013), was used to drive expression of EB3-GFP (*Isl1[ss]*:EB3-GFP). To generate animals in which individual RB peripheral arbors could be observed without overlap from adjacent cells, the EB3-GFP expression plasmid was injected at the single cell stage and embryos with sparse, well resolved neurons were selected for imaging (Figure 1A). Early larval zebrafish lack gills and have low oxygen requirements so they can be immobilized for imaging by embedding anesthetized animals in agarose. We used this mounting strategy to image EB3-GFP comet movements in RB neurons at 24-96 hours post fertilization (Figure 1B and C). Direction of comet movement (comets moving away from the cell body were scored as plus-end-out) was assessed in kymographs generated from the videos (Figure 1D and E).

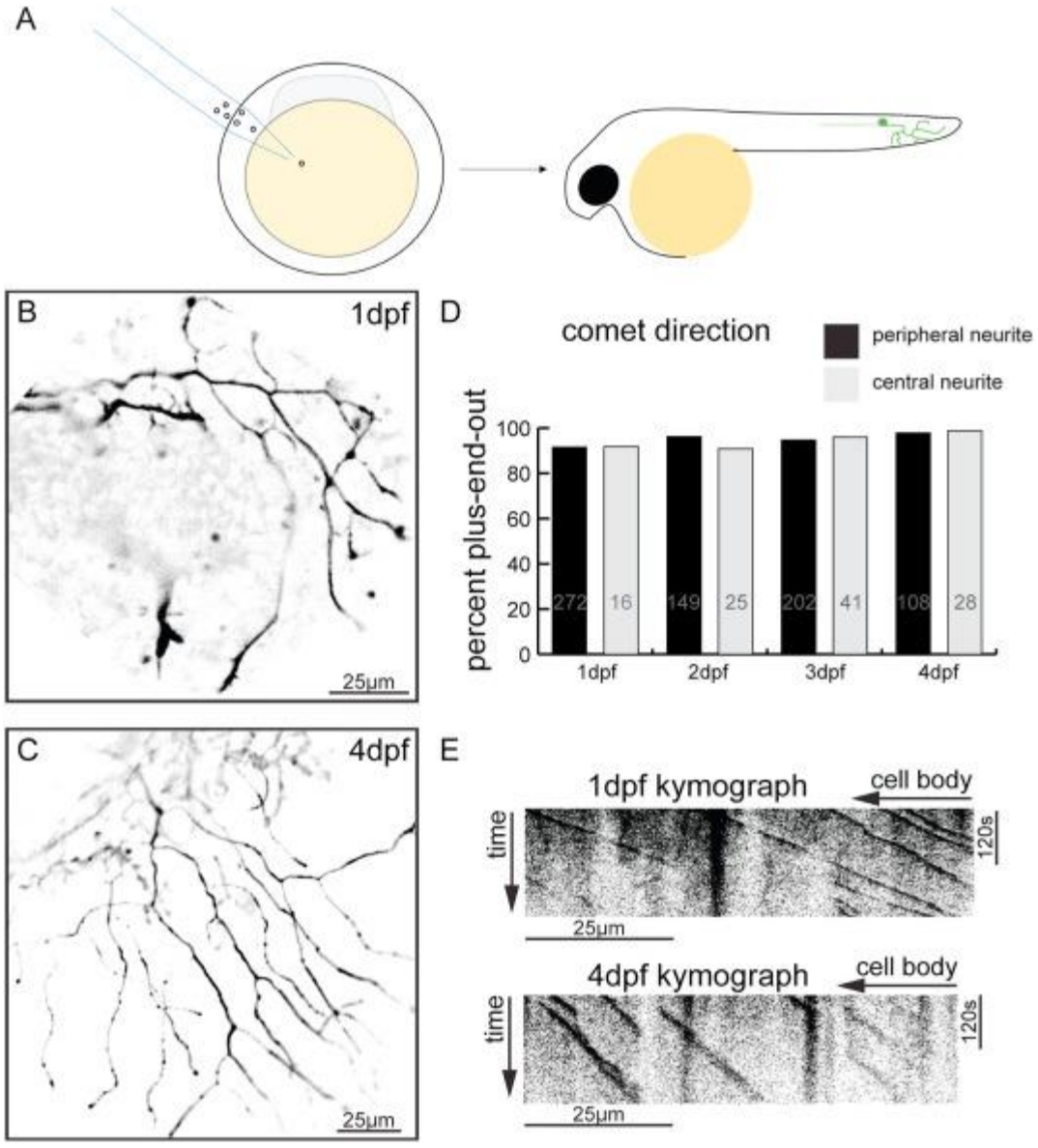


Figure 1. Zebrafish RB neurons have uniform plus-end-out microtubule polarity. A. Schematic illustration of zebrafish egg injection. **B.** Maximum projection of a time-series image of a 1dpf zebrafish RB neuron expressing EB3-GFP controlled by *Isl1[ss]*. **C.** Maximum projection of a time-series image of a 4dpf zebrafish RB neuron expressing EB3-GFP **D.** Graph displaying quantification of comet direction in the central and peripheral neurites of RB neurons at timepoints from 1dpf to 4dpf; number on column is the number of comets counted for that condition. **E.** Sample kymographs of RB neurons expressing EB3-GFP at 1 and 4dpf.

As expected based on the previous analysis of microtubule growth in RB neurons 24 hours post fertilization (Lee et al., 2017), EB3-GFP comets moved away from the cell body at tips of plus-end-out microtubules at this time point (Figure 1D and E and Movie 1). To determine whether the central axon also has plus-end-out microtubule polarity as has been assumed, we assayed EB3-GFP behavior in RB neurites within the spinal cord. These comets also moved away from the cell body (Figure 1D). Thus, at 24 hours post fertilization both neurites have the same microtubule polarity.

During development of Drosophila sensory arbors, dendrites have plus-end-out microtubule polarity during the very first stages of outgrowth (Feng et al., 2019) and then minus-end-out microtubules are added as the arbors develop (Feng et al., 2019; Hill et al., 2012). The mature minus-end-out polarity is not established until the neurons have been in place and functioning for several days (Hill et al., 2012). We therefore hypothesized that RB peripheral neurite polarity might change over several days of maturation. We imaged EB3-GFP in peripheral and central neurites daily up to 96h. At each timepoint the central neurite was plus-end-out and the peripheral arbor was also plus-end-out all the way out to the tips (Figure 1D).

While RB neurons are still present two to three weeks post fertilization (Palanca et al., 2013; Williams and Ribera, 2020), cell death during larval development (Williams and Ribera, 2020) combined with expression in the replacement DRG neurons made labeled RB neurons easier to find in transgenic animals than in sparse mosaic animals. We therefore generated a transgenic zebrafish line expressing LexA under the control of the a previously characterized 305bp fragment (-1036: -731) of the pufferfish *p2x3-2* promoter (Palanca et al., 2013), and EB3-GFP driven by a 4xLexOP, which we will refer to as *P2rx3a*>EB3-GFP. Melanocytes and iridophores migrate into the skin to absorb and scatter light and make imaging of whole animals more difficult as they mature. We therefore generated the *P2rx3a*>EB3-GFP line in a *casper* (*mifta*^{w2}/*mifta*^{w2}, r*oy*^{a9}/*roy*^{a9}) mutant background, which lacks both cell types (White et al., 2008). To further optimize our ability

to image neuron structure and microtubule behavior, we created composite transgenic lines that included other LexA/LexOP transgenes with complementary properties. For earlier timepoints, because *Isl1[ss]* turns on earlier than *p2x3-2* (Palanca et al., 2013) and to include a cell-shape marker, we bred *P2rx3a*>EB3-GFP to a *casper* line containing *Isl1[ss]*>tdTomato to generate a double transgenic fish line. *Isl1[ss]* drives expression transiently, so for later timepoints we generated a separate line by breeding *P2rx3a*>EB3-GFP to *casper P2rx3a*>mCherry. For both combinations, we selected the brightest fish expressing both green and red markers in each generation for imaging (Figure 2). We confirmed that EB3-GFP expression in transgenic lines yielded similar results as transient expression with the *Isl1* promoter by imaging larvae three days post fertilization (Figure 2C and H and Movie 2).

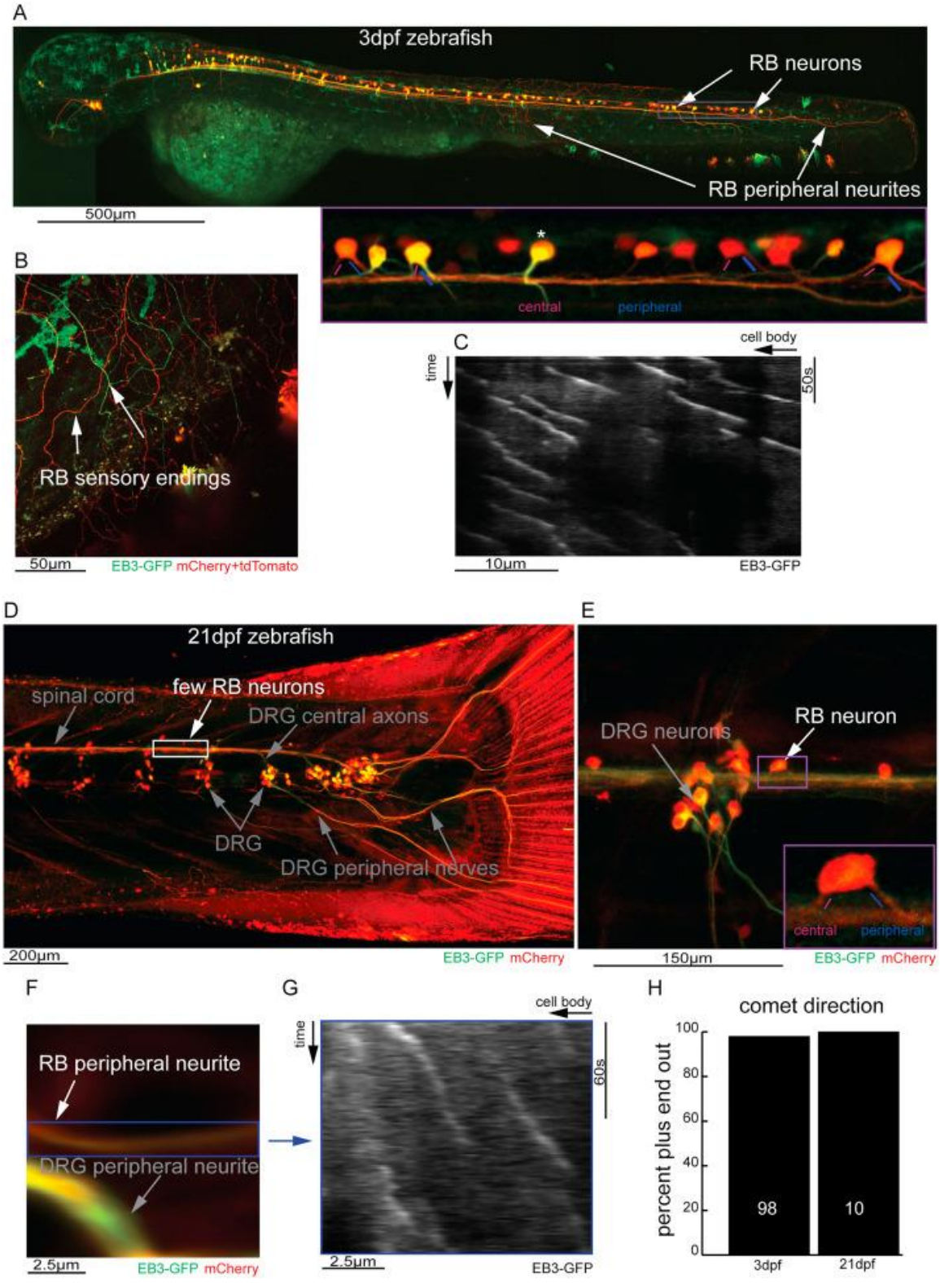

A
3dpf zebrafish
RB neurons
RB peripheral neurites
500µm
B
RB sensory endings
50µm
EB3-GFP mCherry+tdTomato
central
peripheral
C
cell body
time
50s
10µm
EB3-GFP
D
21dpf zebrafish
spinal cord
few RB neurons
DRG central axons
DRG
DRG peripheral nerves
200µm
EB3-GFP mCherry
E
DRG neurons
RB neuron
central
peripheral
150µm
EB3-GFP mCherry
F
RB peripheral neurite
DRG peripheral neurite
2.5µm
EB3-GFP mCherry
G
cell body
time
60s
2.5µm
EB3-GFP
H
comet direction
percent plus end out
100
80
60
40
20
0
98
10
3dpf
21dpf

Figure 2. Zebrafish RB neuron microtubules remain plus-end-out for the life of the cell. A. Overview of 3dpf zebrafish embryo bearing transgenic insertions of *P2rx3a*>EB3-GFP, *P2rx3a*>mCherry and *Isl1[ss]*>tdTomato showing the location and abundance of RB neurons at 3dpf. Enlarged image shows exit of neurites from the cell bodies in the spinal cord. **B.** Higher magnification image showing the morphology of RB neuron sensory endings. Transgenes are not expressed uniformly in all neurons so some cells are labeled red, others green and some both. **C.** A representative kymograph generated from RB sensory endings is shown. **D.** Overview of the posterior portion of a 21dpf zebrafish showing the position of DRG neuron cell bodies, the morphology of the peripheral nerves and the strong reduction in visible RB neurons relative to 3dpf. **E.** higher magnification region showing a rare visible RB neuron in a 21dpf zebrafish. **F.** image of a quantified portion (blue box) of RB sensory neurite in a 21dpf zebrafish. **G.** Sample kymograph of RB sensory neurite in a 21dpf zebrafish neuron expressing EB3-GFP **H.** Graph showing quantification of comet direction in 3dpf and 21dpf RB sensory neurites expressing EB3-GFP; number on column is the number of comets counted for that condition

Using this combination of transgenic lines, we were able to image RB peripheral arbors up to three weeks post fertilization. At this timepoint, viability is compromised when animals are fully embedded in agarose, so we selectively anchored the tail to the coverslip and imaged this region of the animal. As at earlier timepoints, the entire sensory arbor had plus-end-out microtubule polarity (Figure 2G and H and summarized in Table 1). Thus, RB neurons contain only plus-end-out microtubules in central and peripheral neurites throughout their life.

Zebrafish DRG neurons retain bipolar morphology into adulthood.

Because RB neurons are present only early in development, we also wished to examine microtubule organization in DRG neurons that mediate most somatosensation in mature vertebrates. RB and DRG neurons have several significant structural differences. RB peripheral neurites do not bundle into axons prior to entering the skin (O'Brien et al., 2012), while DRG peripheral neurites form nerves that are ensheathed in glia. In adult fish these nerves bring the neurites to the base of the scale, and bundles are also found on the surface of mature scales (Rasmussen et al., 2018). Free sensory endings emerge from these bundles to innervate the skin on the surface of the scale (Rasmussen et al., 2018); RB sensory endings innervate the immature skin prior to scale formation.

Another structural difference that could impact microtubule organization and trafficking is the arrangement of neurites as they exit the cell body. RB neuron cell bodies are located within the spinal cord and typically have ascending and descending central axons that emerge from either side of the cell body. Subsequently the peripheral neurite exits the cell body either directly or from the base of one of the central axons (Andersen et al., 2011; Andersen and Halloran, 2012; Wang et al., 2012). The peripheral axon is larger than either central one, and so is easier to visualize. In most cases at both 3 dpf (Figure 2A) and 21 dpf (Figure 2E), the cell body maintains two exits: one for a central axon and one dominated by the much larger peripheral neurite. An occasional neuron with a single visible initial process was also observed (asterisk in Figure 2A). Rodent DRG neurons also initially grow separate central and peripheral axons from the cell body (Takahashi and Ninomiya, 1987), but later the cell body seems to move aside and a single neurite root is formed (Takahashi and Ninomiya, 1987). Eventually a single fairly long primary neurite emerges from the cell body and gives rise to the central and peripheral neurites in a morphology termed pseudounipolar (Takahashi and Ninomiya, 1987). The description of DRG neurons as pseudounipolar originated in the nineteenth century (Nascimento et al., 2018; Takahashi and Ninomiya, 1987), but the organization of the dorsal root ganglia and resident neurons has not been investigated in zebrafish. We therefore took advantage of the ability to image inside transparent zebrafish to track cellular morphology of neurons in the DRG at different ages.

Very early stages of DRG development have been visualized in zebrafish embryos using the *neuroG* promoter and two neurites were seen emerging from the cell body as in RB neurons (McGraw et al., 2008). To determine whether zebrafish neurons would transition to a pseudounipolar shape as described in larger mammals, we imaged the DRG in 5 week old animals (Figure 3A-E). Surprisingly, most neurons in the DRG maintained two completely separate neurites (Figure 3D), and as well as projecting differentially to either the spinal cord or periphery, these neurites were distinguished by caliber. Central axons were almost always much thinner than

peripheral neurites (Figure 3D). Both central and peripheral neurites had plus-end-out microtubule polarity (Figure 3E). We were able to identify one neuron with a single neurite root exiting the cell body (Figure 3D asterisk) indicating that this morphology is possible, even though less common than true bipolarity. As this raised the possibility that DRG neuron morphology in 5 week animals might still be transitional, we also imaged DRGs in older fish.

