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Review Article

Learning to swim, again: Axon regeneration in fish

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ABSTRACT

Damage to the central nervous system (CNS) of fish can often be repaired to restore function, but in mammals recovery from CNS injuries usually fails due to a lack of axon regeneration. The relatively growth-permissive environment of the fish CNS may reflect both the absence of axon inhibitors found in the mammalian CNS and the presence of pro-regenerative environmental factors. Despite their different capacities for axon regeneration, many of the physiological processes, intrinsic molecular pathways, and cellular behaviors that control an axon's ability to regrow are conserved between fish and mammals. Fish models have thus been useful both for identifying factors differing between mammals and fish that may account for differences in CNS regeneration and for characterizing conserved intrinsic pathways that regulate axon regeneration in all vertebrates. The majority of adult axon regeneration studies have focused on the optic nerve or spinal axons of the teleosts goldfish and zebrafish, which have been productive models for identifying genes associated with axon regeneration, cellular mechanisms of circuit reestablishment, and the basis of functional recovery. Lampreys, which are jawless fish lacking myelin, have provided an opportunity to study regeneration of well defined spinal cord circuits. Newer larval zebrafish models offer numerous genetic tools and the ability to monitor the dynamic behaviors of extrinsic cell types regulating axon regeneration in live animals. Recent advances in imaging and gene editing methods are making fish models yet more powerful for investigating the cellular and molecular underpinnings of axon regeneration.

Contents

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1. Introduction

Fish, our distant vertebrate cousins, are at least as vulnerable as we are to injuries, but their nervous systems have a greater capacity to regrow axons, repair circuits, and recover function. Despite the difference in regenerative ability between mammals and fish, many of the molecular and cellular pathways that regulate axon regeneration are conserved. Fish models have already provided insight into shared mechanisms of axon regeneration and new techniques promise to make them even more powerful systems for investigating how molecules and cells regulate neural repair.

Adult fish regeneration models, which have been established for decades, and the more recently developed larval zebrafish model, have distinct experimental advantages (Table 1). The robust regeneration of optic nerve (ON) and spinal cord axons in larval lamprey and adult goldfish and zebrafish has been exploited to identify factors that promote successful regeneration in the central nervous system (CNS). By contrast, most studies using the larval zebrafish model have focused on axon regeneration in the peripheral nervous system (PNS). The amenability of larval zebrafish to live imaging and genetic manipulation makes them ideal for studying dynamic behaviors of regenerating axons and extrinsic cell types. Adult and larval fish both have well-defined circuits and stereotyped behaviors, facilitating studies of the cell biology underlying axon regrowth and synapse reestablishment, and making it possible to address how anatomical regeneration relates to functional recovery.

Here we review four aspects of axon regeneration studies in adult and larval fish models. First, we discuss efforts to answer one of the most fascinating questions about axon regeneration in the adult fish CNS—why is it so much more successful than axon regeneration in the mammalian CNS? Second, we describe studies of intrinsic growth pathways in fish, which have demonstrated that the molecular basis of axon growth is conserved between fish and mammals. These studies have also identified new molecules associated with regenerative axon growth, providing candidate targets for therapeutic interventions. Third, we review studies in both adults and larvae that assessed the success of functional recovery and mechanisms of circuit re-establishment. Finally, we discuss what has been learned from fish models about interactions of non-neuronal cells with regenerating axons. These studies, many using live imaging in larval zebrafish, have uncovered new roles for extrinsic cell types in PNS axon regeneration and have the potential to reveal much more about dynamic cell behaviors during axon regeneration in both the PNS and CNS.

2. Why do axons regenerate so well in the fish CNS?

What underlies the disparate regenerative abilities of mammalian and fish CNS axons? Exposing mammalian axons to cells of the fish CNS, or fish axons to cells of the mammalian CNS, can distinguish whether differences in regeneration are attributable to neurons themselves or to their surrounding environment. Regenerating axons of both mammalian and fish neurons are repelled by mammalian oligodendrocytes and myelin (Bandtlow et al., 1990; Bastmeyer et al., 1991; Fawcett et al., 1989) but both can grow in the presence of fish oligodendrocytes or fish CNS conditioned media (Bastmeyer et al., 1991, 1993; Schwalb et al., 1995; Schwartz et al., 1985; Wanner et al., 1995). Thus, just as factors in the environment of the mammalian CNS and PNS account for differences in the success of axon regeneration (David and Aguayo, 1981), distinct factors in the mammalian and fish CNS environment account for much of their difference in axon regeneration. Three explanations for the more growth-permissive environment of the fish CNS have been proposed: the absence of inhibitory cues, the presence of factors that block inhibitory cues, and the presence of pro-growth factors.

2.1. Are inhibitory factors present in the fish CNS?

The adult mammalian CNS contains multiple molecules inhibitory to axon growth, including myelin proteins, ECM proteins such as chondroitin sulfate proteoglycans (CSPGs) and Tenascins, and chemorepulsive guidance cues (reviewed by Giger et al., 2010). The mammalian myelin proteins RTN4-A/Nogo-A, myelin-associated glycoprotein (MAG), and oligodendrocyte myelin glycoprotein (OMgp)—all of which have homologs in the zebrafish genome (Lehmann et al., 2004; Shypitsyna et al., 2011; our unpublished observations)—activate the growth-inhibiting Nogo receptor (NgR) complex on regenerating axons (Giger et al., 2010). Although functional studies of fish MAG and OMgp have not been reported, zebrafish Nogo-A homologs have been studied biochemically and genetically. Purified mammalian Nogo and MAG inhibit the growth of fish axons (Abdesselem et al., 2009; Chen et al., 2013), but the fish homolog of the mammalian Nogo-66 domain, which is responsible for growth inhibition in mammals, does not affect growth of fish or mammalian axons (Abdesselem et al., 2009). Zebrafish Nogo-66 can bind to mammalian NgR but fails to activate downstream signaling (Abdesselem et al., 2009), suggesting that zebrafish Nogo-A and its mammalian counterpart may have functionally diverged from each other (Abdesselem et al., 2009; Shypitsyna et al., 2011). Further evidence for functional divergence comes from studies of Rtn4b, a fish

Table 1
Fish models to study axon regeneration.

Organism	Axon models	Pros	Cons
Larval sea lamprey (*Petromyzon marinus*)	Spinal cord	● Basal vertebrate lacking myelin ● Flat, translucent spinal cord facilitates microscopy ● Large, easily identifiable neurons ● Quantifiable swimming behaviors	● Lack of genetic tools ● Lack of live imaging ● "Developmental" environment
Adult goldfish (*Carassius auratus*)	Optic nerve, spinal cord	● Well-described anatomy, electrophysiology ● Well-characterized behaviors	● Lack of genetic tools ● Live imaging difficult
Adult zebrafish (*Danio rerio*)	Optic nerve, spinal cord, motor neurons, posterior lateral line nerve (pLLn)	● Increasing number of transgenic tools ● Genetic models possible (mutants, inducible transgenes, implantable morpholinos)	● Live imaging difficult
Larval zebrafish (*Danio rerio*)	Optic nerve, spinal cord, motor neurons, pLLn, somatosensory neurons	● Numerous transgenes ● Many genetic tools ● Live imaging ● Easily quantifiable behaviors	● "Developmental" environment

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Nogo-A homolog. Whereas loss of mammalian *rtn4* results in only subtle developmental defects and may or may not improve axon regeneration (Kim et al., 2003; Simonen et al., 2003; Zheng et al., 2003), knocking down zebrafish *rtn4b* causes severe brain and spinal cord abnormalities and larval lethality (Pinzón-Olejua et al., 2014). Surprisingly, knocking down *rtn4b* in the adult zebrafish ON reduces, rather than enhances, axon regeneration (Welte et al., 2015), indicating that it does not act as an inhibitory factor in fish.