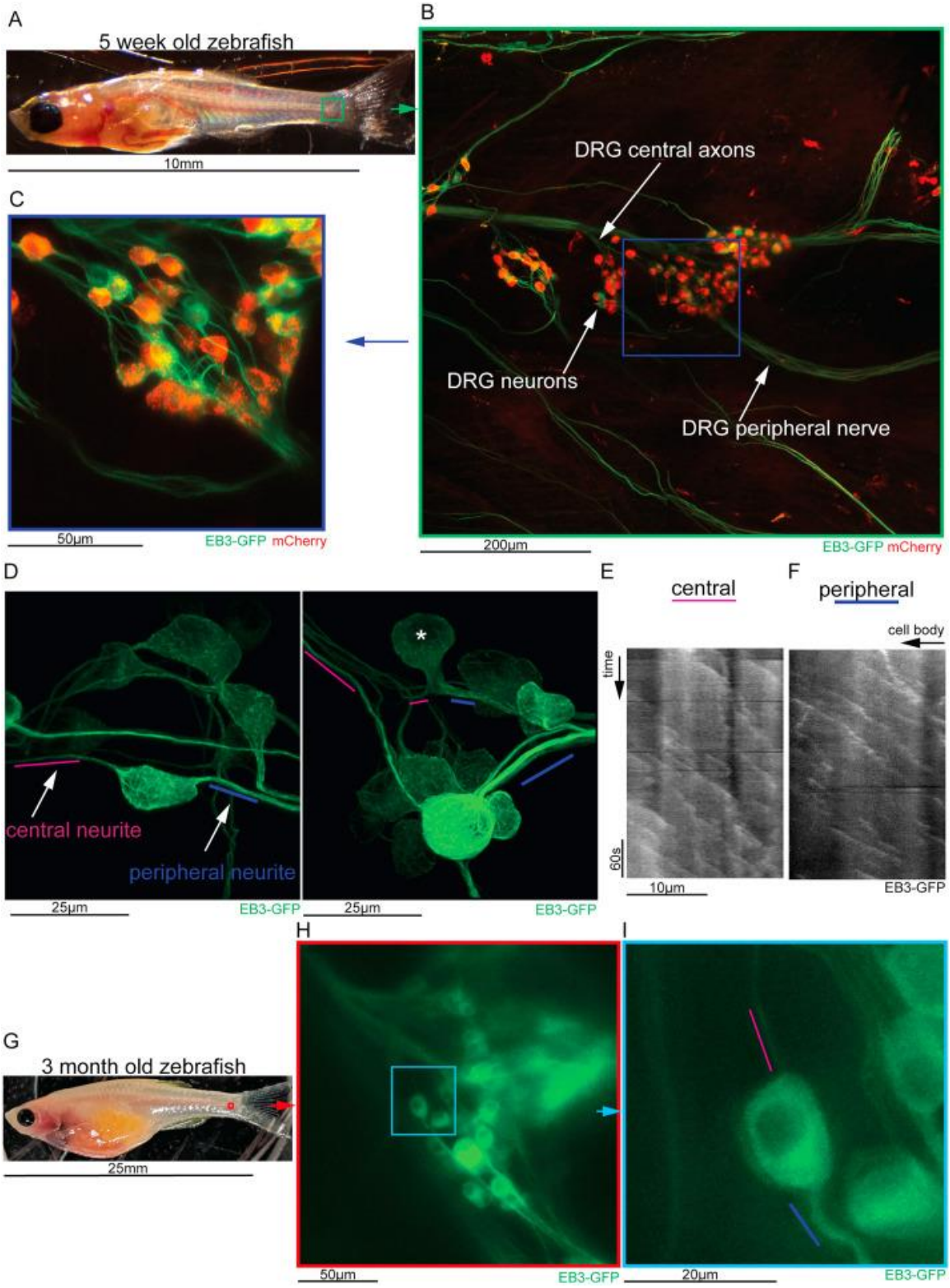


Figure 3. Most zebrafish DRG neurons retain bipolar morphology throughout development.

A. Brightfield overview of 5 week old *casper* zebrafish. **B.** Confocal image of the posterior region of the zebrafish spine showing the spinal cord, DRG neurons and their nerves. **C.** higher magnification image showing DRG neurons cell bodies and their centrally and peripherally projecting neurites. **D.** Airyscan super-resolution images of DRG neurons expressing EB3-GFP, showing clear centrally and peripherally projecting neurites originating from separate locations on the cell body marked pink and blue respectively. Asterisk indicates rare neuron with a cell body projection from which both neurites emerge**. E.F.** Representative kymographs from the centrally and peripherally projecting neurites respectively of 5-week-old zebrafish DRG neurons expressing EB3-GFP **G.** Brightfield overview of 3 month old zebrafish. **H.** 2 photon image of the posterior DRG cluster of a 3 month old zebrafish expressing EB3-GFP **I.** Higher magnification image of a DRG neuron at the edge of the cluster. Central and peripheral neurites are indicated with pink and blue lines respectively

Despite much thicker tissue, we were able to image the posterior most DRG clusters in 3 month old, mating age fish (Figure 3G) using two-photon excitation (Figure 3H, 3I). The weaker Z axis discrimination of two-photon microscopy relative to confocal made it difficult to visualize distinct centrally projecting neurites in the middle of the DRG cluster (Figure 3I), however isolated cells along the edge of the cluster could be clearly seen to be bipolar (Figure 3H, 3I). As in younger fish, the central axon was distinguished from the peripheral neurite by its thinness (Figure 3I). We conclude that in zebrafish most RB and DRG neurons maintain bipolar morphology at maturity, although pseudounipolar cells are also present at low frequency.

Adult DRG peripheral neurites have plus-end-out microtubules in their receptive field.

Having shown that the architecture and microtubule arrangement of RB and DRG neurons is similar near the soma, we wished to analyze more peripheral regions of DRG neurons, including the free sensory endings that extend into the epidermis over the scale surface (Rasmussen et al., 2018). In order to characterize microtubule behavior in mature DRG neurons, we used our *P2rx3a*>EB3-GFP, *P2rx3a*>mCherry transgenic *casper* mutant fish line. The presence of the mCherry was especially helpful in visualizing fine sensory endings.

For imaging, we selected 8-10 month old zebrafish at least 25mm in length (Figure 4A) that are mature adults. In order to avoid potential artifacts from hypoxia or other complicating factors, we intubated the fish using a flow cell based on a previously published design (Xu et al., 2015), but adapted for use with an inverted confocal microscope (Figure S1). This gave us the ability to mount the anesthetized fish on the confocal microscope for hour-long imaging sessions, and recover them afterwards if necessary.

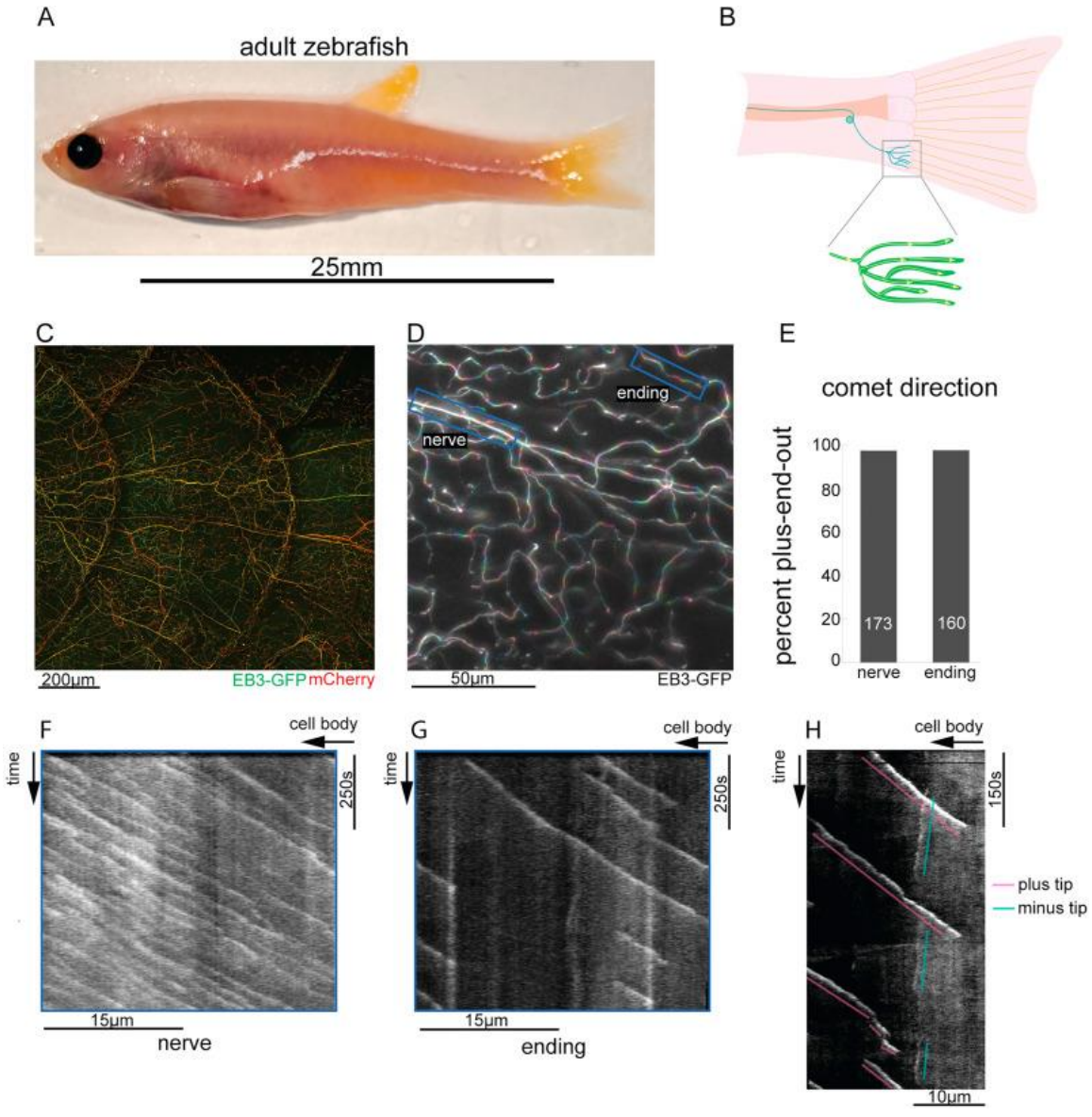


Figure 4. Zebrafish DRG sensory endings have uniform plus-end-out microtubule polarity throughout their entire arbor. A. Brightfield overview of an 8-10 month-old adult zebrafish. **B.** Diagram of a single DRG sensory neuron innervating a posterior scale. **C.** Confocal z-stack of a scale innervated by DRG sensory neurons expressing mCherry and EB3-GFP **D.** False-colored time series z-stack image of the surface of a scale on an 8-10 month-old zebrafish expressing EB3-GFP with successive timepoints batched into colors progressing from the blue to red end of the spectrum, showing a series of rainbows with their red end oriented towards the end of the neurite, reflecting a plus end out microtubule polarity. **E.** Quantification of microtubule polarity from movies of EB3-GFP in DRG nerves and endings in 8-10 month-old zebrafish; number on column is the number of comets counted for that condition **F.G.** Representative kymographs of EB3-GFP in the nerve and sensory endings respectively. **H.** Kymograph generated from a movie of EB3-GFP in a sensory ending in which both growing minus ends (blue) and plus ends (pink) are visible.

We collected EB3-GFP time-lapse movies from neurites bundled within nerves as well as from free sensory endings on the surface of the scale (Figure 4). Although it was difficult to resolve individual neurites in the nerve, all EB3-GFP comets moved in the same direction, away from the cell body, indicating uniform plus-end-out polarity. Similar plus-end-out polarity was observed in the defasciculated and branched free endings on the scale (Figure 4D-G). In single plane timeseries movies we were able to track direction of comet movement and used this strategy for most of our analysis. However, in any individual movie only a short section of neurite was in focus. To more completely visualize microtubule growth in sensory endings, we acquired time series Z-stacks that clearly show EB3-GFP comets moving from the nerve to the tips of sensory endings (Figure 4D and Movie 3). To determine whether other parameters of microtubule behavior might be regionally different in DRG peripheral neurites, we analyzed plus end growth speed from these movies. While speed varied somewhat from fish to fish, we did not observe regional differences (Figure S2). We conclude that even in adult zebrafish, microtubules have plus-end-out microtubule polarity in sensory endings in the skin (Figure 4B).

To further compare microtubule organization in mature DRGs to that in larval RB neurons, we wanted to visualize microtubule minus end behavior. Minus ends in mammalian axons have been difficult to visualize, but are thought to be capped by short stretches of CAMSAP2 (Yau et

al., 2014) or organized by nucleation sites recruited to the side of pre-existing microtubules by the HAUS/augmin complex (Cunha-Ferreira et al., 2018; Sanchez-Huertas et al., 2016). In fine neurites *in vivo*, however, we previously reported that microtubule minus ends do not seem to be capped and instead grow persistently. We characterized this minus end growth most completely in dendritic sensory endings in Drosophila, where we could show that it was important for organization of the microtubule network (Feng et al., 2019). We observed a similar phenomenon in RB sensory endings in zebrafish (Feng et al., 2019). While difficult to detect in more optically challenging adult zebrafish, we did find some EB3-GFP spots that moved in the opposite direction, and slower than plus ends (Figure 4H). We conclude that microtubule minus ends are capable of growth in mature DRG sensory endings. Thus, overall, microtubule organization is similar in RB and DRG neurons.

Sensory neurons maintain a somatic hotspot of microtubule organization

Neurons with well-defined axons and dendrites that have distinct arrangements of microtubules inactivate the central somatic microtubule organizing center (MTOC) as they mature (Lindhout et al., 2021). In contrast, sea anemone neurons with two or three axon-like neurites maintain a somatic MTOC (Stone et al., 2020). To determine whether somatic microtubules in vertebrate neurons with only axon-like processes are organized around a MTOC as they mature, we analyzed somatic microtubules at different developmental time points in zebrafish sensory neurons.

To establish the best strategy to detect somatic MTOCs, we imaged EB1-GFP behavior in mature Drosophila sensory neurons that do not use a single MTOC (Nguyen et al., 2011) and Nematostella tripolar neurons that maintain a single active somatic MTOC (Stone et al., 2020) and Movie 4). By comparing different types of image analysis methods, we found that integrating EB1-GFP fluorescence along a line throughout a timelapse movie worked well to easily distinguish the two types of somatic microtubule organization (Figure 5 A and B). The presence of a somatic

organizer resulted in a strong peak of EB1-GFP fluorescence, while only a broad peak spanning the thick region of the cell was present in the Drosophila neuron (Figure 5A and B). In the Drosophila neuron, we used a centriole protein, fzr, to determine where to place the line to integrate the fluorescence signal as EB1-GFP itself could not be used to identify a peak.

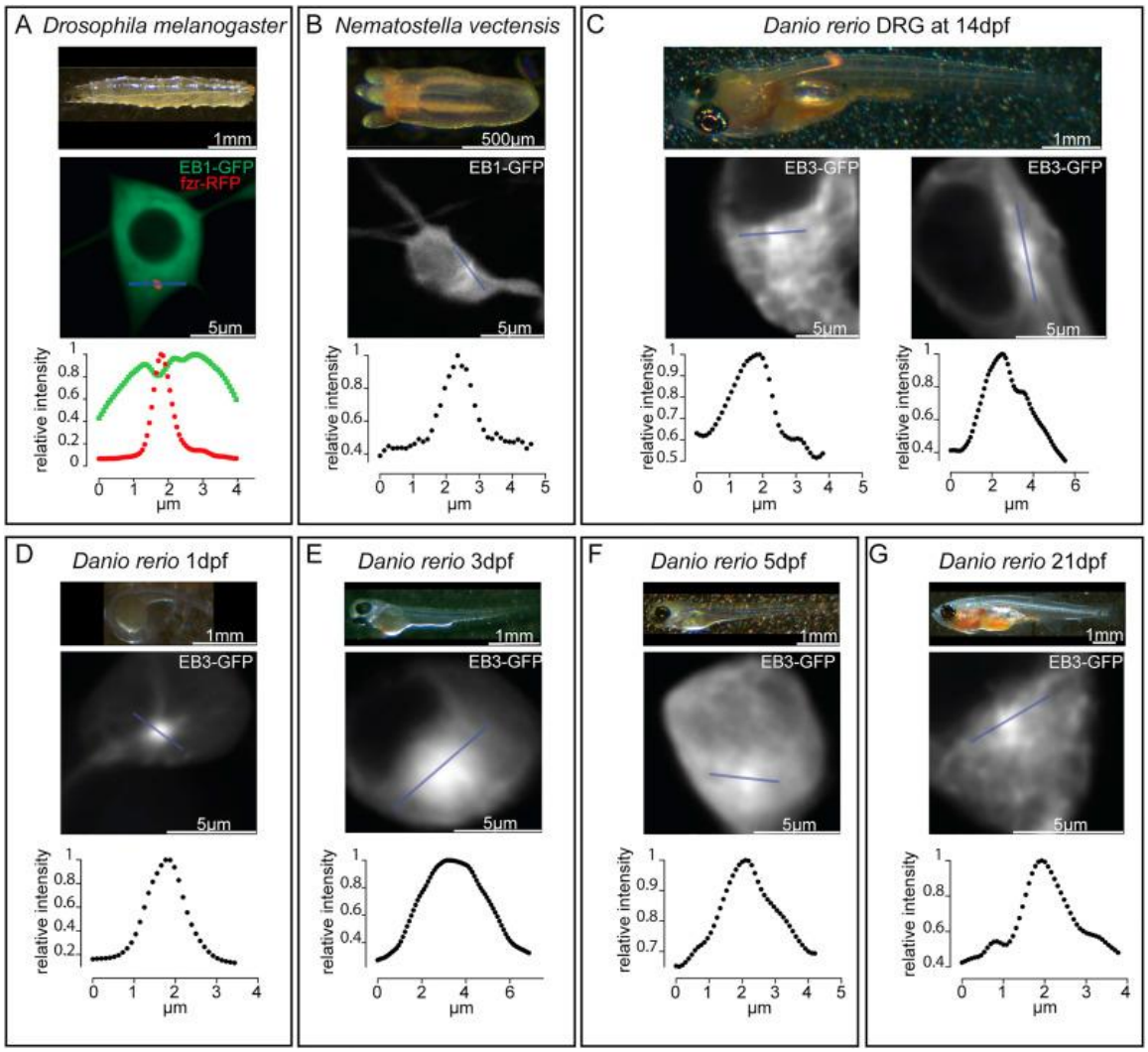


Figure 5. Zebrafish sensory neurons maintain a somatic hotspot of microtubule organization. **A.** Brightfield image of a 3rd instar drosophila larva and image of a ddaE neuron cell body expressing EB1-GFP and fzr-RFP are shown. The blue line indicates the region used to quantify EB1-GFP intensity from a sum projection of a timeseries. The output for fzr-RFP (red) and EB1-GFP (green) is shown in the graph. **B.** A brightfield image of a 4-6 tentacle stage *Nematostella vectensis* polyp and an image of a tripolar ganglion cell expressing EB1-GFP are shown. The blue line was used as the region for summing intensity through the time course and the output is shown

in the graph. **C.** A brightfield image of a 14dpf zebrafish and images of DRG neuron cell bodies expressing EB3-GFP are shown together with lines used to quantify EB3-GFP fluorescence. The graphs show intensity along the line summed through the timecourse. **D.-G.** Brightfield overviews and images of RB neuron cell bodies expressing EB3-GFP are shown from different age fish. In each a line was used to generate summed intensity graphs.