The glial scar created by reactive astrocytes is another major obstacle to regeneration in the mammalian CNS (Giger et al., 2010). Astrocytes (and other cell types) in the glial scar deposit several axon inhibitory molecules at the injury site, including CSPGs (Katoh-Semba et al., 1995; McKeon et al., 1999; Tang et al., 2003). Although there are some reports to the contrary (Alunni et al., 2005; Battisti et al., 1995; Kawai et al., 2001), the fish CNS is thought to lack astrocytes (reviewed by Lyons and Talbot, 2015). Accordingly, immunostaining experiments in adult zebrafish found that CSPGs were unchanged at the lesion following ON injury (Becker and Becker, 2002). Although CSPG levels were not altered by injury, they were expressed at the edges of the optic tract, and enzymatic degradation of CSPGs resulted in pathfinding errors by regenerating axons (Becker and Becker, 2002). Thus, like in mammals, CSPGs are growth-inhibitory in fish; however, they are likely produced by non-astrocyte cell types and may help guide axons rather than block their growth (Becker and Becker, 2002).

Tenascins, another family of conserved ECM glycoproteins found at glial scars, can also regulate axon growth. Inhibition of Tenascin-C in zebrafish results in motor axon guidance defects during larval stages (Schweitzer et al., 2005) and improves axon regeneration and recovery of swimming behavior after spinal cord injury in adults (Yu et al., 2011b), indicating that the CNS environment of fish does not completely lack inhibitors of axon regeneration. Tenascin-R acts as an inhibitory guidance molecule during initial development of the ON (Becker et al., 2003) and in adults is expressed at the margins of the optic tract (Becker et al., 2004b). Thus, similar to CSPGs, Tenascin-R may play a role in guidance during axon regeneration (Becker and Becker, 2002).

2.2. Inhibitors of inhibitory factors in the CNS?

Another way in which the fish CNS could, in principle, support axon regeneration is by expressing factors that neutralize glial inhibitory cells or proteins. Indeed, conditioned media from regenerating goldfish optic nerves (but not from uninjured fish nerves) was found to promote growth of mammalian axons (Schwartz et al., 1985) and decrease the number of rat oligodendrocytes in culture (Cohen et al., 1990). An antibody to an interleukin-2 (IL-2)-like molecule abrogated the effect of fish conditioned media on oligodendrocytes, and recombinant mammalian IL-2 mimicked its effect (Eitan et al., 1992). The cytotoxic effect of IL-2 depends on its dimerization, which is promoted by the activity of a transglutaminase enzyme (Eitan and Schwartz, 1993). Remarkably, injecting transglutaminase into the ON of adult rats improved axon regeneration and recovery of light-evoked responses (Eitan et al., 1994). These findings prompted Schwartz and colleagues to propose that damaged fish nerves secrete an IL-2-like factor toxic to oligodendrocytes, thus allowing regenerating axons to avoid oligodendrocyte-derived inhibitory factors. Although there is no definitive in vivo evidence that oligodendrocytes near injury sites die in fish, this series of studies at least raised the conceptually important possibility that natural antagonists of axon growth inhibitory molecules or cells may contribute to the favorable growth environment of the fish CNS.

2.3. Do positive cues promote axon regeneration in the fish CNS?

Molecules secreted by the regenerating fish ON affect not only oligodendrocytes but also neurons directly. Among these are two distinct biochemically identified factors, Axogenesis Factor (AF)-1 and AF-2, which promote axon growth in cultured fish retinal ganglion cells (RGCs) (Schwalb et al., 1995). AF-1 is secreted by glial cells (Schwalb et al., 1996), requires the activity of a purine-sensitive signaling pathway (Petrausch et al., 2000), and activates expression of several growth-associated proteins (GAPs) (Petrausch et al., 2000). Biochemical purification identified AF-1 as the carbohydrate mannose, which can also promote the growth of rat RGCs in a cyclic AMP (cAMP)-dependent manner (Li et al., 2003).

A screen for cDNAs differentially expressed in damaged and undamaged goldfish ONs identified the retinol-binding protein Purpurin as another positive regulator of ON regeneration (Matsukawa et al., 2004b). Purpurin is required during initial eye development in zebrafish (Nagashima et al., 2009a, 2010), and is upregulated in the retina of both goldfish and zebrafish after injury (Matsukawa et al., 2004b; Tanaka et al., 2007). Treating an uninjured goldfish ON with Purpurin promoted axon growth (Matsukawa et al., 2004b). Kato and colleagues propose that Purpurin secreted by photoreceptors after injury acts as a retinal transporter and promotes ON regeneration by providing RGCs with retinoic acid, which activates pro-growth transcriptional programs (Kato et al., 2013; Nagashima et al., 2009b). The potential involvement of factors like Purpurin and mannose in ON regeneration suggests that the more felicitous environment for regeneration in the fish CNS may result not only from the lack of inhibitory molecules or cells, but also from the presence of positive factors. Some of these factors may merit consideration as potential therapeutics for CNS injuries.

3. Intrinsic pathways regulating axon regeneration in fish

An axon's ability to regenerate is determined not just by factors in its environment, but also by the activity of intrinsic growth pathways (reviewed by Liu et al., 2011). These intrinsic pathways may contribute to the differing success of axon regeneration in mammals and fish, but it is unlikely that a single, consistent distinction between fish and mammalian growth pathways explains the better regeneration of fish CNS axons. Despite the relatively permissive environment of the fish CNS, central axons in fish exhibit widely varying capacities to regenerate (Fig. 1A) (Becker et al., 1997, 2005; Bernstein and Gelderd, 1970; Davis and McClellan, 1993; Rovainen, 1976; Selzer, 1978; Sharma et al., 1993), implying that the activity of intrinsic growth pathways varies even among fish neurons. Moreover, most of the same growth pathways that affect mammalian axon regeneration also affect regeneration of fish axons. Thus, differences in intrinsic growth pathways between fish and mammalian neurons may be quantitative differences in their activity, rather than qualitative differences in their molecular nature. Since it is possible to measure both increases and decreases in axon regeneration in fish, fish models may be particularly suitable for identifying these pathways and investigating how they interact.

3.1. Physiological regulators of axon regeneration

The state of intrinsic growth pathways in any particular neuron is not immutable, but is modulated by the physiological state of the cell or the animal. In most animals, for example, age reduces the success of axon regeneration (Goldberg and Barres, 2000; Liu et al., 2011), reflecting downregulation of pathways that promote axon growth and upregulation of pathways that inhibit it. In a study of adult goldfish, Bernstein (1964) demonstrated this phenomenon with physiological recordings, anatomical tracing, and behavioral analyses: 1-year-old goldfish recovered from spinal cord transection more rapidly than 2-year-old fish, which in turn recovered more rapidly than 3-year-old fish. Age-related decline in axon regeneration has also been observed in the zebrafish CNS (Becker et al., 1997) and PNS (Graciarena et al., 2014). Analogously, the ability of the zebrafish ON to recover from demyelination declines with age (Münzel et al., 2014). Thus, although the adult fish nervous system recovers from injury well compared to

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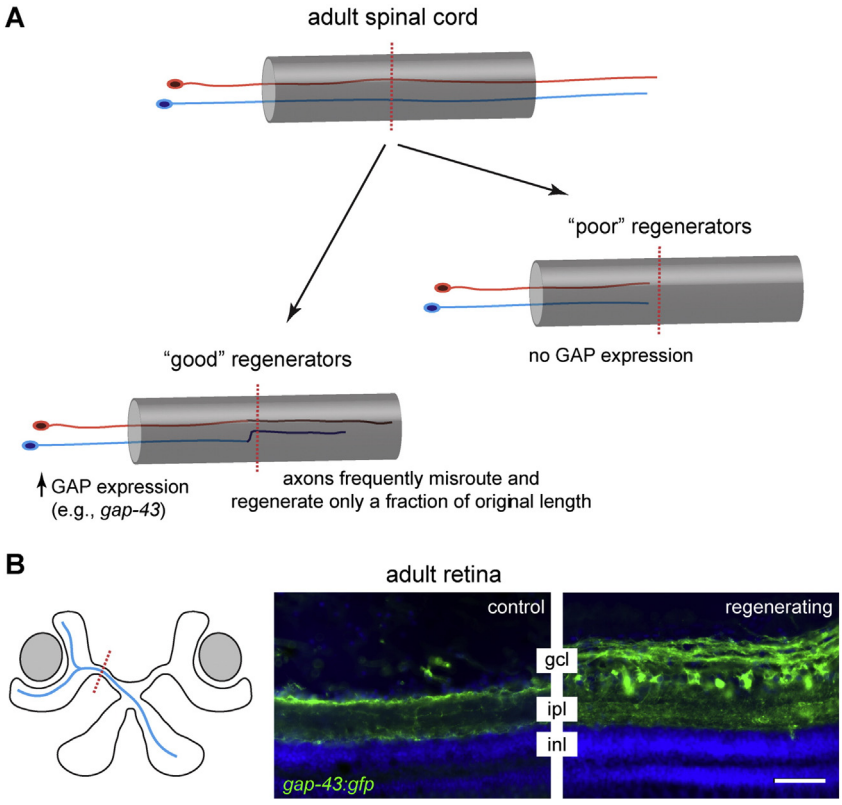

Fig. 1. Adult fish spinal cord and optic nerve axons activate intrinsic pathways to regenerate. A) Axon regeneration in the adult fish spinal cord is variable. Although some axons in the fish CNS upregulate growth associated proteins (GAPs) upon injury and regenerate robustly, they can often misroute and many grow only a fraction of their original lengths. Many axons fail to regenerate altogether, which might reflect a failure to upregulate GAPs. B) Injury-responsive transgenic lines, such as the *gap-43*:*GFP* reporter, provide powerful tools for assessing activation of growth pathways upon injury. For example, upon optic nerve injury (schematic), retinal ganglion cells in the retina (images) upregulate *gap-43*, as demonstrated by the striking increase in GFP expression in the ganglion cell layer (gcl). ipl, inner plexiform layer; inl, inner nuclear layer. Scale bar, 100 μm.
Images were the generous gift of Ava Udvadia (University of Wisconsin, Milwaukee).

mammals, it experiences a similar age-related decline in regeneration that can be exploited to identify genes regulating axon regeneration.

Fish models have also made contributions to addressing the role of neuronal activity in axon regeneration. In adult goldfish, blocking synaptic activity with tetrodotoxin injection disrupts ON regeneration, the re-establishment of functional synapses, and refinement of the retinotectal map (Edwards and Grafstein, 1983; Schmidt et al., 1983; Schmidt and Edwards, 1983). Conversely, applying an electrical field to the spinal cord of lamprey substantially enhances recovery from spinal cord transection (Borgens et al., 1981), and increasing locomotor activity in eels improves spinal cord regeneration (Doyle and Roberts, 2006). The role of activity in axon regeneration is conserved, since blocking activity decreases outgrowth of cultured mouse retinal ganglion cells (e.g., Goldberg et al., 2002) and electrical stimulation of the mammalian spinal cord improves recovery from injury (e.g., Borgens et al., 1987; reviewed by Gordon and English, 2015).

Previous injury causes physiological changes in axons that promote their intrinsic regenerative capacity. This phenomenon, known as a "preconditioning lesion" effect, is mediated, at least in part, by transcriptional responses that prime the cell for axonal growth (reviewed by Hoffman, 2010). In mammals, preconditioning lesions of peripheral nerves accelerate regeneration after a second lesion to the same peripheral nerve (Gutmann, 1942), and a preconditioning injury to a peripheral sensory nerve allows regeneration of central axons of those neurons (Neumann and Woolf, 1999). In the early 1980s, Grafstein and colleagues similarly demonstrated that a preconditioning lesion accelerates ON regeneration in goldfish (Edwards et al., 1981; Lanners and Grafstein, 1980; McQuarrie and Grafstein, 1981). Subsequent studies have documented preconditioning lesion effects in the spinal cord of lamprey (Zhang et al., 2004) and in peripheral lateral line nerves of adult zebrafish (Graciarena et al., 2014), demonstrating that the preconditioning phenomenon is widely conserved, if not universal, among vertebrate neurons.

3.2. Conserved molecules and pathways regulate intrinsic axon growth potential

The second messenger cAMP is upregulated by injury and cAMP-dependent pathways play important roles in the preconditioning lesion effect (reviewed by Hannila and Filbin, 2008). Like in other vertebrates, CNS axon regeneration in lamprey and goldfish is improved by treatment with cAMP analogs (Jin et al., 2009; Lau et al., 2013; Pale et al., 2013; Rodger et al., 2005), which likely act by activating Protein Kinase A (Pale et al., 2013; Rodger et al., 2005). The ability to identify specific neurons and image them in live zebrafish larvae enabled a vivid demonstration of cAMP's potential for promoting axon regeneration (Bhatt et al., 2004). Bhatt et al. (2004) lesioned larval spinal cords and monitored anatomical and functional regeneration of Mauthner cell axons, which project from the hindbrain to the caudal spinal cord to mediate fast escape responses. Injured Mauthner cell axons regenerated only about a third of the time in control neurons. Strikingly, introducing a cAMP analog directly into Mauthner cells induced robust axon regeneration in almost all treated neurons, as documented with confocal microscopy, calcium imaging of circuit activity, and behavioral analysis.

Intrinsic growth capacity is also affected by negative regulators, which increase with age to reduce regeneration. Phosphatase and tensin homolog (PTEN) and suppressor of cytokine signaling 3 (SOCS3), two negative regulators of axon regeneration in mice (Liu et al., 2011), appear to play conserved roles in adult zebrafish. Inhibiting one of two zebrafish PTEN orthologs with PTEN-targeting morpholinos improved

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regeneration of adult zebrafish spinal cord neurons (Liu et al., 2014), while pharmacological inhibition of PTEN's downstream effector, mammalian (or mechanistic) target of rapamycin (mTOR), reduced cytokine-enhanced ON regeneration in zebrafish (Diekmann et al., 2015a). Similarly, SOCS3, which decreases axon regeneration in mammals by inhibiting Jak/Stat signaling downstream of growth-promoting cytokines (Smith et al., 2009), inhibits axon regeneration in the zebrafish ON (Elsaeidi et al., 2014). Fish Socs3a also acts by antagonizing the Jak/Stat pathway but, in contrast to mammalian SOCS3, it does not block axon regeneration altogether (Elsaeidi et al., 2014).