We imaged DRG neurons when the *p2x2-2* promoter became active and they began to express EB3-GFP, and found that they displayed hot-spots of EB3-GFP fluorescence similar to that in the Nematostella neuron (Figure 5C). We could not image DRG neuron cell bodies at sufficient resolution to determine whether the MTOC persisted throughout the life of the cell. We therefore used RB neurons, which we showed above have similar microtubule organization and somatic structure to DRG neurons, to image somatic microtubule organization throughout the cellular lifespan.

In order to visualize RB neurons in the first week after fertilization, we crossed the *P2rx3a*>EB3-GFP, *P2rx3a*>mCherry double transgenic *casper* line to the *P2rx3a*>EB3-GFP, *Isl1*>tdTomato double transgenic *casper* line and selected the brightest embryos displaying both markers for imaging. For later timepoints we in-crossed the *P2rx3a*>EB3-GFP, *P2rx3a*>mCherry *casper* line, and selected the brightest embryos with both markers for imaging. Timepoints were taken every day after fertilization for the first two weeks, and again at three weeks post fertilization. For embryos, EB3-GFP comets could be seen radiating from one point in the soma (Movies 5 and 6). At later time points, the analysis strategy developed by comparing Drosophila and Nematostella cell bodies enabled detection of a hotspot of EB3-GFP fluorescence. At all timepoints a peak of fluorescence, like that in the Nematostella neurons, was apparent. Example neurons at different ages are shown (Figure 5D-G). Even at three weeks, when RB neurons are rare and DRG neurons much more prevalent (Figure 2D and E), a clear EB3-GFP hotspot was present. For each zebrafish condition at least 4 cells were analyzed with the exception of the rare 21dpf RB neurons, where 3

were analyzed. All cells had clear summed EB3-GFP peaks similar to the examples shown. This analysis suggests that vertebrate sensory neurons maintain an active somatic MTOC even when mature.

Discussion

To address the long-standing question of whether branched receptive arbors of vertebrate sensory neurons have cellular dendritic features, we used live cell imaging to probe microtubule organization in two types of zebrafish neurons. We found that both RB and DRG neurons have central and peripheral axons with uniform plus-end-out microtubule polarity. Even the branched sensory endings that innervate the skin and receive and integrate signals have the axonal plus-end-out polarity, and this arrangement is maintained at all stages of maturity. In addition to showing that a key defining feature of dendrites, the presence of minus-end-out microtubules, is absent from receptive regions of sensory neurons, we characterized some additional aspects of their microtubule and cellular organization. Mature polarized neurons in mammals (Stiess et al., 2010) and Drosophila (Nguyen et al., 2011) do not use a centrosomal MTOC to organize somatic microtubules. However, the recent finding that Nematostella neurons with only axon-like neurites maintain an active somatic MTOC (Stone et al., 2020) raised the possibility that vertebrate sensory neurons with exclusively axon-like neurites might do the same. Indeed, we found that somatic microtubule organization in RB and DRG neurons more closely resembles that in unpolarized Nematostella neurons than Drosophila polarized neurons (Figure 5). The presence of a somatic MTOC does not, however, mean that microtubules are all anchored at this point. The observation that growing minus ends are present in RB neuron (Feng et al., 2019) and DRG neuron (Figure 4H) sensory endings confirms that microtubule minus ends are present far from the somatic MTOC.

In addition to characterizing key aspects of microtubule organization in vertebrate sensory neurons *in vivo*, we also examined how their neurites emerge from the cell body. Invariably, mature DRG neurons are described as pseudounipolar, meaning that they have a single neurite originating from the cell body that branches to give rise to the central and peripheral axons. However, this description relies on analysis of relatively few animal species. We therefore took the opportunity to expand our understanding of the basic cellular morphology of sensory neurons to another animal. Surprisingly, we found that, while pseudounipolar RB and DRG neurons were present, they were outnumbered at all life stages by true bipolar cells that had small central axons and much larger peripheral axons originating directly from the cell body. Our work provides important cellular context for understanding vertebrate sensory neuron biology.

One particularly intriguing aspect of vertebrate sensory neuron cell biology is how central and peripheral axons become structurally and functionally distinct when they have the same microtubule polarity. Nematostella neurons with similar microtubule polarity, all neurites plus-end-out, do not sort different cargoes to each neurite; instead all neurites have both pre- and post-synaptic structures (Stone et al., 2020). Indeed, there are some hints that polarized sorting may not be particularly efficient in DRG neurons. The receptor that senses noxious heat, VR1, is not only found in sensory endings, but also localizes to projections within the spinal cord (Tominaga et al., 1998). However, it is clear that RB and DRG central and peripheral axons do have critical differences. For example, their caliber is clearly different as they emerge from the cell body or primary neurite (Figure 3) and during development they are influenced differentially by specific transcription factors and centrosome position (Andersen et al., 2011; Andersen and Halloran, 2012; Tanaka et al., 2011). Developing central and peripheral axons also respond to different signals (Liu and Halloran, 2005), suggesting different receptors may be present on the two processes. Mature central and peripheral axons are differentiated by their response to injury. While severing of the DRG peripheral axon elicits transcriptional reprogramming and regeneration, severing the central

axon does not (Liu et al., 2011; Rishal and Fainzilber, 2010) suggesting that injury sensing machinery is differentially distributed. How might proteins be differentially targeted to the two axons? One possibility is that there is no specific targeting, only specific retention based on different interactions of the two neurites with surrounding cells. Another, not mutually exclusive possibility, is that other microtubule features, for example post-translational modifications that can steer some kinesins specifically into axons of polarized mammalian neurons (Tas et al., 2017), distinguish the peripheral and central axons and guide cargoes selectively to each.

The lack of any region of the sensory arbor with minus-end-out microtubules separates vertebrate sensory neurons from invertebrate ones. Invertebrate sensory neurons with branched receptive fields in Drosophila and C. elegans have minus-end-out microtubules in at least part of the arbor (Harterink et al., 2018; Rolls et al., 2007; Stone et al., 2008). In contrast, we show that two types of vertebrate sensory neurons do not. As most neurons in bilaterian animals have axons and dendrites with distinct microtubule polarity (Rolls and Jegla, 2015) we hypothesize that this is the most efficient way to generate two different types of neurites. However, this efficiency may be counterbalanced by other factors in specific scenarios. One such example might be the PVD neuron in C. elegans. It has a branched sensory dendrite arbor (Tsalik et al., 2003) that has minus-end-out microtubules in the large anterior process and plus-end-out microtubules in the smaller, but morphologically similar, posterior process (Harterink et al., 2018). Both processes emerge from a single neurite exiting the cell body. While this arrangement means that the two dendrite regions have different microtubule organization, it allows for a continuous parallel microtubule array throughout the main trunk of the dendrite. In the case of vertebrate sensory neurons, the long distance between the cell body near the spine and sensory endings in the skin may make kinesin-based anterograde transport along a uniform microtubule array important. Axonal kinesins are optimized for fast, processive transport (Soppina et al., 2014), and so this may make the tradeoff with reduced sorting efficiency to neurites with different microtubule organization worthwhile.

While this rationale applies primarily to axons in the nerve, reorganizing microtubule polarity in the sensory endings may be either mechanistically difficult or of little benefit for cargo distribution.

The three neuron types in which microtubule organization has been found to be similar in all neurites, Nematostella tripolar ganglion neurons (Stone et al., 2020), RB neurons and DRG neurons (Figure 5), all seem to maintain a distinct somatic MTOC. While more examples would be helpful to determine the significance of this observation, one possibility is that a centrosomal MTOC is used to establish and maintain plus-end-out polarity in all neurites. Microtubules would not need to remain attached to the MTOC for this to work. The HAUS/augmin complex can recruit nucleation sites to the sides of existing microtubules (Petry et al., 2013), and plays a role in axonal microtubule organization (Cunha-Ferreira et al., 2018; Sanchez-Huertas et al., 2016). While this arrangement has been described as generating branched microtubule arrays, the majority of nucleation sites generate new microtubules in parallel to existing ones (Petry et al., 2013) and so could propagate polarity from a plus-end-out arrangement emerging from the MTOC throughout the neurites.

Methods

Drosophila maintenance and imaging

Drosophila were maintained at 25C on cornmeal agar fly food as described (Feng et al., 2019). To generate larvae for somatic microtubule imaging virgins from the line 221-Gal4, UAS-EB1-GFP (Stone et al., 2008) were crossed to males from the line UAS-fzr-mRFP (Basto et al., 2006). Adults were transferred into a fresh food vial every 24 hours, and larva that were 72-96h old were selected for imaging. Larvae were mounted under number 1.5 glass coverslips secured with tape, and a 63x 1.4NA oil immersion objective was used for imaging at 1.27 seconds per frame.

Nematostella vectensis maintenance and imaging

Nematostella were maintained in glass bowls in 12 g/L Instant Ocean and fed brine shrimp daily. Animals were kept in the dark at room temperature. Animals used for spawning were >6months in age and females were kept in separate bowls in order to control timing of fertilization of the eggs. They were exposed to light for 10-12 hours using a standard fluorescent light box to initiate spawning. Egg sacs from female bowls were removed and mixed with water from bowls containing males for 20-30 minutes within in one hour of spawning to achieve synchronized fertilization. Fertilized embryos were then isolated from the egg sacs by gently agitating in 12g/L Instant Ocean containing 2% L-cysteine, pH 8.5. Embryos were then rinsed 3-5X with 12g/L Instant Ocean and immediately transferred to Falcon 353007 60mm polystyrene dishes for injection. Embryo injections were carried out as previously described (Stone et al., 2020). Animals were injected with a plasmid containing the Nematostella EB1-GFP and mCherry controlled by the pan-neuronal elav promoter (NvElav-EB1-GFP-2A-mCherry) and screened for expression of the NvEB1-GFP transgene 1-2 weeks post injection using an AxioZoom.v16 microscope.

For imaging, whole, live Nematostella polyps at the 4-6 tentacle stage were anesthetized in 2% urethane in 12g/L Instant Ocean until tentacles were completely extended. They were then mounted on a metal slide with a 12mm hole in the center as described (Stone et al., 2020). The underside of the hole was covered with an air permeable membrane and the top side of the hole was covered with a piece of nylon mesh. Polyps were transferred to the top of the nylon mesh in a droplet of 12g/L Instant Ocean containing 2% urethane and covered with a 12mm round coverslip. Animals were imaged on a Zeiss LSM 510 confocal microscope. NvEB1-GFP videos were taken with a 63x 1.4NA oil immersion objective and captured at 1 frame per 2.88 s.

Zebrafish lines, maintenance and rearing

The following zebrafish lines were used in this paper (see table of reagents for full formal genotypes). Lines were not necessarily homozygous for transgenes, but were propagated by selecting the brightest fish expressing both markers in each generation.

P2rx3a>EB3-GFP, *P2rx3a*>mCherry, *mifta*^{w2}/*mifta*^{w2}, *roy*^{a9}/*roy*^{a9}

P2rx3a>EB3-GFP, *Isl1[ss]*>tdTomato, *mifta*^{w2}/*mifta*^{w2}, *roy*^{a9}/*roy*^{a9}

Wild type AB (for *Isl1[ss]*:EB3-GFP injection and imaging)

Adult zebrafish were maintained in an Aquaneering habitat at a density of 3 fish per liter, sex separated and mated weekly, fed brine shrimp twice daily, given a 14h light 10h dark light cycle, and water was kept at 28.5C with a pH of 7.5. Young fish were raised in glass bowls in a 28.5C incubator with a 14h light 10h dark light cycle and received once daily 50% water exchanges of 28.5C Blue Water (0.6g/L Instant Ocean salt, 0.01mg/l methylene blue) until day 5, when their bowls were transferred from the incubator to the room in which the Aquaneering colony was kept. Animals were fed rotifers from 5 dpf to 3 months. Brine shrimp were added to their diet from 2 weeks of age. At 3 weeks post fertilization the fish were transferred from bowls into the Aquaneering habitat and maintained on a slow drip. All experiments were approved by the Institutional Animal Care and Use Committee, including procedures for pain management and euthanasia. For adult experiments male and female fish were used. For larval experiments no distinction was made due to the lack of sex chromosomes and the lack of apparent sexual differentiation at early developmental timepoints. Adults used in experiments ranged from 8 to 10 months old.

Overview of microscopy

Brightfield overview images of younger fish, the drosophila larvae, and the nematostella polyp were generated using a Zeiss Axiocam 105 color camera mounted on a Zeiss Stemi 305 dissection scope. Brightfield overview images of 3 month and older fish were obtained using a Google Pixel 2 camera phone. Confocal images of fish injected with *Isl1[ss]*>EB3-GFP plasmid were obtained using a Zeiss LSM 780 with a 20x air objective. All other zebrafish confocal fluorescence images were generated using an inverted Zeiss Axio Observer.Z1 LSM 800 with Airyscan confocal microscope outfitted with a 10x 0.3 NA air objective, a 20x 0.8 NA air objective and a 40x 1.2 NA water objective, each of which was used depending on the level of detail and field of view required for the experiment. All 2-photon images were generated on a Leica SP8 confocal microscope with a Spectra-Physics Insight X3 using a 25x 1.0 NA 2.6mm WD water objective. For details of Nematostella and Drosophila EB1-GFP imaging see above.

Live imaging of adult zebrafish

Imaging of adult fish was performed in a flow cell adapted from a previous design (Xu et al., 2015) by adding a hole in the bottom what was sealed with a coverslip to adapt it for an inverted microscope (Figure S1). The flow cell was carved from single block of aluminum by the Penn State physics department machine shop. Pumping force was provided by a Rainin Dynamax RP-1 peristaltic pump. A closed recirculating loop was constructed by connecting the pump, flow cell, and fluid reservoir with polyethylene tubing. Flow rate was calibrated to ~6ml/minute of 0.016% MS-222 in 0.6g/l Instant Ocean. Fish were immobilized with 4% low melt agar. Time series Z-stacks were generated by selecting fast scan settings such that 5 focal planes with ~2um

separation could be imaged with a cumulative frame time of 3.45 seconds. This allowed for imaging of EB3-activity in a >100mm*100mm*10mm volume that contained the fine sensory endings. To generate a movie focal planes at each time point were merged in ImageJ via the "maximum projection" function.

Imaging of non-adult transgenic fish lines

Fish younger than 2 weeks were fully embedded in 1.5% low melting agar in a 4 well Ibidi µ-slide. Fish older than 2 weeks were anesthetized, and bathed in enough liquid to raise level several millimeters over the fish into a well of the µ-slide. Only the tail was immobilized by submerging the tip of a micropipetter filled with 45C 1.5% low melting agar directly over the tail region and carefully squirting molten agar onto the tail before it solidified. This left the gills free and facilitated recovery of the fish. All fish used in the data set successfully recovered after imaging before being euthanized.

Fish ERG EB3-GFP speed analysis

EB3-GFP videos of fish DRG neurons were analyzed using the “multiple kymograph” feature of ImageJ. Areas of the neurites were selected that were clearly part of the nerve innervating that portion of the scale, as well as the terminal sensory endings. Additionally, we analyzed the thin neurites before the final branching event that defined them as terminal, and a medium thickness region that while not part of the main nerve, may still have contained more than one neurite. Speed was determined manually by scoring the resultant kymographs and comparing displacement along the X and Y axis over the run of the comet that was captured

Somatic MTOC analysis

Time series images were collected for each of the cells in question. A line was drawn through the brightest EB region for all cells except those in Drosophila where fzr-mRFP was used to guide the line. ImageJ was used to perform a "sum slices" projection on the EB video, which integrates all EB activity into a single frame, creating a high dynamic range image with the brightest point reflecting the spot with greatest EB activity. A line was then drawn through the brightest point and ImageJ's "plot profile" function was used to generate the output data. Lines used are indicated on each of the example images.

Microinjection of zebrafish eggs with *Isl1[ss]*:EB3-GFP and live imaging

Zebrafish eggs at the one-cell stage were placed in a 1.5% agarose injection mold. A solution of DNA (10 - 50 ng) mixed with phenol red was prepared and loaded onto an injection needle. Injections were performed on a Zeiss upright microscope (Carl Zeiss) using a Narishige M-152 micromanipulator (Narishige Group). Injected eggs were transferred to a petri dish with artificial seawater and a few drops of methylene blue and incubated at 28.5 degree Celsius. After a few hours, unfertilized eggs were discarded using a pipette. The following day, embryos were screened for fluorescence.

Embryo mounting was carried out as described previously (O'Brien et al., 2009). Embryos were treated with 5% phenylthiourea (PTU) in Ringer's solution to inhibit pigment formation. GFP-positive zebrafish embryos at 1 day post fertilization (dpf) were dechorionated with forceps and anesthetized in 0.02% tricaine. Embryos at 2, 3 and 3 dpf were directly anesthetized in 0.02% tricaine. A mounting chamber was prepared by fixing a Teflon ring on to a cover slip with vacuum

grease. Anesthetized embryos were dropped into 1% low melting agarose solution, maintained at 42 degree Celsius in a heating block. An embryo was then transferred to the mounting chamber along with a drop of agarose. Using a probe, the embryo was positioned correctly for imaging. After the agarose hardened; the chamber was filled with PTU Ringers solution with 0.02% tricaine. The top of the ring was then greased and closed with a glass slide. See imaging overview section for microscopy details.

Plasmid construction

pEX_P2rx3a.E1b:LexA-VP16.Sv40.4xLexOP.E1b:EB3-GFP.Sv40 plasmid has been described (Feng et al., 2019).

Construction of plasmid expressing EB3-GFP under the *Isl1[ss]* promoter: All cloning steps were performed using the Multisite Gateway system (ThermoFisher Scientific) and Tol2kit (Kwan et al. 2007). Dr. Reinhard Koster provided the vector pCSEB3-GFP containing the human EB3 protein tagged with GFP (Distel et al. 2010). The EB3 fragment was amplified from pCSEB3-GFP using the forward primer 5'-GGGGACAAGTTTGTACAAAAAAGCAGGCTGCCACCATGGCTGTCAATGTGTACTCCAC-3' and 5'-GGGGACCACTTTGTACAAGAAAGCTGGGTAGTACTCGTCCTGGTCTTCCTG-3' as the reverse primer and then recombined with pDONR221 to generate a middle entry clone. The *Isl1[ss]* promoter (Sagasti et al., 2005) was amplified using the forward primer, 5'-GGGGACAACTTTGTATAGAAAAGTTGAACAAAAGCTGGAGCTCCACC-3' and 5'-GGGGACTGCTTTTTTGTACAAACTTGTCTTTCAGGAGGCTTGCTTC-3' as reverse primer and was recombined with pDONRP4-P1R to generate a 5' entry vector. All entry clones and the

destination vector pDestTol2pA2 were then subjected to an LR reaction to create the final expression vector containing *Isl1[ss]*>EB3-GFP, which was sequenced to confirm the presence of all three inserts.

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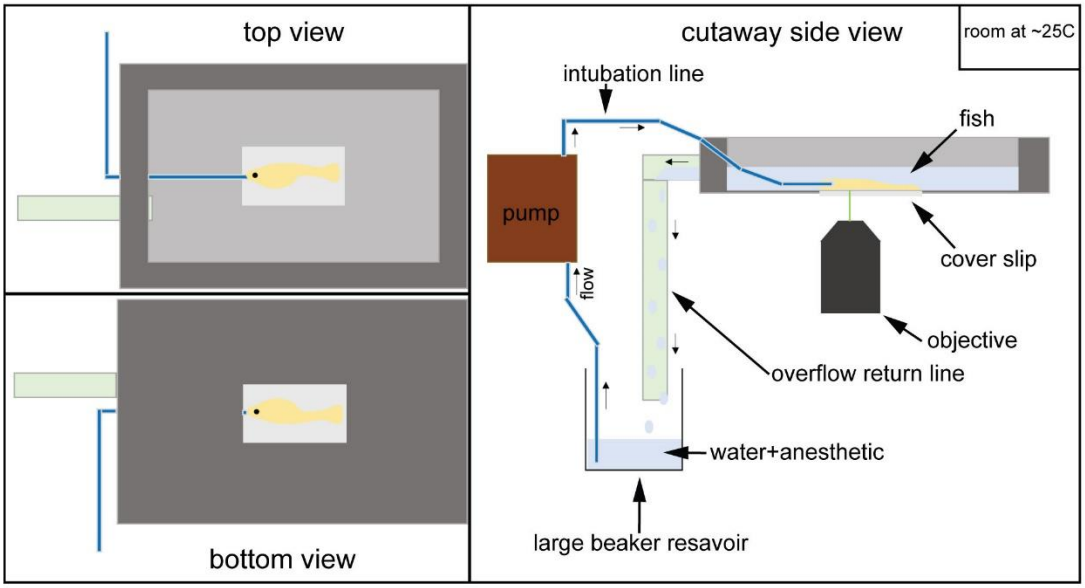
Chapter 3 supplemental information

SI figures

A

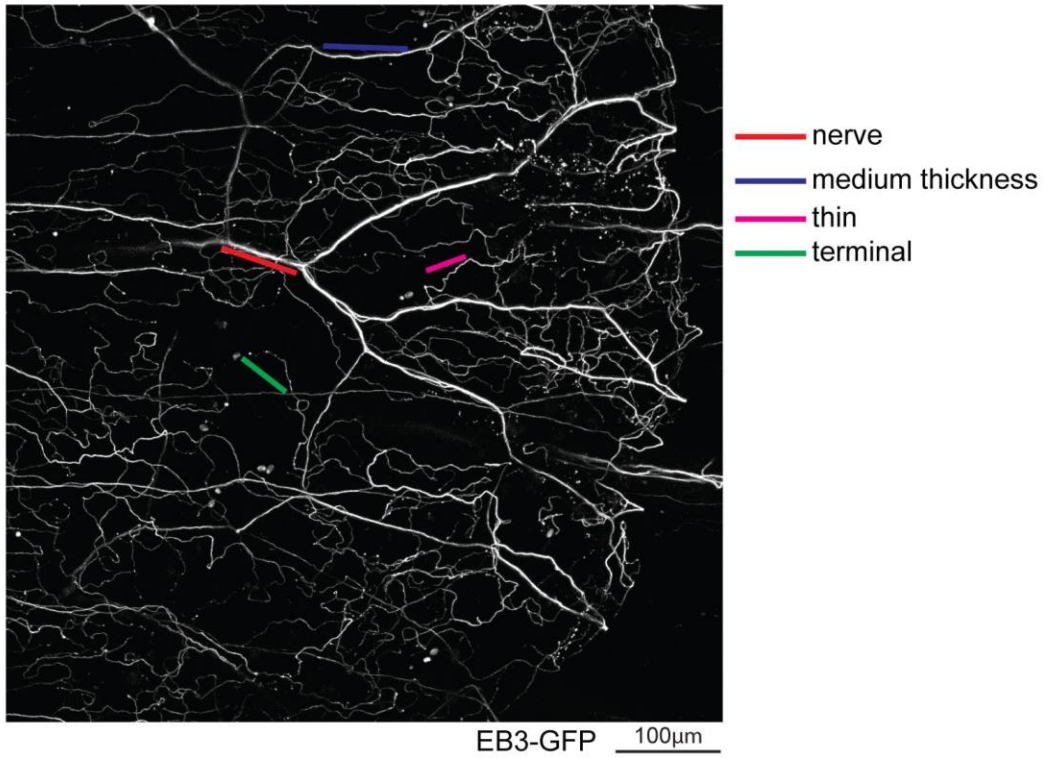


B

Figure S1. Chamber for imaging adult zebrafish.

A. A diagram of the intubation and imaging chamber is shown. **B.** An adult fish mounted for imaging is shown on the microscope. The objective can be seen through the coverslip under the tail. The thin intubation tube is positioned in the mouth to flow water over the gills.

A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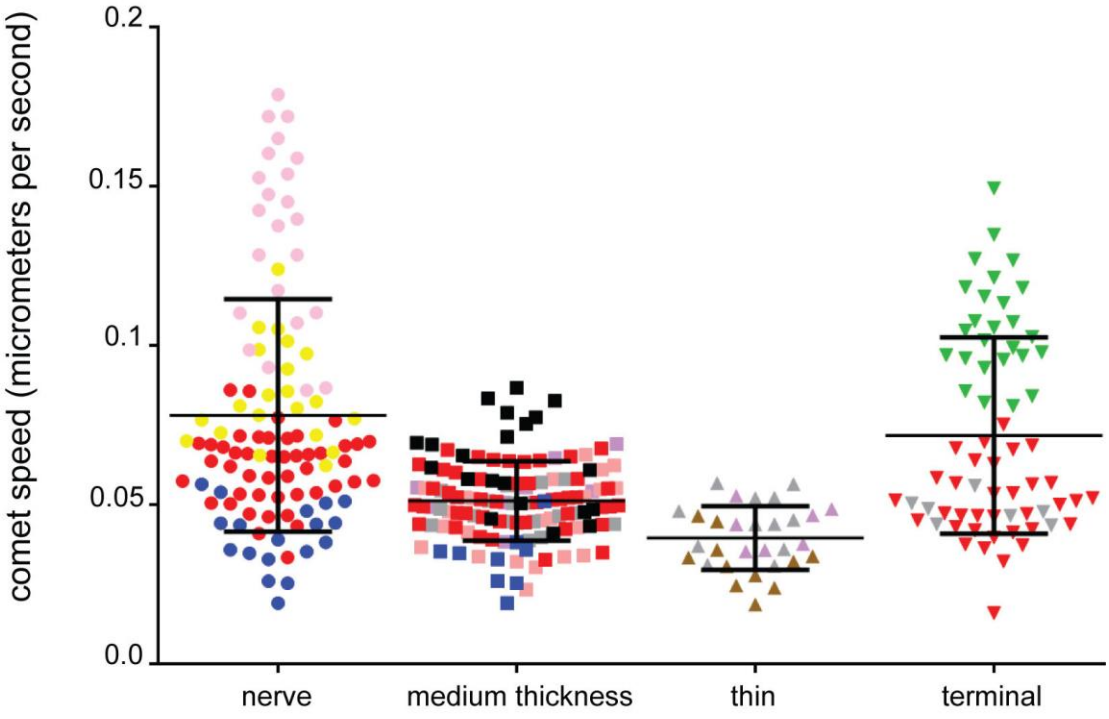
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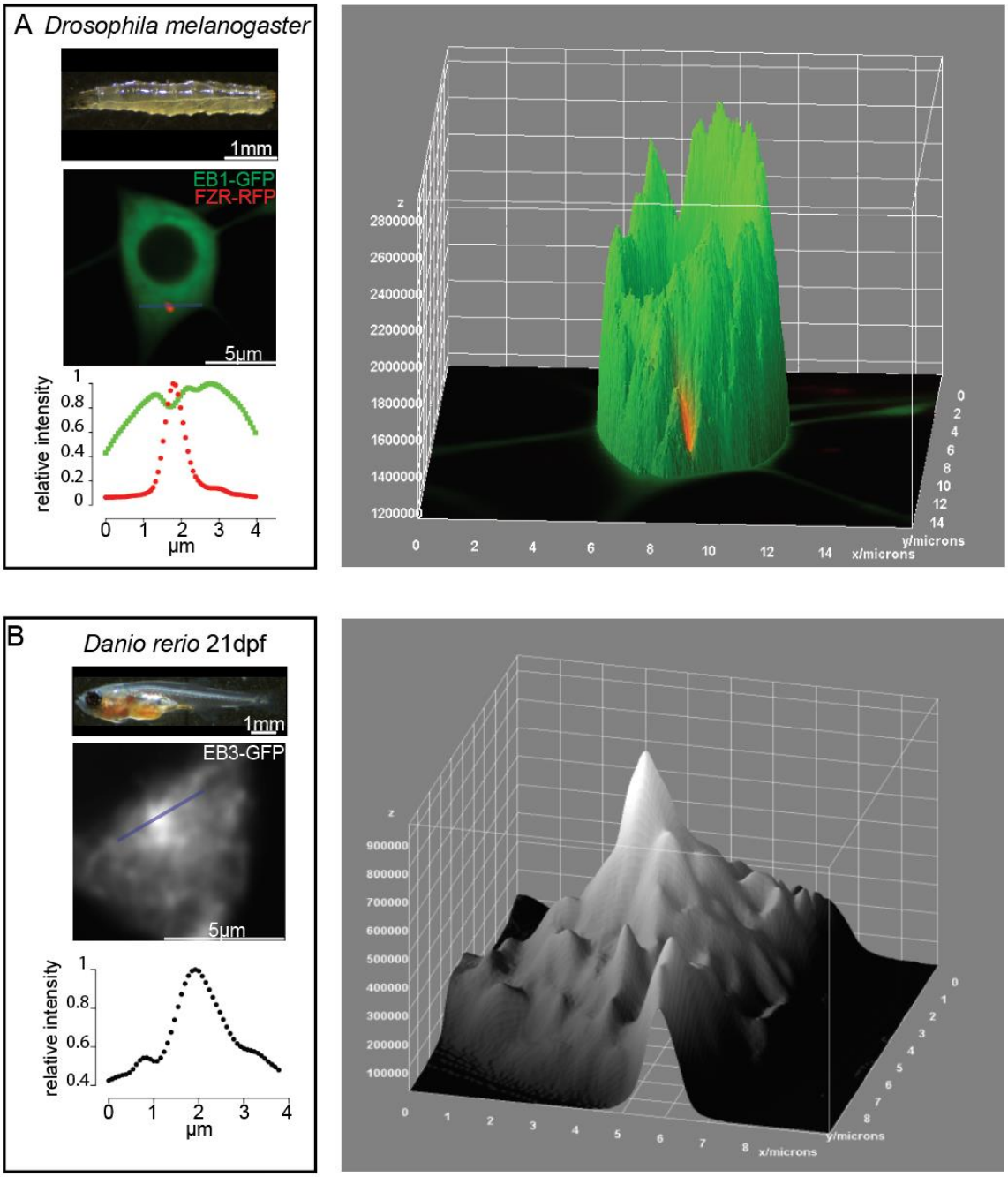
Figure S2. Regional analysis of plus end growth speed in DRG peripheral neurites. **A.** An image of a scale with fluorescently labeled DRG neurons is shown. The regions used for analysis of EB3-GFP speed are shown. **B.** Growth speeds generated from kymographs of EB3-GFP are plotted in different regions of the DRG peripheral neurite. Each color represents a different fish. Between fish differences overwhelmed any regional variation.

Chapter 3 supplemental information (unpublished)

An alternate method of graphing cumulative EB activity in neuronal cell bodies

The analysis of the cell body MTOC was difficult to graphically illustrate, and I wanted to show an alternate method which I feel effectively highlights the fact that in the drosophila neuron the centriolar marker is not positioned in an area of the cell that is enriched for EB1 activity, nor that there is any particular enrichment of activity in the drosophila neuron cell body. A 3D projection of intensities in the cell body from the summed image of the video is much more informative, but cannot be fully effective in a static format, as the observer cannot rotate the image to see the other side.



Unpublished figure S1. An alternate method of intensity visualization A. Right panel is the sum projection of an EB1-GFP and fzr-RFP time course video and profile plot of the line shown EB intensity is negatively correlated with fzr intensity. Right panel is a 3D projection of the intensities in the neuronal cell body for each channel. **B.** Left panel is the sum projection of an EB3-GFP video and profile plot of the line shown, showing the presence of a hotspot of EB activity. Left panel is a 3D intensity projection of intensity in the cell body for EB3-GFP

Preface to chapter 4

The following chapter is selected data from a project that has been submitted to The Journal of Neuroscience, but not yet been published. It exists as a bioRxiv submission and can be found here (https://www.biorxiv.org/content/10.1101/2021.07.03.450951v1.full) under the title "The MAP3Ks DLK and LZK direct diverse responses to axon damage in zebrafish peripheral neurons". I am the second author of this paper, and have included in this chapter only the experiments I performed for this project. Figures one and two are characterization experiments performed to inform analysis and the approach used for injury, while figures three and four are a pre-submission version and the figure version directly from the manuscript respectively. Figure legend for figure 3 is adapted to reflect different panel content of the pre-submission version. The figure legend for figure 4 is directly copied from the manuscript. Because the paper was written only in part by me, I have not included text from the manuscript other than the figure legend for figure 4, and relevant methods, which are identical, and are not anticipated to change in the final published product as a result of the review process. The contribution to the experiments herein by the other authors is that they generated and performed initial characterization on the mutant alleles (as described in the BioRxiv manuscript), and provided us with the mutants to for us to breed into our lines transgenic and Casper fish lines.