The conservation of regeneration regulators, like cAMP, PTEN and SOCS3, suggests that although fish CNS axons regenerate better than mammalian axons, intrinsic molecular mechanisms of regeneration are largely the same in all vertebrates. Moreover, the fact that regeneration can be improved with preconditioning lesions and cAMP, or by inhibiting PTEN and SOCS3, belies the simplistic notion that fish axons optimally activate intrinsic growth pathways. It is likely that a balance of growth promoting and inhibiting factors determine each axon's ability to regenerate, and this balance may contribute to differences in the ability of axons to regenerate not only between fish and mammals, but also between different cell types in fish.

3.3. Identifying new molecules regulating axon growth potential

Searching for genes and proteins associated with axon growth (growth-associated proteins, GAPs) or specifically with regeneration (regeneration-associated genes, RAGs), has been a fruitful strategy for identifying candidate regulators of axon regeneration (reviewed by Patodia and Raivich, 2012). In fish, several RAGs and GAPs have been discovered in screens for transcripts or proteins that increase after injury to the adult ON or spinal cord. These include a tubulin (Heacock and Agranoff, 1976), several cell adhesion molecules (Becker et al., 1998; Bernhardt et al., 1996; Paschke et al., 1992), the microtubule interacting zRich proteins (Heacock and Agranoff, 1982; Pathi et al., 2012; Wilmot et al., 1993), the lipid microdomain-associated Flotillins, Reggie-1 and Reggie-2 (Munderloh et al., 2009; Schulte et al., 1997), the microRNA miR-133b (Yu et al., 2011a), and the transcription factors Kruppel-Like Factor (KLF) 6a and Klf7a (Veldman et al., 2007, 2010). Unbiased expression profiling of neurons responding to axon damage continue to lengthen the list of GAPs in zebrafish (Hui et al., 2014; Lemmens et al., 2015).

Adult fish have proven to be excellent models for identifying genes correlated with regenerative growth, but likely only some of these genes play functionally significant roles in regeneration. Since many GAPs are required during initial development, testing their function in the regeneration of mature nervous systems requires a method for knocking them down specifically in adult tissues. To address this problem, Becker et al. (2004a) developed a technique in which gelfoams soaked in morpholino antisense oligonucleotides targeting specific genes are implanted directly into damaged CNS tissue. Using this technique, several studies have demonstrated that inhibiting specific genes, including tubulins (Veldman et al., 2010), the cell adhesion molecule L1.1 (Becker et al., 2004a), the Flotillins (Munderloh et al., 2009), the microRNA miR-133b (Yu et al., 2011a), and KLF transcription factors (Veldman et al., 2007), compromises axon regeneration. Although morpholino knockdown experiments should be interpreted cautiously (Kok et al., 2015; Rossi et al., 2015; Schulte-Merker and Stainier, 2014), this method provides a simple initial test for the function of new candidate genes in axon regeneration.

Could the GAPs and RAGs identified in adult fish models serve as potential therapeutic targets in mammals? In a few cases, homologs of fish genes required for regeneration have also been implicated in mammalian axon regeneration. For example, overexpressing or knocking down Flotillins in rat neurons have both been reported to accelerate regeneration (Koch et al., 2013; Santiago et al., 2013), perhaps reflecting growth-promoting and -inhibiting functions for these genes, which may regulate trafficking at growth cones (reviewed by Stuermer, 2012). Similarly, KLF transcription factors regulate axon regeneration not just in fish (Veldman et al., 2007, 2010), but also in the mouse ON and spinal cord (Blackmore et al., 2012; Moore et al., 2009). In fish, Klf6a and Klf7a are required for axon regeneration (Veldman et al., 2007). In mice, overexpressing Klf6 or Klf7 improves the growth of RGC axons (Moore et al., 2009) and overexpressing Klf7 fused to a transcriptional activation domain promotes the growth of corticospinal neurons (Blackmore et al., 2012). By contrast, Klf4 and Klf9 are inhibitors of ON axon regeneration in mice (Moore et al., 2009), demonstrating that a balance of growth-promoting and -inhibiting KLF factors regulate axon regeneration (reviewed by Moore et al., 2011). These findings validate the use of fish models as a source for candidate mammalian regeneration genes.

While it has been possible to find new GAPs based on expression changes in adult fish, another strategy will be necessary to identify pathways that sense injury and activate these genes to promote regeneration. A forward genetic screen for mutants defective in recovery from injury is theoretically possible, but may be too laborious to be feasible in fish models. A more reasonable approach would be to screen for fish mutants that fail to upregulate GAPs after injury. Transgenic zebrafish lines that express GFP under the control of regulatory elements for growth-associated protein 43 (*gap43*) (Diekmann et al., 2015b; Kusik et al., 2010; Udvadia, 2008) and α1-tubulin (Goldman et al., 2001; Senut et al., 2004), display markedly increased GFP expression after ON injury (Fig. 1B). Knocking down the transcription factor MASH1/Ascl1a reduces GFP expression in *gap43* transgenic reporter fish and disrupts ON regeneration, while overexpression of MASH1 in injured rat neurons enhances regeneration (Williams et al., 2015). This finding provides proof-of-principle that activation of injury-sensing pathways can be detected with *gap43* reporter fish. Transgenic GAP reporter lines could be used to test the function of additional candidate pathways, such as the dual leucine-zipper kinase (DLK) mitogen-activated protein kinase (MAPK) pathway, which has yet to be studied in fish, despite its central role in sensing axon damage in other animals (reviewed by Tedeschi and Bradke, 2013). More importantly, these transgenic reporters may be ideal tools for forward genetic screens to identify new regeneration factors.

4. How do circuits rewire? Regenerating synapses and restoring function

The ultimate goal of axon regeneration interventions is to rebuild synapses and recover function of damaged circuits. Fundamental questions about circuit recovery are just beginning to be addressed in vertebrates. For example, do regenerating axons use the same guidance cues as in development to find their synaptic targets? Do they form synapses that are similar in number, size, and molecular composition to those formed during development? Do axons rewire with their original synaptic partners or do compensatory circuits contribute to functional recovery? Following spinal cord injury, fish rapidly recover “normal” swimming behavior (Becker et al., 1997; Bernstein, 1964; Bernstein and Gelderd, 1970; Davis and McClellan, 1993; Hooker, 1932; Oliphint et al., 2010; Rovainen, 1976; Tuge and Hanzawa, 1937). However, careful analysis of axon regeneration suggests that at least half of all transected axons never regenerate (Becker et al., 1997, 2005; Bernstein, 1964; Bernstein and Gelderd, 1970; Davis and McClellan, 1993; Oliphint et al., 2010; Rovainen, 1976; Selzer, 1978; Sharma et al., 1993). Moreover, those axons that do regenerate frequently adopt aberrant trajectories and extend only a few millimeters, a small fraction of their original length (Becker et al., 1997; Becker and Becker, 2001; Oliphint et al., 2010; Rovainen, 1976; Wood and Cohen, 1981). This dichotomy presents an opportunity to investigate mechanisms that regulate synapse reestablishment and the requirements for functional recovery.