Chapter 4

The regenerative properties of fish DRG sensory neurites

Introduction

In the vertebrate nervous system, touch sensory function is mediated primarily by DRG (Dorsal Root Ganglion) sensory neurons. The function of these sensory neurons is critical to survival and quality of life. Two examples of this are the amputation frequency of diabetics who have previously lost sensation in their feet, and the reduced lifespan of humans born with a congenital insensitivity to pain. Sensory neuron function in any given tissue requires sensory neuron innervation to that tissue, and there is at least some data to suggest innervation may effect healing or maintenance of tissue in some species (Farkas & Monaghan, 2017). Unlike neurons in the brain that are within the protective enclosure of the skull, sensory neurons must extend out through peripheral nerves to the skin surface where they are vulnerable to damage.

A great deal of work has been done on the regeneration of DRG neurons, with attempts to enhance the regenerative process of peripheral nerves through which they travel being documented hundreds of years ago (Terzis, Sun, & Thanos, 1997), and continuing today, with over 50 publications on NCBI in the last year on the topic of DRG regeneration. Yet despite the importance of a functional sensory system and the vulnerability of these neurons, little has been done to investigate the regeneration of the DRG neurites at the point where they innervate the skin, and so the applicability of the large body of prior work to this particular question is unclear. Cell culture experiments cannot properly assess the regenerative properties of the branched sensory endings because DRGs do not develop specialized or branched neurites in culture. In-vivo work has been done in the form of sciatic nerve injury (Shin et al., 2012), but the sciatic nerve contains motor as well as sensory neurites, and the metrics used in this such as the sciatic

function index (SFI) test walking ability, and are thus more of a motor than sensory metric (Shen & Zhu, 1995). This means in order to properly evaluate DRG neuron sensory neurite regeneration, in-vivo work is needed, with an assay that can specifically monitor sensory neurite damage and regeneration in the skin itself.

DRG sensory neurons are not configured like other vertebrate neurons (Haberberger, Barry, Dominguez, & Matusica, 2019). A stereotyped neuron is generally described as having a single axon that synapses with, and transmits signals to its post synaptic target. It is also described as developing a branched dendrite close to its cell body, that exists to form post synaptic connections with the axons of other neurons and integrate synaptic input from them (B. Alberts, 2007). While DRG sensory neurons also have a single axon that projects to and synapses with a post synaptic target to which they signal, the neurite which receives input for that signaling is quite different from a classical dendrite in that its receptive field is not for synaptic input, but rather mechanosensation. Because the cell bodies of DRG sensory neurons are located just outside the spine, their sensory neurite is are equal in length to the distance from the ganglia outside the spinal segment to the target they innervate. In humans this can be up to a meter or longer from the bast of the spine to the end of the toes. For giraffes or blue whales, the distance would be considerably longer. These neurites (just like axons) form long linear projections through tissue, before arborizing at their target. Like invertebrate sensory neurons, the arbors that form are also unlike classic dendritic arbors in that they form no synaptic connections, and instead rely on receptors activated by vibration, stretch, thermal and chemical stimuli for their input (Vandewauw, Owsianik, & Voets, 2013). That said, despite these differences, they still possess a receptive field, and fulfill a dendrite like role in signaling by integrating input from their local environment.

Because of this apparent mixture of axonal and dendritic characteristics, we recently investigated an important aspect of the axonal vs dendritic character of DRG neuron sensory neurites (Shorey et al., 2021). Vertebrate neurons have a polarized microtubule cytoskeleton, with plus-end-out microtubule polarity in their axons, and mixed microtubule polarity in their dendrites.(Baas, Deitch, Black, & Banker, 1988) This is not a trivial distinction. A polarized microtubule cytoskeleton facilitates polarized transport (Harterink et al., 2018; Rolls, 2011). Polarized transport is what enables local differences in cellular function, such as the differences between an axon and a dendrite.

Because of this we decided to examine the microtubule polarity in DRG sensory neurons in order to determine the degree of dendritic vs axonal character in the sensory neurites. We found that despite their branched morphology and role in signaling, they have a axonal, plus end out microtubule polarity throughout not only the linear portion, but throughout the entirety of the sensory neurite, including the arborized portion at the surface. Despite the differences between this sensory neurite and a classical axon, this finding lends significant weight to a cellular perspective classifying DRG neuron sensory neurites as axons.

The identity of a neurite as axonal or dendritic is critically important to understanding the process by which it regenerates. Axons use a well characterized molecular pathway to sense damage and regenerate(Hammarlund, Nix, Hauth, Jorgensen, & Bastiani, 2009). This pathway is highly conserved, and senses injury in such genetically distinct organisms as C. elegans, Drosophila, and mice (Holland et al., 2016). Discovered in C. elegans, the pathway uses a Dual Leucine Zipper Kinase (DLK) MAP3K as an initiator of a signaling cascade involving pre and post transcriptional responses (Byrne et al., 2016; Hammarlund et al., 2009) that result in the regeneration of a new axon. It has also been shown to be involved in axon injury signaling in mice, though while aspects of the known downstream pathway in axon injury were disrupted in

the mutant, regeneration was still observed (Itoh, Horiuchi, Bannerman, Pleasure, & Itoh, 2009; Shin et al., 2012). While other answers are possible, this is most likely because of an evolutionarily recent copy event in vertebrates that has produced a DLK homolog given the name Leucine Zipper Kinase (LZK). This protein has been shown to also be involved in regeneration responses, and much stronger suppression of post axon damage phenotypes have been observed when both genes are inhibited (Welsbie et al., 2017). The sequence similarity between these two proteins lends weight to an argument that axon regeneration in vertebrates still uses this pathway, but that it simply has two partially redundant initiators. However, the DLK pathway is not the only known method for neurons to sense and respond to injury. In Drosophila, while axon regeneration has a strict DLK dependence, dendrites regenerate in a DLK independent manner (Stone, Albertson, Chen, & Rolls, 2014). Because all axons to date have been observed to be plus end out, this would imply that the DRG neuron sensory neurites would regenerate in a DLK+LZK dependent manner via the established axon regeneration pathway. Clarifying the status of DLK pathway involvement in the regeneration of DRG sensory neurites in both the distal sensory endings and proximal nerve would serve as a step towards potentially explaining scenarios where DRG neurons fail to maintain innervation or recover from injury. Additionally, it could help anticipate whether drugs that interact with the DLK+LZK pathway might affect sensory arbor repair and maintenance, and shed light on the mechanism and effects of new therapeutic approaches involving that pathway (Ma et al., 2021).

While mice have DRG neurons with sensory projections innervating their skin, the high absorbance, scattering and autoflorescent background of their skin creates obstacles visualizing the fine sensory endings, and performing fine laser-based injury. The Zebrafish is an optically friendly and genetically tractable vertebrate model that also has DRGs that innervate their fins and scale surface (Palanca et al., 2013). This allows us to directly visualize DRG neurites from

the surface, all the way back to the cell body. Established methods allow for extended imaging of animals which are both anesthetized and immobilized (Rasmussen, Vo, & Sagasti, 2018), and it has been demonstrated (at least at the embryonic stage) that zebrafish are amenable to precision subsurface laser-based injury that still allow recovery for later time points (Rieger & Sagasti, 2011). In this study we examine the regenerative capabilities of DRG sensory neurites immediately above and below the scale surface, as well as proximal to the cell bodies in both wild type and DLK+LZK mutants to determine the potential role of DLK+LZK signaling in the regeneration of DRG neuron sensory neurites.

Results

Zebrafish DRG neuron sensory neurites exhibit robust regeneration

In order to productively determine genetic criteria for regeneration of DRG neurites, we first sought to establish that they do regenerate after laser injury, and in what time frame this occurs. For both simplicity and the hope that linear sections of neurite would simplify quantification, we targeted the caudal tailfin.

Initial characterization of regeneration was performed using a previously published transgenic insertion, P2rx3a>mCherry, which drives LexA under the control of the P2rx3a promoter and enables cell visualization with the resultant expression from a downstream LexOP:mCherry (Palanca et al., 2013) in 8-11 month old fish in a *nacre* mutant background as these lack melanocytes to complicate imaging (Lister, Robertson, Lepage, Johnson, & Raible, 1999).

Post cut images were taken immediately after cutting, and fish were re-imaged at 24hpi (Figure 1A), 48hpi (Figure 1B) and 72hpi (Figure 1C). While fish displayed some degree of

regeneration by 24hpi, the large difference in extension between 0-24hpi and 24-48hpi implies that either regeneration occurs at radically different rates over time, or more likely that there is a lag period before regeneration begins and the 24hpi time point is shortly after this. Cut neurites were able to fully regenerate to innervate the entire length of the tail by 72hpi. We concluded that in our laser injury assay, after a lag phase the DRG sensory neurites of fish with intact DLK and LZK loci displayed robust regeneration that would enable us to probe the factors upon which that regeneration depended.

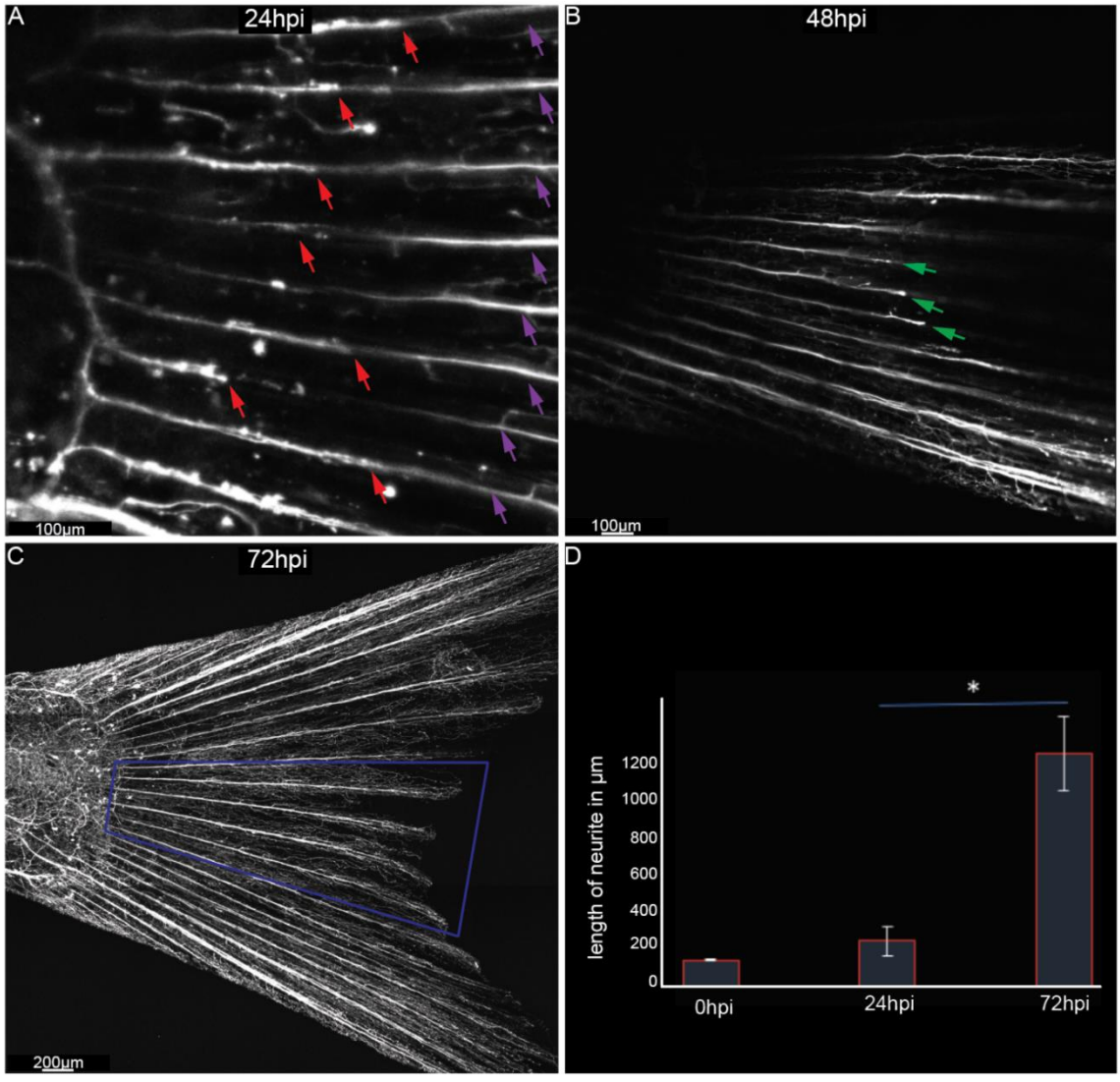


Figure 1: Regeneration of zebrafish DRG neuron tail innervation. A. stumps of injured neurites in the base of the tail in a 9 month old zebrafish 24 hours after injury, showing almost complete degeneration of neurites on the distal side of the cut stumps ladled with red arrows, uninjured neurites on the other side of the tail labeled purple arrows. **B.** regenerating stumps of damaged neurites at 48hpi re-innervating lost territory. C. Overview of tail of fish 72hpi, showing full recovery of innervation. D. Quantification of neurite length at 0, 24, and 72hpi.

Zebrafish DRG neuron sensory neurites at the scale surface display robust regeneration.

While the optical accessibility and linear nature of neurites in the tail were appealing for injury assays, the thinness of the tail causes difficulty presenting the data gathered, as only thin optical section can be represented without causing confusion from the uninjured neurites on the other side, and this restricts images to isolated Z planes lacking full context of their location in the animal. To avoid this we transitioned the injury assay to the scales covering the body of the fish. For this assay we used a previously published P2rx3a>EB3-GFP insertion, which drives LexA under the control of the P2rx3a promoter and enabled visualization of cells via it's downstream LexOP:EB3-GFP (Feng et al., 2019; Shorey et al., 2021) in a *casper* mutant background (White et al., 2008). In this genetic background and location on the animal DRG sensory neurites displayed a similar time course of regeneration to those in the tail, with some extension being visible at 24hpi, and the bulk of regeneration having finished by 72hpi (Figure 2B).

While regeneration reliably occurred at the scale surface it seemed to be robust but inexact, with most neurites recovering the majority of their previous length, but with large variations in exact position (Figure 2 blue through red arrows), with some even extending past their initially observed length (Figure 2 yellow arrow). This may be clarified by the observation that even in our first session imaging any given fish we were consistently able to find multiple neurites already in the process of degeneration (Figure 2A pink arrows) and also neurites that despite not being visibly associated with a degenerating distal portion, nor having been injured as

part of the assay, nevertheless extended over the course of the assay to cover new area (figure 2 grey arrows). Together, these details inform the standard we will use for “normal regeneration”: that the majority of innervation is recovered, rather than having returned to an exact pre injury morphology.

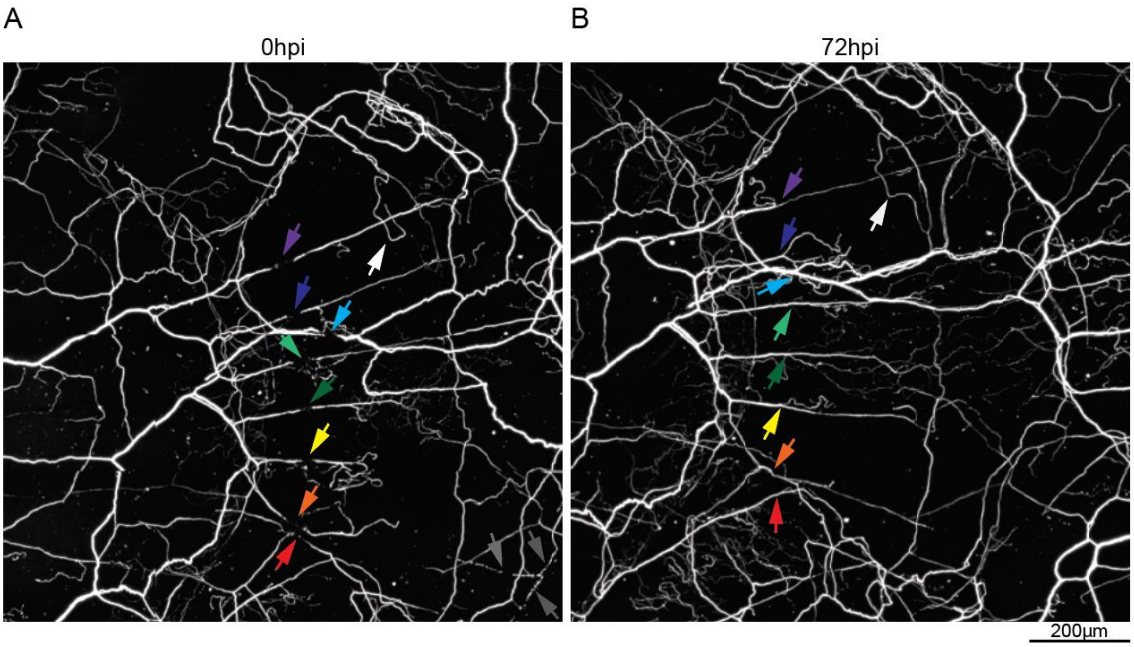


Figure 2: DRG neurons at the scale surface undergo robust but inexact regeneration. A. view of EB3-GFP expressing zebrafish scale immediately after laser-based injury to both surface and subscale nerves. Violet through red arrows indicate laser injuries, light-blue indicates sub-scale nerve injury, grey arrows indicate neurites degeneration from prior damage, white arrow indicates neurite lacking any distal degenerating pieces, but engaging in outgrowth between timepoints. **B.** Same scale, at 72hpi. Note injuries violet through red have all extended past the cut site, and that those previously extending to the edge of the scale have extended similarly. Note that exact position varies, and that in the case of yellow and orange arrows the neurites have extended further than the pre-injury state.

Zebrafish DRG sensory neurites regenerate in a DLK+LZK independent manner, both above and below the scale.