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4.1. Functional measures of regeneration

Although functional recovery in fish is generally successful, just how well do circuits recover? Zebrafish larvae can recover normal swimming behavior and the stereotyped startle response within 9 days after spinal cord injury (Briona and Dorsky, 2014). Similarly, behavioral recovery following complete spinal cord transection in larval lamprey is robust, with ~ 90% recovery of movement (Oliphint et al., 2010). Nevertheless, mild differences in several functional measures, including swim speed, tail beat frequency, and body wavelength persist (Oliphint et al., 2010). This robust recovery may partly reflect the fact that the environment has not substantially changed since initial development and intrinsic growth potential has yet to decline. Not surprisingly, recovery of function in older animals is more variable. Adult zebrafish can also regain swimming behavior following spinal cord transection (Becker et al., 1997), but some do not recover and these animals tend to show less axon regeneration. By some measures the majority of adult zebrafish recover from spinal cord lesion (Becker et al., 2004a; Fang et al., 2012; Vajn et al., 2014), but rigorous functional tests uncover substantial persistent deficits in sustained swimming behaviors (van Raamsdonk et al., 1998), likely resulting from a failure to coordinate movement of proximal and distal body segments. In contrast to spinal cord lesions, adult zebrafish appear to fully recover several visual behaviors following ON injuries (Kato et al., 2013). The time required for recovery correlates with the complexity of the behavior assayed, ranging from a short recovery period to regain the optokinetic response (14 days; when some regenerated axons reach the tectum), to a medium recovery period for the optomotor response (25–30 days; when large numbers of regenerated axons reach the tectum), to a long recovery period for chasing behavior (90–100 days; when synaptic refinement is complete). These behavioral measures can be compared with structural studies of axon and synapse regeneration to determine how anatomical recovery relates to functional recovery.

4.2. Axon guidance during regeneration

To reestablish functional synaptic connections, regenerating axons must first navigate to their targets. Axon regeneration is usually successful in the larval zebrafish PNS and requires guidance cues, the extracellular matrix, and guidepost cells (Isaacman-Beck et al., 2015; Pujol-Martí et al., 2014; Rosenberg et al., 2014; Villegas et al., 2012). Larval motor neurons, for example, re-navigate choice points and re-target the correct muscle territory with remarkable precision (Isaacman-Beck et al., 2015). Perturbing ECM protein modifications that likely regulate the distribution of guidance cues (Isaacman-Beck et al., 2015), ablating Schwann cells that likely express guidance cues (Isaacman-Beck et al., 2015; Rosenberg et al., 2014), or genetic removal of the Netrin receptor deleted in colorectal carcinoma (DCC) (Rosenberg et al., 2014), all disrupt the regeneration of larval motor axons. Remarkably, initial development appears largely unperturbed in these mutant scenarios, indicating that either distinct or redundant cues promote initial development.

The adult environment presents a greater challenge to regenerating axons. It is not clear if regenerating axons encounter the same set of cues that guided them during development or if these cues are missing or redistributed, causing the misrouting of re-growing axons. In the adult fish ON, Eph receptor tyrosine kinases and their ephrin ligands play similar roles during development and regeneration of the retinotectal map. Ephs and ephrins are upregulated during ON regeneration in both zebrafish (Becker et al., 2000) and goldfish (King et al., 2003, 2004; Rodger et al., 2000). Functional disruption of Eph/ephrin signaling inhibits the restoration of the retinotectal map in goldfish (Rodger et al., 2004), indicating that this guidance pathway is required during development and adult regeneration. By contrast, Class 3 semaphorins (Sema3), which play guidance roles during development, may form part of the non-permissive regeneration environment of the mammalian CNS by acting as both repellents and pro-apoptotic factors (reviewed by Pasterkamp and Verhaagen, 2001). Spinal cord transection in lamprey increases *sema3* expression near the site of injury shortly after transection (Shifman and Selzer, 2007), suggesting that it could contribute to the failure of some lamprey spinal axons to regenerate. Indeed, exogenous addition of Sema-3A inhibits ON regeneration in goldfish by increasing RGC apoptosis, decreasing axon growth, and disrupting debris clearance by macrophages (Rosenzweig et al., 2010).

In a clever set of experiments, Wyatt et al. (2010) tested if degenerating CNS axons provide guidance cues for regeneration by analyzing adult *robo2* mutant zebrafish. Robo2 is required for ON pathfinding during development (Fricke et al., 2001), and pathfinding defects in *robo2* mutants persist into adulthood (Wyatt et al., 2010). Following ON lesion, regenerating axons did not grow along the erratic paths established during development and instead showed relatively normal pathfinding, suggesting that regenerating axons do not necessarily use the same guidance factors as in development and that degenerating ON tracts do not provide guidance for regenerating axons.

4.3. Synaptic regeneration

ON injury in goldfish has been a useful model for studying synapse regeneration, since the formation of a rough retinotectal map is activity independent (reviewed by Matsukawa et al., 2004a; Meyer, 1983; Schmidt and Edwards, 1983). Moreover, unlike in mammalian ON injury models, regeneration occurs in the absence of cell death (Meyer et al., 1985), so any synaptic deficits are likely attributable to poor regeneration. Goldfish ON regeneration proceeds in four steps: a preparation period (1–5 days), an axonal elongation period (7–40 days), a synaptic refinement period (35–80 days), and a functional recovery period (5–6 months) (Meyer and Kageyama, 1999). Zebrafish ON regeneration occurs through a similar sequence, albeit with faster kinetics (Kato et al., 2013). To analyze synapse regeneration in the goldfish tectum, Meyer and Kageyama (1999) labeled a small number of retinal neurons with horseradish peroxidase (HRP) after nerve crush, then visualized tectal synapses by electron microscopy. Surprisingly, the initial synapses formed were exclusively at the incorrect (anterior) end of the tectum. During the middle stages of regeneration, synapses were found in equal numbers at the correct and incorrect portions of the tectum. During late regeneration, anterior synapses were eliminated and the remaining synapses were exclusively at the correct (posterior) end of the tectum. Thus, reestablishment of the retinotectal map involves correction of large-scale synaptic errors. This process contrasts with normal development of the retinotectal map in fish, which progresses through an orderly sequence of ingrowth and synaptogenesis (Stuermer et al., 1990).

The giant reticulospinal (RS) axons of the larval lamprey, which project from the hindbrain into the spinal cord, have also been excellent models for understanding the cell biology of synapse regeneration following injury. RS axons in the spinal cord form both electrical and chemical (glutamatergic) synapses and can be easily identified and individually labeled by intracellular injection of HRP. Following injury, approximately 30–50% of RS axons grow across the lesion and animals regain swimming behavior after 3–4 weeks. However, regenerated axons make a variety of pathfinding and branching errors, and fail to regenerate both electrical synapses and postsynaptic chemical synapses (Wood and Cohen, 1979). Moreover, regenerated synapses can occur in ectopic locations (Wood and Cohen, 1981). In an impressively thorough study, Oliphint and colleagues characterized the size, number, axonal distribution, and composition of regenerated CNS synapses (Oliphint et al., 2010). Regenerated synapses contained all the major molecular (actin, synapsin, and SV2) and ultrastructural (SV cluster, active zone, and postsynaptic dendrite) characteristics of RS synapses but were less complex, contained fewer vesicles, and had smaller active zones than controls. Only 1–2% of the total RS synapses distal to the lesion regenerated by 10–12 weeks post-injury. Similarly, regeneration of

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dopaminergic and serotonergic synapses in the zebrafish spinal cord is limited to ~50% of unlesioned controls immediately caudal to the injury (within 0.75 mm) and background levels further caudal (3.5 mm) (Kuscha et al., 2012). Despite these synaptic deficits, swimming behavior in lesioned lamprey and zebrafish recovers robustly, suggesting that only a small fraction of original synapses are sufficient to support function, or that plasticity of compensatory circuits contribute to recovery.

4.4. Reestablishment of synaptic specificity

The transgenic tools available in larval zebrafish present an opportunity to examine the specificity of regenerated synapses, as exemplified by studies of synaptic specificity in the posterior lateral line nerve (pLLn). Axons of the pLLn innervate mechanosensory organs called neuromasts, which contain hair cells of two opposing polarities. Importantly, hair cell polarity and hair cell-axon synapses are visible by light microscopy in living and fixed animals. 2–4 lateral line axons innervate a neuromast in larval zebrafish; individual axons innervate hair cells of only a single polarity (Faucherre et al., 2009; Nagiel et al., 2008). Careful ablation experiments were performed to eliminate all but a single lateral line axon (Pujol-Martí et al., 2014). The spared axon expanded its receptive field to synapse with hair cells of both polarities. However, upon regeneration of the injured axons, the spared axon pruned back to its original synaptic partners. This finding suggests that axons of the PNS can rewire their synapses and that competition between axons is an important dictator of synaptic specificity. The molecular mechanisms of this synaptic selectivity have yet to be elucidated, but this and other larval zebrafish circuits–including parts of the CNS where circuits are well-defined, such as the retina (e.g., Yoshimatsu et al., 2016)–are ideal models for exploring the molecular basis for reestablishing specific synaptic connections during regeneration.

5. Dynamic cell behaviors during axon regeneration

Axon regeneration in vivo requires axons to navigate a dynamic cellular environment that can significantly influence regenerative outcomes. Fish models have provided novel insights into roles of these extrinsic cell types in both PNS and CNS axon regeneration. In particular, the ability to image dynamic processes in live zebrafish larvae, combined with the increasing availability of cell type-specific transgenic tools, has recently revealed several previously unknown behaviors of extrinsic cells during axon degeneration and regeneration.

5.1. Coordination of axon regeneration with wound healing

Axon injury often occurs in combination with damage to surrounding tissues. Repair of axons and damaged tissues must be coordinated for proper reinnervation but the widespread, nonspecific damage created by traditional lesion paradigms has made this issue difficult to study. The ability to sever single, transgenically labeled axons in larval zebrafish while minimizing damage to surrounding tissues (Lewis and Kucenas, 2013; O'Brien et al., 2009b; Rosenberg et al., 2012; Villegas et al., 2012) offers a unique opportunity to understand how axon regeneration is coordinated with tissue repair.

Given the relatively robust axon regeneration of peripheral axons in zebrafish larvae, it was surprising that the cutaneous endings of touch-sensing trigeminal peripheral axons failed to regenerate into their former skin territories after precise laser axotomy in three-day old larvae (Fig. 2A, B, C) (O'Brien et al., 2009a). Live imaging revealed that although axotomy promoted some growth of these trigeminal axon terminals, they actively avoided their original territories, likely reflecting the deposition of inhibitory factors in the skin that limit structural plasticity after initial development (O'Brien et al., 2009a). However, if axotomy was combined with damage to neighboring epidermal cells in the skin, a scenario much more likely to mimic a real injury, trigeminal axons regenerated robustly (Fig. 2D) (Rieger and Sagasti, 2011). Epidermal wounds in zebrafish produce a gradient of hydrogen peroxide (H_2O_2) that depends on the dual oxidase (Duox)-dependent enzyme (Niethammer et al., 2009). Exogenous application of H_2O_2 promoted peripheral sensory axon regeneration after precise laser axotomy, whereas Duox inhibition reduced regeneration, suggesting that the H_2O_2 gradient promotes axon regeneration (Rieger and Sagasti, 2011). These studies revealed the need for coordinated regeneration of multiple cell types during wound healing.

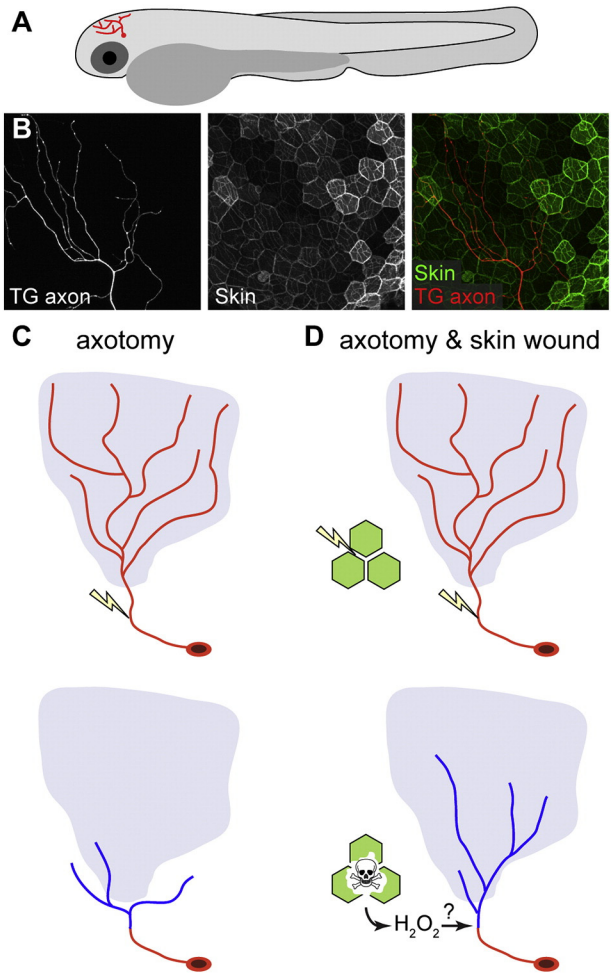

Fig. 2. Skin wounding promotes regeneration of peripheral sensory axon arbors. A) Schematic of a zebrafish larva with a single trigeminal (TG) peripheral arbor labeled. B) A single larval TG sensory peripheral axon innervates many skin cells (visualized with a PH-PLC-GFP reporter expressed in the skin). C) Precise axon damage stimulates some regenerative growth, but axons avoid their former territories. D) Damaging nearby skin cells provides a short range regeneration-promoting signal that depends on H_2O_2 production.
Images generated by Marci Rosenberg (UCLA).

5.2. Cleaning up after axon degeneration

Phagocytosis of debris following Wallerian degeneration (WD) of the distal axon is considered a prerequisite for successful axon regeneration. Accordingly, preventing axon degeneration, and thus removal of the distal axon fragment, by expression of the Wallerian Degeneration Slow (Wld^S) protein delays or alters axon regeneration in mice and fish (Bisby and Chen, 1990; Brown et al., 1992; Chen and Bisby, 1993; Martin et al., 2010). Identifying cell types involved in axon debris clearance, characterizing their behaviors, and investigating the molecular pathways they use to clear debris is thus critical for fully understanding tissue repair. Phagocytosis is a dynamic process, which makes live imaging in zebrafish a particular attractive approach for studying it.