Having established the robust regeneration of DRG sensory neurites in the skin, we next sought determine whether their ability to regenerate relied upon the core axon injury sensing pathway (Hammarlund et al., 2009). In order to assess whether DRG neuron sensory neurites display DLK/LZK dependence for regeneration at or near the scale surface, we bred fish lines containing P2rx3a driven florescent proteins for cell visualization to mutant lines containing the *dlk*^{la231} and *lzk*^{la232} mutations. The mutants were generated by a CRISPR/Cas9 mediated deletion. *dlk*^{la231} is a 519bp in frame deletion that removed most of the kinase domain, and *lzk*^{la232} contains separate 28bp and 30bp deletions in exon 1, which cause a frameshift before the kinase domain (for full details see prepublication). Variations existed between assay conditions with regard to which transgenes were present and the status fish regarding the *roy*^{a9} (which reduces iridophores) and *mifta*^{w2} (which reduces melanocytes) mutations which together comprise the *casper* background (White et al., 2008). See methods and table of reagents for full details.

While the nerve underneath the scale is morphologically and functionally similar to a classic axon, and being linear experiences damage in an all or nothing context, the neurites at the scale surface are quite different in that they are damaged more frequently and often by a matter of small degrees. Because of these differences, we allowed for the possibility that they might exhibit differential dependance on DLK+LZK for regeneration. We used the UV pulse laser to perform cuts at the scale ridges (Rasmussen et al., 2018), as well as the nerve under the scale providing innervation to the adjacent posterior scale (figure 1, left panel, and examined the results of injury to both sites control and mutant conditions.

Because of the robust but inexact nature of regeneration in wild type neurons, and the limitation that reliably removing 100% of innervation to a scale with cuts to the surface was not feasible, we focused on the experimental goal of determining the strict dependence on DLK+LZK for regeneration, as opposed to characterizing potential subtle changes in the behavior of mutant cells. Regeneration was assessed with a binary metric of whether cut neurites mostly regenerated to their previous coverage by 96hpi. For injuries under the scale and at the scale surface this was the case in wild type (Figure 3A top panels), both homozygous single mutants, and the homozygous double mutant (Figure 3A bottom panels). As regeneration still occurred under these conditions, heterozygous mutants were not examined. Given that regeneration still occurred, even in the homozygous $dlk^{la231}/\ dlk^{la231}$ and $lzk^{la232}/\ lzk^{la232}$ double mutant condition we conclude that contrary to expectations based upon their microtubule polarity, regeneration of DRG neuron sensory neurites at and below the scale surface does not, separately or in combination, require DLK or LZK.

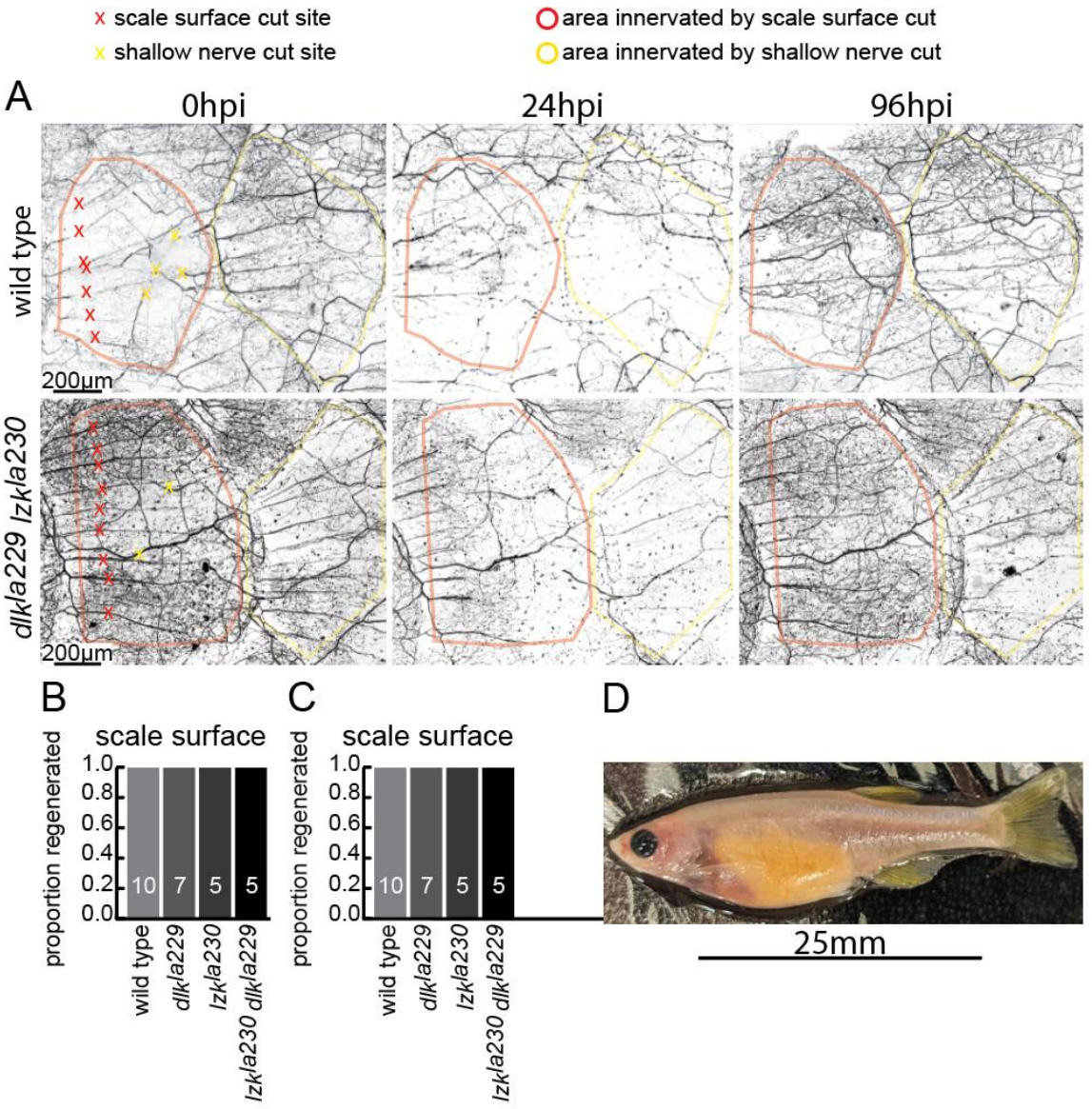


Figure 3: In adult zebrafish at both the scale surface and the nerve under the scale, DRG neurons at regenerate in a DLK+LZK independent manner. A. At 0HPI 8-11 month old zebrafish were subjected to UV pulse laser mediated ablation at multiple points along the scale surface, as well as at the nerve feeding the innervation of the posterior scale, in an attempt to knock out innervation to both scales. Wild type and double mutant fish were in a *casper* background and doubly transgenic for P2X3>EB3-GFPand P2X3>mCherry. Single mutants are in an otherwise wild type background, and singly transgenic for P2rx3a>LexA.LexOP:mCherry. Cut sites are indicated with a red X for scale surface cuts, and a yellow X for under scale nerve cuts, and the area of innervation corresponding to each outlined in red and yellow respectively. At 24HPI each fish was imaged to confirm the success of each cut, and the corresponding loss of innervation after the DRG arbors had degenerated. Note the lack of innervation relative to 0HPI in the outlined areas of the same color. At 96HPI fish were imaged to show the degree of regeneration that had occurred since the 24HPI timepoint. Note the increase in innervation from

the 24HPI timepoint, and the general similarity in terms of coverage to the 0HPI timepoint. **B.** Quantification of the proportion of fish of each genotype indicated regenerating to general equivalence after 96 hours from cuts at the scale surface. **C.** Quantification of the proportion of fish of each genotype indicated regenerating to general equivalence after 96 hours from to the nerve under the scale. D. Representative image of injury age *casper* fish.

Zebrafish DRG sensory neurites regenerate in a DLK+LZK independent manner even when injured proximally to the cell bodies.

As we observed from our pre injury condition, the very accessibility of neurites at the scale surface contributes to their frequent damage and resultant need to regularly, perhaps even continuously regenerate. Even under the scale is an area that frequently requires regeneration as zebrafish lose scales from regular life events such as mating. This would relegate these types of injury to being commonplace rather than traumatic. In order to examine whether the apparent DLK+LZK independence we observed in the regeneration of DRG sensory neurites at or near the surface was due to an alternate as yet uncharacterized regenerative program developed for exposed topical portions subjected to frequent damage, we sought to cut at a site sufficiently proximal to the DRG cell bodies that injury there would not be a regular event and would require regeneration of almost the entire neurite. To do to this we needed a cut site that was as close to the cell bodies as possible while still providing assurance that either off target effects of the cut itself, or some degree of acute axon degeneration, would not endanger the cell bodies.

We had previously shown that in *casper* mutant fish due to the lack of both melanocytes and iridophores, it is possible to image the posterior DRG cell bodies of adult fish via 2 photon microscopy (Shorey et al., 2021). Unfortunately, performing precision ablation at this depth in adult fish proved problematic. However, in 4-5 week old *casper* mutant fish, posterior DRG sensory neuron cell bodies can be imaged in 1 photon, and are amenable to reliable precision ablation with a 2 photon source laser (presumably due to the shorter wavelength involved, we

were unable to perform ablations at this depth with the Micro-Point UV pulse laser used in the surface experiments). DRG sensory neurons innervating the body of the animal have grown to full coverage at this point, though they do not display fully mature morphology at the scale surface. However, DRG sensory neurons innervating the tail seem to display fully mature adult morphology, and have excellent optical accessibility proximal to their cell bodies, with the proximal neurites running nearly perpendicular to the angle of imaging.

At this age, tests in transgenic c*asper* fish showed we could reliably ablate near the posterior DRG cell bodies without apparent neuronal death. Having validated the feasibility of the assay, we migrated both P2rx3a transgenes and the dlk^{la231}/ dlk^{la231} and lzk^{la232}/ lzk^{la232} mutations into a c*asper* mutant background to generate a doubly transgenic fish line that was a homozygous null mutant at 4 separate loci. Due to the lack of visible phenotype from the non *casper* mutations, line founders were identified by tissue sampling and diagnostic PCR

In the doubly transgenic c*asper* mutants, which either had wild type DLK and LZK genomic loci, (control) or dlk^{la231}/ dlk^{la231} + lzk^{la232}/ lzk^{la232} mutations (experimental) we ablated ~100um distal to the posterior DRG neuron cell bodies (figure 4A), and while at 24hpi, degeneration of the severed neurites was not complete, we were able to observe more distal sections totally lacking innervation into which regenerating neurites were growing (figure 4B). By 96hpi these were observed to have regenerated all the way to the tip of the fish tail, fully recovering their coverage prior to injury (figure 4C). Using the same metrics of whether the injured neurites seemed to have regenerated back to their full coverage we observed no difference between the wild type DLK and LZK, and the dlk^{la231}/ dlk^{la231} + lzk^{la232}/ lzk^{la232} conditions (Figure 4D). With the caveat that the proximal cuts were in a pre-adult fish, identical results at cut sites from the surface, under the scale, and all the way back to 100um off the cell body indicates that the entire sensory neurite of DRGs not only regenerates in a DLK+LZK independent manner, but

is able to successfully conduct pathfinding all the way back to its original target at a time-point long after its original outgrowth.

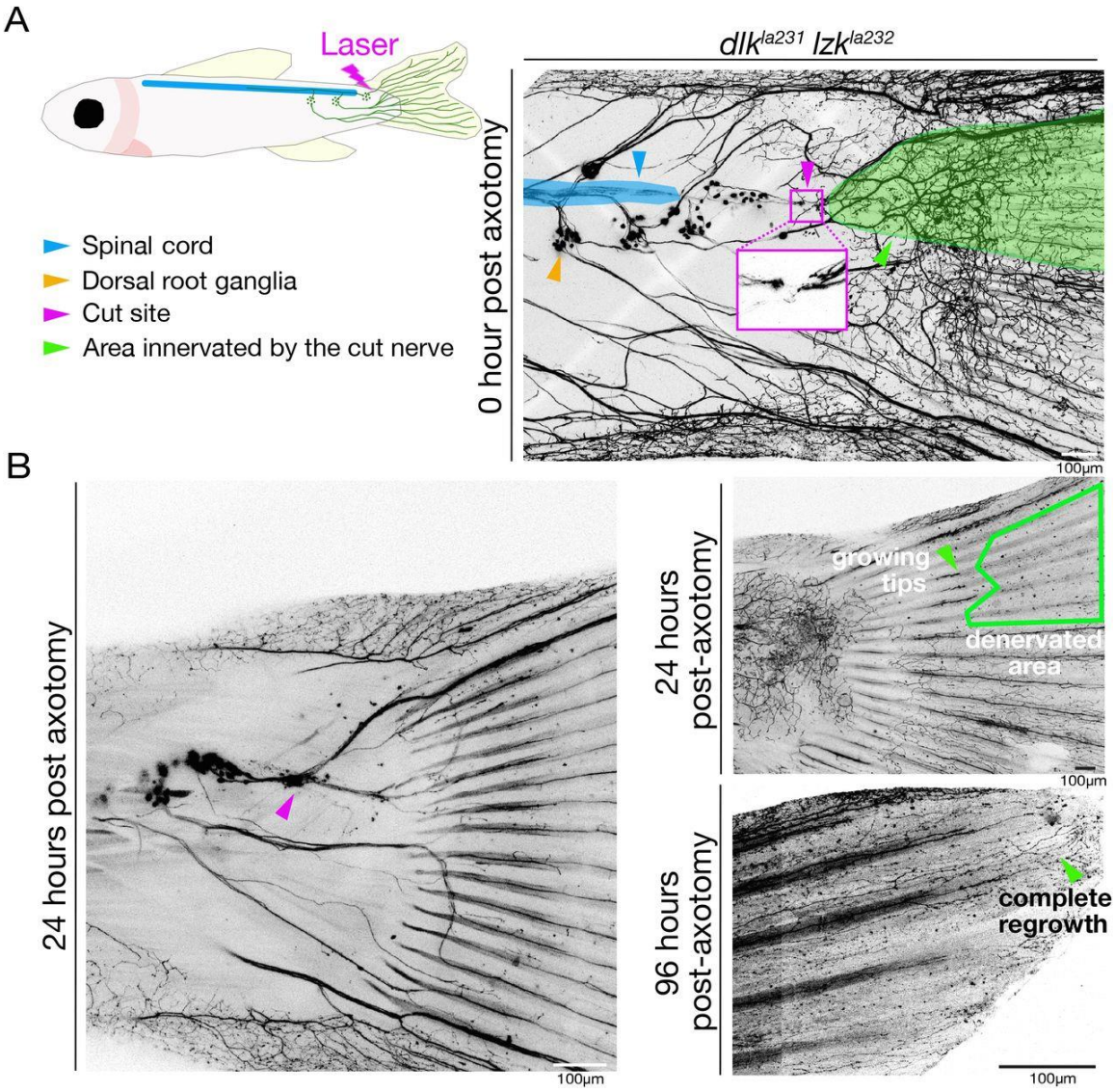


Figure 4: DRG axons innervating the juvenile tail regenerate in wildtype and *dlk*^{la231} *lzk*^{la232} mutants. A) Top left: Cartoon of juvenile casper fish, showing tail-innervating DRGs in P2rx3a:LexA;4xLexAop:mCherry transgenic fish. Lightning bolt indicates axotomy site. Blue indicates spinal cord. Legend refers to the image on the right. Top Right: 2-photon overview showing DRG cell body position, sensory nerve layout and an example of axotomy location in a 4-5 week-old zebrafish. Inset shows the axotomy site of the caudal-most

DRG peripheral nerve. Green highlights separated arbors of the severed DRG nerve, which will degenerate after axotomy. B) Bottom left: Homozygous *dlk*^{la231} *lzk*^{la232} mutant experimental animal 24 hours post-axotomy showing that axons have grown past the axotomy site. Bottom right: Fin of the same animal 24 and 96 hours post-axotomy at different magnifications. At 24 hpa, axons have grown into the fin, but have not yet reached the fin tip. By 96 hpa, axons have reached the fin tip. Axons regenerated in all wt (n=5) and all *dlk*^{la231} *lzk*^{la232} mutants (n=6). Scale bars: 100μm.

Discussion

In order to examine the degree of axonal character of DRG sensory neurites with regard to their regeneration requirements, we used laser microdissection and live imaging of whole fish at multiple ages (including adults) that were all able to be recovered for repeated imaging at later time points to track neuronal regeneration. Besides demonstrating that wild type DRG sensory neurites regenerate and properly engage in pathfinding, whether injured above the scale surface, below the scale surface or proximal to the cell body, we characterized the timeframe over which this regeneration occurs. Successful regeneration of injuries above the scale is expected as these regions are contact surfaces subject to regular insults. Successful regeneration and pathfinding following injuries immediately below the scale is also unsurprising as scale loss is a regular part of fish life, and prior work has documented the ability of DRG neurons to innervate a regrown scale (Rasmussen et al., 2018). That this function still works properly in the absence of potential chemical cues from the tissue involved in the removal and regrowth of a scale is noteworthy, as is the ability to successfully regenerate almost the entire sensory neurite after injury near the cell body, and successful path finding along a large portion of the length of the animal, at a developmental stage long after the time when they would do so as part of normal development.

We demonstrated robust regeneration of DRG neuron sensory neurites from all three cut locations in a *dlk*^{la231}/ *dlk*^{la231} + *lzk*^{la232}/ *lzk*^{la232} homozygous double mutant background. While

DLK+LZK dependent regeneration would be the expectation for a neurite with uniform axonal (plus-end-out) microtubule polarity, previous characterization of sensory neurite regeneration from sciatic nerve injury in mouse shows at least some degree of DLK independent regeneration (Shin et al., 2012) (though LZK was not modulated). Unfortunately, studies showing increased suppression of post axotomy phenotypes from DLK+LZK blockade only provide some additional context, as they were done in an optic nerve crush model, on a different cell type in the CNS (Welsbie et al., 2017). It would however, not be inconceivable that due to the high frequency of damage to sensory neurites, that a different pathway may have developed to deal with sensing injury and initiating outgrowth. The DLK pathway has been shown to involve caspase activation (Chen et al., 2016), and continuous damage would mean continuous caspase activation, which could be detrimental to cell survival. The simplest example of which would be to establish continuous outgrowth kept in check by repressors at the innervation targets (whose effects could be global or local), which would be relieved as soon as damage caused the new tip of the neurite at the proximal side of the cut to no longer be close enough to a suppressor to be effected. Though such a simple explanation is appealing, the ~24 hour delay between injury and regeneration would argue against this being the mechanism as a local level of control. Alternatively, it is known that dendrites also regenerate via a pathway not involving DLK (Stone et al., 2014) and it is also possible that this is the pathway used by these DRG neuron sensory neurites. Unfortunately, while the distance of this alternate pathway has been established, we lack enough information on it to design experiments to determine whether it is in play here.

While technical restrictions prevented us from isolating and damaging the very finest of the terminal sensory endings at the scale surface, a reasonable extrapolation of their DLK+LZK dependence can be derived from their pre-injury condition: In wild type pre-injury fish, we were able to observe multiple sites of minor preexisting damage in every fish observed (Figure 2A). Given post injury degeneration is mostly complete by 24hpi, this indicates that all of these

injuries occurred within the past day. Multiple sites of damage per fish per day means thousands of injuries had already occurred to our 8-11 month old fish. This suggests that regeneration is part of the maintenance of the terminal sensory endings, and that a background that was deficient in regeneration would all else equal, have a corresponding deficiency in innervation. No such deficiency was observed in any of the mutant backgrounds we examined. As such, while not definitively ruled out, it would at the very least be quite surprising if these sections were to display a DLK+LZK requirement for their regeneration, especially when the rest of the neurite did not.

While surprising, we have no data to suggest that this result is specific to fish, rather than being more broadly applicable to vertebrate DRGs. However, these results could explain why in mammals DLK inhibitors were found to be protective against peripheral neuropathy, as opposed to slowly inducing it by causing failure to be able to regenerate from the accumulated damage involved with daily use of extremities (Ma et al., 2021). Further work in determining what pathway is involved in DRG regeneration would greatly benefit from more information on the pathway by which dendrites regenerate, as this seems the next most likely possibility for how their regeneration is controlled, after continuous outgrowth kept in check by target innervation.

Methods

Tail injury regeneration characterization:

One fish was anesthetized in 0.16% MS-222, and immobilized with 4% low melting point agarose. Injury was performed with an Andor MicroPoint UV pulse laser through a 1.2NA 40x

water immersion objective. All images shown were collected on a Zeiss LSM 800 Axio Observer Z.1. Images documenting injury and regeneration were taken at 0hpi, 24hpi, 48hpi and 72hpi. Images were taken using a 0.8 NA 20x air objective.

Scale surface regeneration characterization:

One fish was anesthetized in 0.16% MS-222, and immobilized with 4% low melting point agarose. Injury was performed with an Andor MicroPoint UV pulse laser through a 1.2NA 40x water immersion objective. All images shown were collected on a Zeiss LSM 800 Axio Observer Z.1. The fish was intubated with 0.16% MS-222 at a flow rate of ~6ml/minute and immobilized with 4% low melting point agarose. Images documenting injury and regeneration were taken at 0hpi, 24hpi, 48hpi and 72hpi. Images were taken using a 1.2NA 40x water immersion objective.

Cutting DRG nerves in juvenile fish

R4-5 week-old fish of the genotype P2rx3a:LexA,4xLexOP:EB3-GFP (not shown in the figure), P2rx3a:LexA,4xLexAop:mCherry, *roy*^{a9}/*roy*^{a9}, *mifta*^{w2}/*mifta*^{w2}, containing either wt or homozygous *dlk*^{la231} and *lzk*^{la232} were anesthetized in a VWR polystyrene petri dish, filled halfway with 0.16% tricaine in 0.6 g/L Instant Ocean salt solution, and immobilized by applying agarose to their midsection only, leaving both the head's respiratory apparatus, and tail free. Fish were then imaged on a Leica SP8 microscope equipped with an InSight X3 unit from Spectra-Physics. A 25X (NA= 1) water immersion objective with a working distance of 2.6mm was used to image the posterior spinal cord of the fish, and an ROI was chosen to restrict the cut site to the width of the nerve and positioned ~100 μm from the posterior-most DRG, along the posterior projecting nerve. The tunable laser was set to 900nm, and both the tunable and fixed wavelength 1045nm

lasers were set to 100% on the slowest speed setting and scanned for ~1 second. The 2-photon overview showing the nerve stumps after cut was performed on the aforementioned SP8, all other images for this experiment were obtained with a Zeiss LSM 800 Axio Observer Z.1 with a 20X air objective (NA=0.8).

Cutting scale nerves

All fish were between 8 and 11 months old. The single *dlk*^{la231} and *lzk*^{la232} mutants were transgenic for P2rx3a:LexA,4xLexAop:mCherry, and did not possess mutant *roy* or *mifta* alleles. Wildtype and double mutant fish were in a *roy*^{a9}*/roy*^{a9}, *mifta*^{w2}*/mifta*^{w2} background and doubly transgenic for both P2rx3a:LexA,4xLexAop:EB3-GFP and P2rx3a:LexA,4xLexAop:mCherry^{la207}. All images shown were collected on a Zeiss LSM 800 Axio Observer Z.1. For wildtype, a 25X a multi-immersion objective was used (NA= 0.8), and for the double mutants a 20X air objective (NA= 0.8) was used. Laser injury was performed using an Andor Micro-Point UV pulse laser.

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Chapter 5

Conclusions

Model system selection in neurobiology

There is no best model organism for neurobiology. Even if we were to eliminate the approval process in order to allow unrestricted direct experimentation on humans, some experiments would still be done in other systems for technical reasons. Our work here has leveraged multiple model systems in order to take advantages of their particular strengths as they apply to specific experimental goals. Doing simultaneous axon and dendrite cuts would have been prohibitively difficult in any mammalian model. Even in a transparent fish system amenable to an all-optical damage and observation approach, it would have been far slower doing individual embryo injections to generate sparse motor neurons, and without the abundance of existing lines to facilitate more analysis like RTNL-1 localization, would have required significant subcloning efforts. At the same time, despite the speed and tool availability of drosophila, the DRG polarity assessment simply could not have been performed in that system because drosophila don't have DRG neurons. While mice do have DRG neurons, despite the advancements in the mouse system since the original EM-hook data was acquired, the same logistical barriers to getting EM data in the fine segments with their wandering orientation remain. Exciting recent advancements in infrared fluorescent protein development may make optical interrogation of nerve fibers in the skin of a living mouse possible, but currently the fluorescent proteins in that spectrum are dim, and in the context of EB imaging, one cannot simply compensate for dimness by increasing expression level that way that one can with a cell shape marker, as the amount of probe that can

localize at the polymerizing tip of a microtubule saturates, and further levels only serve to make things worse by increasing background. Additionally, all infrared proteins I am aware of that could be considered for that experiment require some sort of cofactor, such as biliverdin, and even researchers previously claiming their proteins are able to function with the endogenous levels of biliverdin in mice found have shown that at most half the protein is bound and fluorescent in best case cell types (Shemetov, Oliinyk, & Verkhusha, 2017), and that in neurons this amount can drop to drop to less than a quarter of their already low value for brightness (Piatkevich et al., 2017). Optical polarity assessment in mice could possibly be performed on the neurons innervating the low-background surface of the cornea with existing tools, but that would only give us information in trigeminal neurons, though these are closely related to DRG sensory neurons. Similar issues prevent the assessment of regeneration from being as practical in mice, with traditional labeling methods staining would be necessary to track reinnervation of the skin. This would limit post regenerative timepoints to one per animal, with no ability to confirm the post injury degeneration of fibers to guarantee that innervation at a later time point was new growth. Here, the infrared proteins could possibly be made to work as cell shape markers under a strong promoter or possibly leveraging something like the Fireworks system (Alexandrov, Shu, & Steitz, 2017), though the latter would require a great deal of subcloning. Even if novel approaches like these enable the visualization of fibers in the skin, scar formation is powerfully inhibitory to growth in a nerve, and due to their skin's optical properties (or even after surgery to bypass the skin, the greater depth at which work would take place), mice would be less amenable to leveraging the precision of laser-based injury (Song et al., 2012). Fish allowed for the optical determination that gave us orientation of fibers in their native context, and allowed us to look at individual DRG cell bodies *in vivo* in real time, as well as perform precision injuries with an informative number of timepoints tracking degeneration and regeneration.

Full removal of all neurites from a mature neuron

In chapter two I showed that mature neurons can recover from the removal of all neurites. Having used precision laser ablation to remove both the dendrites and axon of mature Drosophila sensory neurons, and observed their robust recovery, we can infer that while axon and dendrite regeneration programs have separate transcriptional programs they initiate, these do not appear to be incompatible, or even limited by competing for common resources. In fact, based on outgrowth and percent of cells engaging in extension, there may be some degree of synergy between the two pathways. Mature Drosophila peripheral neurons can seemingly regenerate both dendrites and axons without any confusion about which is which, and do so from proximal injuries leaving little to no stump to regenerate from. This is interesting because while dendrite regeneration involves adding new neurites, regenerating from a proximal axon injury in Drosophila peripheral neurons usually involves converting a dendrite into an axon (Stone, Nguyen, Tao, Allender, & Rolls, 2010). Though we cannot rule out that the injured neuron didn't first make a dendrite and then convert it, the mixed polarity during regeneration (Song et al., 2012) would complicate determining whether this is the case. Though admittedly speculative, this potentially intersects with the other experiments we performed in the context of regenerating neurites with mixed or unclear identity. Neurites with a mix of axonal and dendritic characteristics could hypothetically mix and match aspects of their damage response. If the pathways had instead been found to be mutually incompatible, this would be much less likely. Even if this is not the case, a greater probability seems that neurites with mixed axonal and dendritic characteristics could employ a corresponding mix of the factors logistically involved with each type of neurite outgrowth. In this light we could see an explanation for how DLK is clearly involved in the regeneration of mouse DRGs but not strictly dependent (Shin et al., 2012), though in that scenario, simpler answers do exist, like the intact LZK locus, or possibly

even a role for MAP3K9 (Figure 2). While the observation of robust, concurrent multi compartment regeneration, with proper neurite specification in mature neurons, is interesting, care should be exercised in extrapolating this result to other systems. The description of the two regenerative programs not being inherently incompatible may very well be true across other species, but many other neuron types are much less tolerant of damage, such as mouse retinal ganglion cells (RGCs), where a large fraction of each undergo apoptosis following axon injury (Welsbie et al., 2017). Whether also damaging their dendrites would be pro-survival because of engaging additional regenerative machinery as is hinted by the ddaE neuron results seems no more likely than the possibility that deprivation of both axonal and dendritic target derived growth factors would push the cell further towards death. However, while I'm hesitant to describe anything that would push the research community towards a greater trust of neuronal cell culture, the ability of DA neurons to properly respecify compartments after injury, not just with expected morphology but microtubule polarity and ER localization seems encouraging in the context of how primary neuron cell culture is initiated. In this scenario, depending on the portion of the brain and the developmental stage in question, not all cells that end up being plated would previously have been, or neurons so immature they had not yet extended neurites. Our data could be taken as a slight reassurance that this does not make the neurons critically divergent simply because of having been plated after the shearing of their neurites during the disassociation process.

The finding that after axotomy, ddaE neurons have an inverse relationship between the number of additional branch points created and the outgrowth of the axon could be interpreted several ways. One interpretation is that activation of the DLK pathway by cutting the axon consistently triggers growth of the cell, and that cells not undergoing outgrowth to the same degree in the axon simply do so elsewhere, especially in the outlier cases of neurons with minimal extension of a converted axon. Traces could be performed to determine whether linear

outgrowth is fairly constant, but distributed throughout the cell instead of taken up by the extending axon. Another interpretation is that ddaE neurons, which do not grow their dendritic arbors to coverage like ddaC neurons (Grueber, Jan, & Jan, 2002), and instead regenerate to dendritic complexity (Stone, Albertson, Chen, & Rolls, 2014), must have some type of feedback which indicates to them how complex their arbor is, and something in the series of alterations to the cell's composition that occurs as a result of the axon regeneration process causes this signal to report low as the cell converts a dendrite, resulting in compensation by the neuron. In this scenario, perhaps cells more successful in axon regeneration manage to exit the phase where the conversion is initiated sooner and induce less perturbation of the arbor. While not a perfect solution, it might be possible to investigate this hypothesis by performing distal axotomies, which engage extension earlier, and do not require converting a dendrite (Rao & Rolls, 2017). Because of the difficulty of tracking axons in a bundle, this experiment would need some way to make the neurite in question resolvable, either by being the only fluorescently labeled neuron, or by being a different color than the other labeled neurons, whether by a sparse activation of a heat shock dependent Brainbow recombination cassette (Veling et al., 2019), or use of a light dependent recombinase such as FLPV(Yao et al., 2020), expressed at sufficiently low levels that only deliberate irradiation o the cell during the injury process would result in the downstream generation of a label unique to that cell.

Vertebrate sensory neuron polarity

In chapter three, I answered the question of how the microtubule cytoskeleton is polarized in DRG neurons, specifically at the branched portion innervating the skin. I did this via an optical approach that bypassed the need for electron microscopy, and allowed analysis of intact live neurons. In order to track polymerizing microtubule tips, we generated a transgenic line that

expresses EB3-GFP under the P2rx3a promoter. While not pan-sensory, in its native context this promoter drives expression of a channel involved in nociception, allowing us to visualize a number RB and DRG sensory neurons (depending on the age of the animal) that innervate the surface of the fish. By performing time lapse imaging of this EB3-GFP in different parts of the neurons we were able to discover several things of interest. First, we established that both RB and DRG sensory neurons have uniform plus-end-out microtubule polarity throughout their centrally projecting neurite (as expected) as well as their large branched sensory neurite. In the case of the DRGs there was some evidence suggesting this from the EM-hook experiments performed in the mouse trigeminal nerve, but only the nerve shaft was examined (Topp, Meade, & LaVail, 1994). Here we have confirmed that the polarity is uniform throughout the entire sensory neurite, resulting in what is to my knowledge, (in the absence of a basal body) the unique juxtaposition of axonal plus-end-out microtubule polarity, and the retrograde signaling that is this neurite's purpose. Insight into why this is the case may be gleaned by examining what most sets these DRG sensory neurons apart from others which are configured differently: their length. While projection neurons in the CNS can be quite long, the sensory neurite of DRG a neuron can be easily over a meter in humans, and I feel safe in assuming a great deal longer in mammals like giraffes and blue whales. It is possible that plus end out growth and transport are faster and necessary to efficiently cover the comparatively vast distance between the cell body and the end of the sensory arbor. DRGs are also probably damaged much more frequently than other neuron types, if only because of their location, and it is possible that that plus end out growth facilitates rapid recovery from incessant injuries as well. This finding of uniform plus-end-out microtubule polarity, makes sense in the context of our other discovery: that RB and DRG neurons have a highly active MTOC in the cell body from which microtubules polymerize out into both the central and peripherally projecting neurites. While I don't think it possible that such microtubules span the entirety of the neurites in question, a continuous stream of plus tips

extending into the neurite could easily assist in the establishment of a plus end out arbor, which could later be maintained by positive feedback loops. Normally neurons transition from a centrosomally organized cytoskeleton to a non-centrosomally organized skeleton around the time they begin to develop mixed polarity dendrites (Lindhout et al., 2021). The lack of need to do so in these cells could explain why the central organization is allowed to persist, as this format has to date has also been observed in the all-plus-end-out neurons of Nematostella vectensis. While we have no data on the trafficking or position of nucleation machinery into these neurites, our observation of EB3-GFP labeled minus ends and the cell body MTOC raises the possibility that with sufficient stability, an arbor could be grown and maintained as a function of severing events generating additional plus and minus ends throughout the neurite (Figure 1), especially with additional support from augmin mediated microtubule dependent microtubule nucleation (David et al., 2019) shown to be involved in maintaining axonal polarity (Sanchez-Huertas et al., 2016). This offers at least one possible answer of how this system could be established and then maintained. Additionally, though the significance is unclear, fish DRG neurons are seemingly a bit different from mammalian DRGs in that they primarily stay bipolar throughout development. This should serve as a reminder that while these are vertebrates with DRG neurons, they are more evolutionarily diverged from humans than mice and, and care should be taken when assuming which aspects of their biology will be the same.