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Numerous studies in mammals have shown that macrophages, blood-derived "professional" phagocytes, eat axon debris in the PNS (reviewed by Vargas and Barres, 2007). However, few if any studies in mammals have imaged the dynamics of macrophage behavior during WD in live animals. Zebrafish present a uniquely accessible system with which to study and manipulate the interactions of macrophages with degenerating axons in vivo. Genetic removal of macrophages delays debris removal following axotomy of the pLLn or motor nerves (Lewis and Kucenas, 2014; Villegas et al., 2012). Live imaging of macrophage responses to motor axon injury revealed that they are recruited to the site of laser axotomy prior to the onset of WD and phagocytose debris shortly after WD begins (Fig. 3Di,ii) (Rosenberg et al., 2012). Macrophages were recruited normally following axotomy of Wld^S-expressing motor neurons and displayed a novel "scanning" behavior, in which they repeatedly probed the intact but disconnected distal axons with membrane extensions (Rosenberg et al., 2012). These observations suggest that macrophages can distinguish injured, degenerating axons from injured but intact axons and that their recruitment does not require WD. Schwann cells near the lesion are strong candidates for regulating these macrophage behaviors since mammalian Schwann cells produce chemoattractants that recruit macrophages to injured nerves (Vargas and Barres, 2007). Studies of the requirement of zebrafish Schwann cells in macrophage recruitment, however, have come to differing conclusions (Pope and Voigt, 2014; Rosenberg et al., 2012).

"Non-professional," non-blood derived phagocytes, including peripheral glia, are increasingly recognized as important regulators of axon debris clearance. Live imaging of axon degeneration in the zebrafish PNS revealed that Schwann cells and perineurial glia phagocytose and degrade axon debris (Fig. 3Dii) (Ceci et al., 2014; Lewis and Kucenas, 2014; Rosenberg et al., 2014; Xiao et al., 2015). Perhaps more surprisingly, epithelial cells of the epidermis were recently found to phagocytose degenerating peripheral axon endings of somatosensory neurons (Rasmussen et al., 2015). Live imaging of specific fluorescent reporters in the epidermis during axon degeneration revealed the entire process of phagocytosis, from the internalization of axon debris by F-actin-rich protrusions, to the trafficking of debris through endocytic compartments, to the degradation of debris in lysosomes (Rasmussen et al., 2015). Together these studies establish zebrafish larvae as a leading model for studying phagocyte behaviors during neural repair.

5.3. Bridging cells promote axon regeneration

CNS injuries in fish promote glial proliferation and migration into the lesion site (Blaugrund et al., 1993; Cohen et al., 1994; Dervan and Roberts, 2003; Goldshmit et al., 2012; Lurie et al., 1994). In the mammalian CNS, astrocyte reactive gliosis at injury sites invariably blocks regeneration (reviewed by Cregg et al., 2014). Glial behaviors may differ markedly in fish; some studies have proposed that glial cells at the injury site promote regeneration by providing a bridging substrate for growing axons (Cohen et al., 1994; Goldshmit et al., 2012; Lurie et al., 1994), but other studies suggest that axons may cross the lesion site before glial cells (Blaugrund et al., 1993; Dervan and Roberts, 2003). This discrepancy may be due to the techniques used to visualize cells in fixed tissues. Resolving the sequence of events at a CNS injury site may thus require time-lapse imaging of axons and glia in live adult fish.

Goldshmit and colleagues detailed the morphogenesis of glial bridge formation in adult zebrafish following spinal cord lesion (Goldshmit et al., 2012). Following an initial proliferative and migratory phase, glia that reached the lesion site elongated along the AP axis to develop a bipolar morphology, as had been suggested by immunostaining for intermediate filaments and microtubules in lamprey (Lurie et al., 1994). At these stages, axons near the lesion grew perpendicular to the axis of the spinal cord (Goldshmit et al., 2012). At 2–3 weeks post injury, glia within the lesion elongated further to bridge the lesion and initial axon regeneration occurred along glial bridges. The final phase of lesion repair involves dismantling the glial bridge and remodeling the central canal.

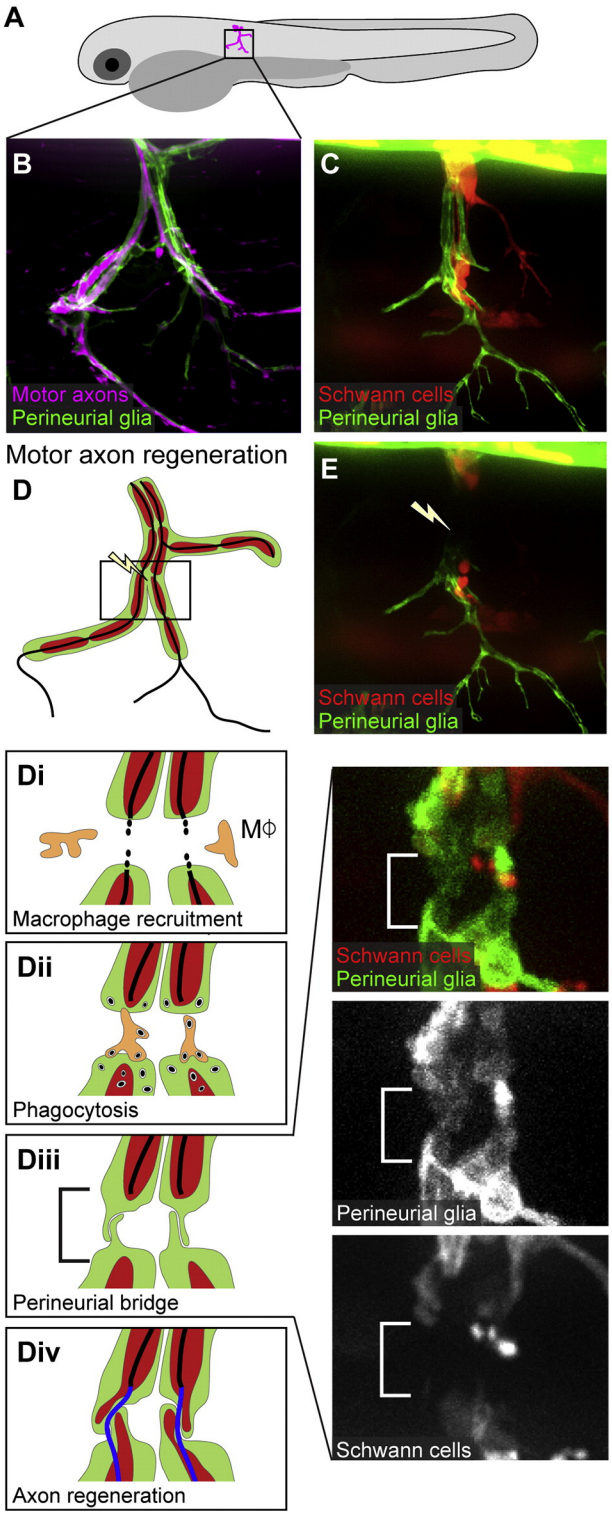

Fig. 3. Glial bridging behaviors visualized in live zebrafish. A–C) Larval motor nerves in fish are surrounded by perineurial glia (B) and contain Schwann cells (C). D–E) Nerve injury stimulates the recruitment of macrophages (Mϕ) (Di), which, along with perineurial glia and Schwann cells, contribute to phagocytosis of axon debris (Dii). Perineurial glia and, later, Schwann cells bridge the nerve gap (Diii), allowing motor axons to regenerate across the lesion site (Div).
Images were the generous gift of Gwendolyn Lewis and Sarah Kucenas (University of Virginia).