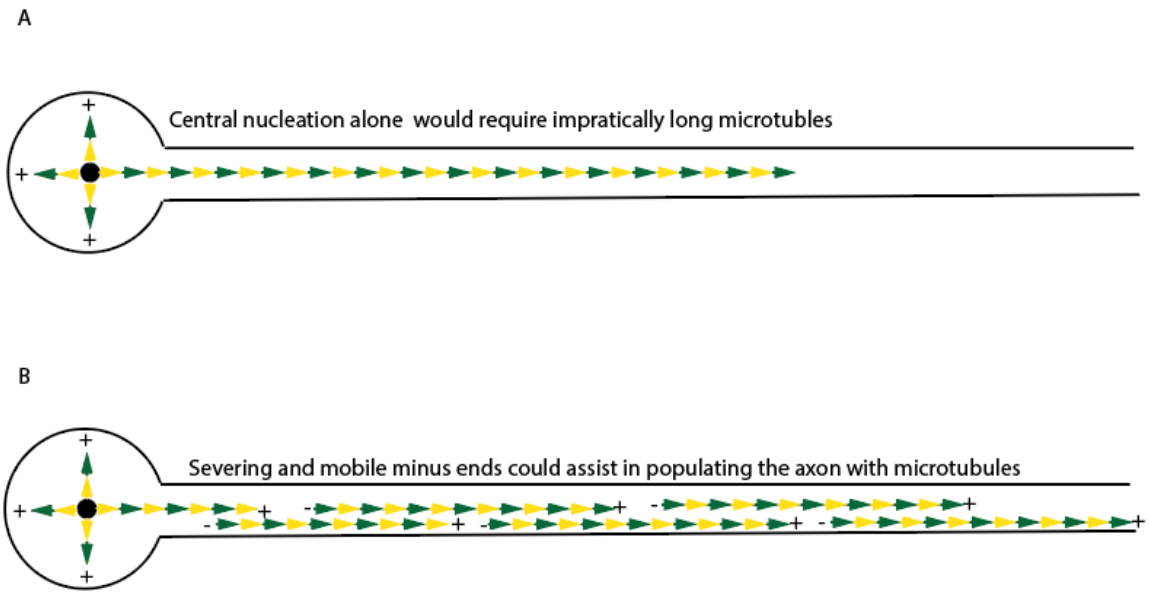

Figure 1: Mobile minus ends enable alternate cytoskeletal configurations. A. diagram of a plus end out neuron with cell body nucleation, showing the microtubule cytoskeleton limited by the length of a single unbroken microtubule. **B.** diagram of a plus end out neuron with cell body nucleation, no longer limited by the length of a microtubule due to the ability to use severing to generate new microtubules and minus end growth to allow extension from both ends.

Sensory neuron regeneration

There are two potential strategies for maintaining neuronal function: avoiding damage in the first place, and being able to recover from damage that does occur. Unlike CNS neurons, which in vertebrates are buried within the protective confines of the spine and brain, the neurites of peripheral sensory neurons need to be positioned in very vulnerable locations to properly perform their function. Within the CNS a neuron may go its entire life without ever needing to regenerate its axon, and in fact, as evidenced by the increase in expression of inhibitory non-cell-autonomous factors as mammals develop, may not even be able to do so once the animal reaches maturity. DRG sensory neurons on the other hand, are potentially damaged to one degree or

another with even normal levels of contact, and absolutely damaged on the occasions when the skin they innervate is damaged or removed via burn, impact, abrasion, or excision. If peripheral regeneration suffered from the same lack of regenerative capability that is enforced in the mature mammalian CNS, every bruise, scrape, cut, or even sunburn would result in the permanent loss of at least some sensory function to that area, resulting in an inescapable progressive loss of sensory function in the skin surface over time. That this is not what happens tells us that these neurons have evolved robust regeneration systems to keep up with the litany of insults to which they are subjected in performance of their function. The discovery that the sensory neurons of zebrafish DRG sensory neurons have axonal, plus-end-out microtubule polarity suggested that they would use the same pathway as other axons to sense damage and initiate regeneration. Interestingly DRG sensory neurite regeneration persisted in a background with homozygous knock-out of both DLK and LZK. This is surprising both from the context of the consistency with which plus-end-out neurites rely on the DLK pathway for regeneration, as well as the observed phenotypes of DLK knockdown in mouse DRGs. While we can confidently, and accurately declare this as "DLK independent regeneration" (as technically, all regenerating occurring in a DLK mutant is DLK independent, even if mediated by LZK) there are two scenarios we need to consider when attempting to draw meaning from this experiment. While not necessarily the most likely, the first is mechanistically the simplest and also the easiest to investigate: evidence from invertebrates suggests that in addition to DLK, there is at least one other MAP3K which has a role in axon regeneration. Experiments in mutating MLK-1 (homolog of slpr in drosophila, or MAP3K9 in mammals) showed a similar loss of regenerative function as the DLK-1 mutant, and crosstalk was observed between what MAP2Ks they signaled to, as well as the MAPKs signaled by the MAP2Ks (Hammarlund, Nix, Hauth, Jorgensen, & Bastiani, 2009; Nix, Hisamoto, Matsumoto, & Bastiani, 2011). This crosstalk at the MAP2k level turns out to be important; while PMK-3 (p38) had originally been identified as the necessary MAPK, KGB-1 (JNK) was later found to be

jointly necessary, and it is because of this crosstalk that both are activated by either DLK-1 or MLK-1. In light of this, it is unsurprising that overexpression of either DLK-1 or MLK-1 was sufficient to compensate for the lack of the other. In drosophila, slpr also signals through the DLK MAP2K MKK7 and is able to activate BSK (JNK) and p38 by doing so (Stronach & Perrimon, 2002), That both slpr and MKK7 also bind JIP (Yasuda, Whitmarsh, Cavanagh, Sharma, & Davis, 1999) implies that it could also be trafficked in the same pattern as DLK, suggesting that like in C. elegans, even if not jointly required for axon regeneration it may be able to compensate for DLK deficiency, and that dependency in this context is then simply a matter of how much of each the cell expresses (Figure 2). The finding that in some contexts the DLK and MLK genes are jointly necessary and with sufficient expression can also compensate for each other is potentially important in light of two facts: First, while the redundancy of DLK and LZK in enabling motor axon regeneration in fish shows that gene redundancy for axon regeneration exists at the MAP3K level, these are both DLK derivatives derived from a recent copy event; LZK is not a MLK-1 homolog. Second, pan-cellular knockout of DLK in mice is lethal, whereas homozygous DLK+LZK mutants in fish show only minor developmental phenotypes. This implies that some aspect of the signaling normally associated with DLK is being performed by some other gene, which is not LZK. The involvement of another gene in an aspect of this signaling pathway, as well as the redundancy demonstrated in fish at least suggests that if MLK was expressed in sufficient levels it could be enabling regeneration. This is especially worth considering as one of the mutants bears a premature stop codon, and as such may induce upregulation of sequence related transcripts as a result (Z. Ma et al., 2019). While there is significant divergence between DLK or LZK and MLK, it is unclear what degree of similarity is sufficient for upregulation of related genes demonstrated to result from mRNA transcripts with premature stop codon mutations (Z. Ma et al., 2019).

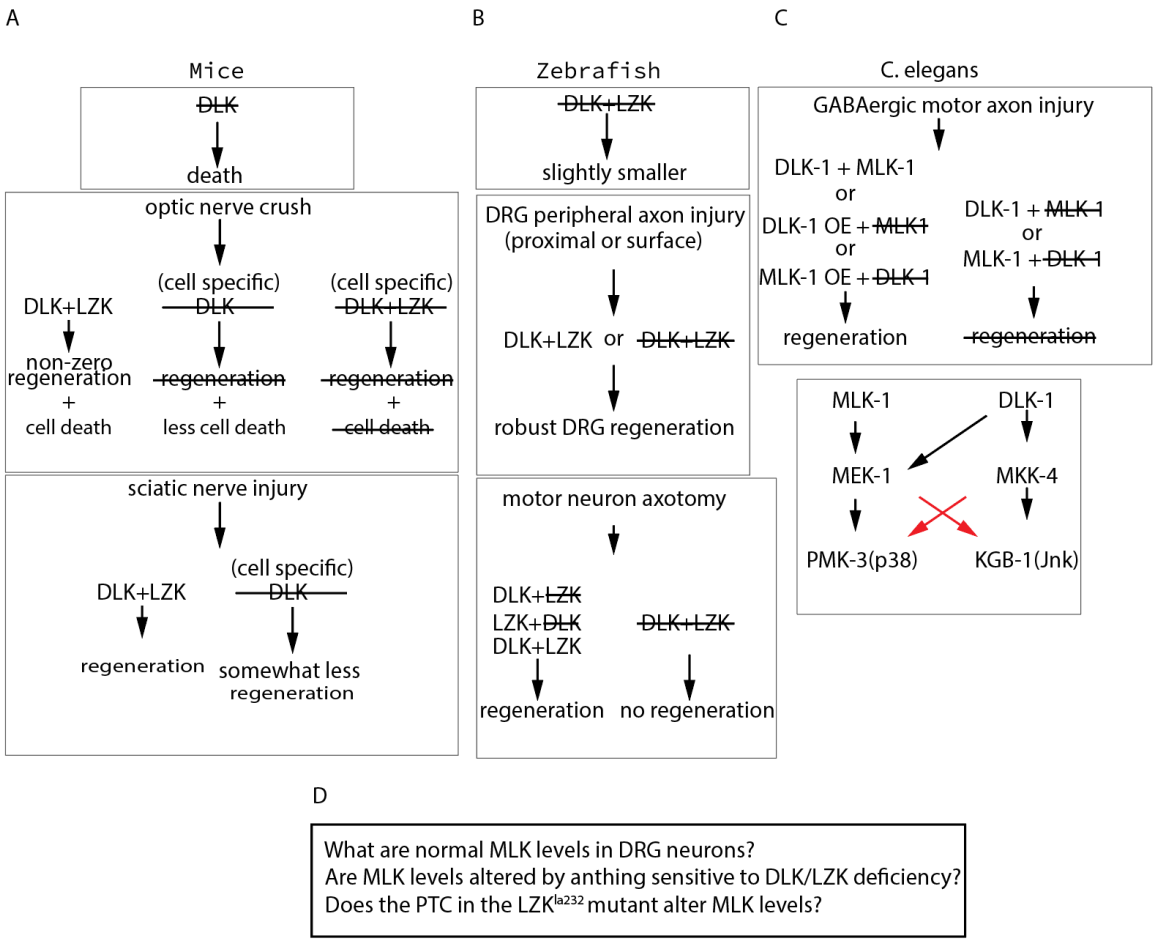

Figure 2: Varying results of DLK deletion across species. A. Diagram indicating the response of mice to DLK knockout, notably death in a pan cellular scenario, and mild to moderate effects on regeneration. **B.** Diagram indicating zebrafish response to both DLK and LZK deletion, notably the redundancy for motor neuron regeneration, and the independence of DRG sensory neuron regeneration. **C.** Diagram indicating the crosstalk at the MAP2K level between DLK-1 and MLK-1 in C. elegans that results in either joint dependency or interchangeability between the two for regenerative purposes, depending on expression level. **D.** A series of questions to address before definitely ruling out DLK-type signaling in the role or DRG neuron regeneration.

While MLK involvement would be the simplest answer, is it also the case that fish DRGs may need even more regenerative capacity than those of mammals, as while both animals suffer high frequency minor impact and abrasions, fish frequently lose entire scales through such regular activities as mating. I speculate that maximum regenerative capacity would be achieved by a system that would be a poor fit for the complexity of the CNS and the need to maintain discrete

circuits, but an excellent option for the simplicity of the sensory system in the periphery: a basal state of outgrowth that is only suppressed by contact with targets of innervation (Figure 3). This model is supported by the observation that the rate of linear extension seen in DRG axons regenerating far from their final target is much higher than seen at the scale surface. Outside the context of scale removal, I am unclear on how high frequency minor damage to the sensory arbor could be effectively addressed by the DLK pathway. If removal of five micrometers of neurite in an arbor of thousands of micrometers was sufficient to activate DLK response, these cells would have a near continuous state of DLK activation. Alternatively, if such an injury was not sufficient to activate DLK signaling, an arbor could potentially be chipped away bit by bit without triggering a regenerative response, leading us to the scenario of a continued loss of function. The DLK pathway makes excellent sense for mediating periodic damage to simple structures like unbranched axon shafts, but how it would work in the case of a highly branched terminal is less clear. Insight into this may be gained by examining the DLK activation, or regeneration dependence, on small pieces of branched axon terminals of more traditional axons (perhaps in the periphery, like the drosophila neuromuscular junction.), or areas so close to the tip as to be potentially governed by synaptic plasticity. Lastly, almost the entirety of our understanding of neuronal regeneration comes from the early steps involved in axon damage signaling, and at a broader level, the process of outgrowth and guidance we can correlate to the factors in initial outgrowth and guidance. Neurons also have dendrites, and at least in drosophila those neurites are capable of robust regeneration. It is not completely out of the question that these cells use whatever undiscovered non-DLK-type pathway that dendrites use to recover from injury.

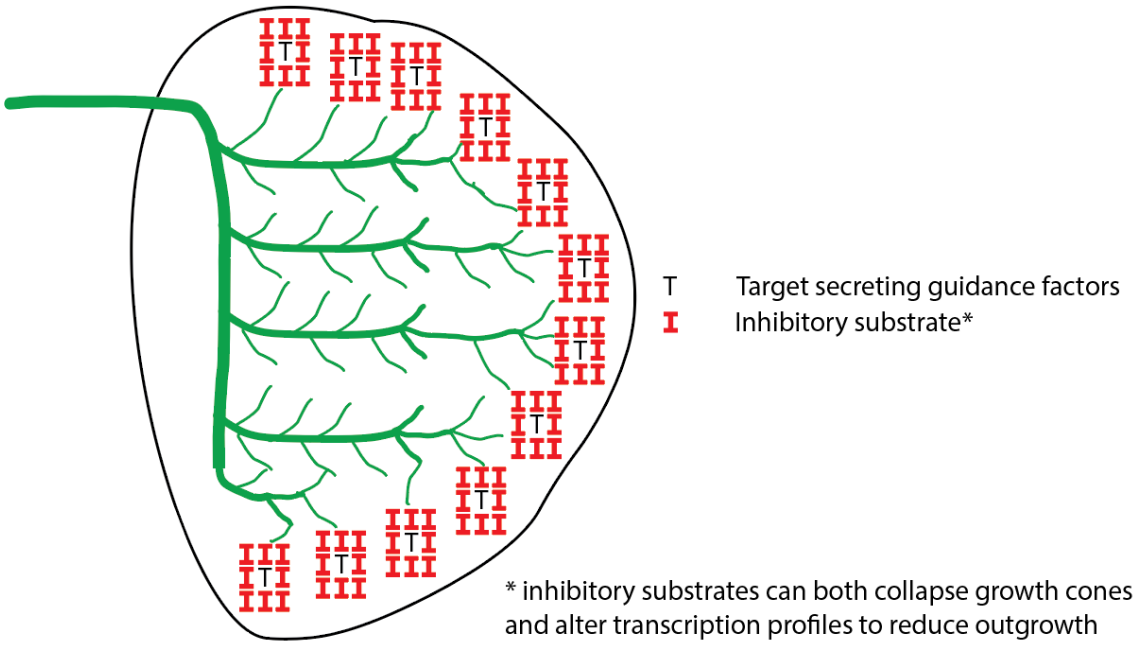

Figure 3: A model for a DLK-independent system of innervation/regeneration. Cartoon of a nerve innervating a fish scale with outgrowth towards targets secreting guidance factors surrounded by inhibitory substrates, resulting in a method of outgrowth completely regulated by target innervation.

If asked to truly reach to construct a model, I would suggest the most likely case is that, based on the redundancy of DLK and LZK in fish motor axon regeneration, and the robust phenotypes seen RGC survival when adding LZK knockout to DLK knockout (Welsbie et al., 2017), that the partial phenotype seen in the sciatic nerve injury of DLK knockout mice would strengthen to become definitive regeneration failure. If this were not the case, I would insist on examining if whatever regeneration was left responded to a MAP3K 9 inhibitor. However, even if this were shown to be the case, for the reasons previously described, I would not necessarily anticipate the surface innervation to respond to the same modulations. Clarification of both of these scenarios could give significant insight into therapeutic approaches used to enhance regeneration in humans, as if fish do have a novel method of outgrowth, in a idea scenario, their

aggressive regeneration could potentially be mimicked by pharmacologically manipulating humans DRGs to a similar state. More realistically, it could be used to anticipate whether therapeutics modulating DLK (and DLK-type) signaling would have problematic side effects in maintenance of the touch sensory system, despite having at least short-term benefits (J. Ma et al., 2021) and either embrace or avoid this approach as indicated.

Closing points

This study was made possible by prior work that has shown drosophila and zebrafish to be excellent models for *in vivo* live imaging, and the tools, genetic and otherwise, for doing so. Without the ability to perform live imaging at multiple timepoints to document injury and recovery, or track microtubule polymerization in in-vivo, answering the questions we asked would have been difficult, potentially to the point of impracticality. Because of the approaches these models enabled, we were able to investigate the interactions between multiple neuronal regeneration pathways. We were also able to resolve what has been an open question since the 1980s about how the microtubule cytoskeleton of sensory endings is structured. With that knowledge we were then able to probe the regenerative properties of sensory neurons in a vertebrate model, and found that at least in fish DRGs, regeneration is not controlled in the manner that we anticipated based on the microtubule data. Lastly, given the robust regenerative capacity of both zebrafish DRG and drosophila DA neurons, I'm given hope that there is nothing fundamental about neurons which makes robust regeneration impractical or problematic, and that with sufficient work we can improve injury outcomes by finding ways to transiently imbue human neurons with greater regenerative capacity.

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