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What are the mechanisms of glial bridge formation in the CNS? The fibroblast growth factor (Fgf) *fgf3* was previously shown to be upregulated following spinal cord injury (Reimer et al., 2009). Goldshmit et al. (2012) found that Fgf ligands, receptors, and downstream effector genes were upregulated over a broad time window following injury in both glia and neurons surrounding a lesion site. Gain- and loss-of-function experiments demonstrated that Fgf signaling activated a MAPK pathway and was required for the initial steps of glial proliferation and migration. Fgf signaling was also required for glial bridge morphogenesis, and blocking bridge formation led to failure of axon regeneration and a decrease in functional recovery. The mechanism of glial bridge formation may be conserved, since exogenous Fgf2 application improves recovery after mammalian spinal cord injury and promotes the formation of cellular bridges containing glia with bipolar morphology (Goldshmit et al., 2014).

The glial bridging behaviors documented in the fish CNS resemble Schwann cell bridging in the mammalian PNS (reviewed by Jessen et al., 2015). Nerve injuries in the PNS induce the differentiation of the distal, denervated Schwann cells into Bands of Büngner that span the injury site (Jessen et al., 2015). Recent genetic and live imaging studies in larval zebrafish have confirmed that this behavior occurs in fish and have provided molecular insights into the process. Following transection of the pLLn, either by laser or electrical ablation, Schwann cells proliferate and gradually lose expression of myelin proteins and the junctional component Claudin-K (Ceci et al., 2014; Xiao et al., 2015). Live imaging of animals expressing markers of axons and Schwann cells showed that axons only regrow across the lesion site once Schwann cells bridge the gap (Ceci et al., 2014; Xiao et al., 2015). Glial bridge formation appears to involve actin-rich membrane protrusions (Xiao et al., 2015). Early blockade of the ErbB signaling pathway, which is required for Schwann cell migration and differentiation (Lyons et al., 2005), did not affect axon growth rates but caused abnormal pathfinding and frequent axon retraction that led to stunted growth and defasciculation (Ceci et al., 2014; Villegas et al., 2012; Xiao et al., 2015). Pharmacological inhibition of ErbB signaling after glial migration significantly delayed the initial stages of axon regeneration, suggesting that ErbB signals are required to promote the early phases of regeneration (Ceci et al., 2014).

Peripheral nerves are encased in an outer layer of perineurial cells, which have long been proposed to participate in axon regeneration (reviewed by Kucenas, 2015). Until recently, however, the role of perineurial glia in axon repair has been unclear. The motor nerves of larval zebrafish are a simple system for investigating the interactions between perineurial glia, Schwann cells, and regenerating axons (Fig. 3). Both Schwann cells and perineurial glia are required for motor axon regrowth, since genetically removing either cell type resulted in aberrant or incomplete axon regeneration (Lewis and Kucenas, 2014; Rosenberg et al., 2014). Live imaging of animals expressing markers of both perineurial glial and Schwann cells demonstrated that perineurial glia bridge the injury gap prior to Schwann cells (Fig. 3D) (Lewis and Kucenas, 2014). Perineurial bridge formation required Schwann cells but not axon fragmentation (Lewis and Kucenas, 2014). These results suggest that following axotomy Schwann cells release factors that stimulate bridging behaviors in perineurial glial essential for axon regeneration.

5.4. More cellular behaviors to explore in fish?

So far most studies of axon regeneration in larval fish have focused on peripheral axons, but central axon regeneration can be imaged just as effectively. Since central axons exhibit more variable regeneration (e.g., Bhatt et al., 2004), studying them would provide the potential to document both pro- and anti-regenerative behaviors by extrinsic cell types. Few, if any, studies have addressed the role of oligodendrocytes in larval zebrafish axon regeneration, but it may be informative to compare their behavior to mammalian oligodendrocytes, which potently inhibit regeneration (Bandtlow et al., 1990; Bastmeyer et al., 1991; Fawcett et al., 1989). Microglia, blood-derived immune cells of the CNS, are the most likely candidates to clear axon debris after injury in the CNS, and an electron microscopy study suggested they participate in phagocytosing axon debris following ON injury in goldfish (Battisti et al., 1995). Several tools for imaging and manipulating microglia are available in zebrafish larvae and have been effectively used to study their role in neuronal cell corpse engulfment (Mazaheri et al., 2014; Peri and Nüsslein-Volhard, 2008; Shen et al., 2016; Sieger et al., 2012), but have yet to be used to study microglial behaviors during repair of CNS axons. Astrocytes regulate axon pruning, debris clearance, and regeneration in *Drosophila* and mammals (Chung et al., 2013; Cregg et al., 2014; Tasdemir-Yilmaz and Freeman, 2014). If astrocytes are truly absent from the fish CNS, other cell types, such as radial glia, may have evolved compensatory behaviors (Lyons and Talbot, 2015). The rapidly expanding suite of tools for creating transgenic reporters should make it possible to develop markers that will more definitively determine if fish have astrocytes and to document the roles of radial glia in repair.

The ability to image axon regeneration and cell interactions in live animals is arguably the main advantage of larval fish models over other axon regeneration models. If it were possible to apply a similar live imaging approach to the adult CNS, fish models would be even more powerful—perhaps the best vertebrate model system for illuminating the black box between injury and recovery. Live imaging in adult fish is much more challenging than in larvae, since adult fish are highly pigmented and difficult to anesthetize for long periods of time. But live imaging in adults is not impossible, as demonstrated by pioneering studies in adult goldfish that revealed dynamics of regenerating dye-labeled optic fibers through cranial flaps (Dawson et al., 2015; Dawson and Meyer, 2001, 2008; Johnson et al., 1999). A recent innovation in anesthesia that allows continuous imaging of adults for at least two days (Xu et al., 2015), and the discovery of several zebrafish mutants that lack adult pigmentation (Hsu et al., 2013; White et al., 2008), should facilitate time-lapse imaging of fluorescently labeled nerves in intact adults, at least in superficial parts of the animal. Combining these approaches with multiphoton microscopy for deeper light penetration may even allow imaging of regenerating CNS axons, without resorting to the invasive cranial windows or open preparations used for live imaging in adult mice (e.g., Lorenzana et al., 2015; Misgeld et al., 2007; Xu et al., 2007).

Ideally, live imaging would be combined with specific genetic manipulations, but, until recently, methods for interrogating gene function in adult zebrafish neurons have been limited to the application of antisense morpholinos via gelfoams or transgenic overexpression. The development of genome editing technologies with the CRISPR/Cas9 system (Gonzales and Yeh, 2014) should allow for increasingly complex genetic experiments in zebrafish and could even make genetic manipulation possible in traditionally "non-genetic" fish species, such as lamprey (Square et al., 2015). In parallel, high-resolution mapping of expression patterns in Gal4 and Cre "enhancer trap" lines in the CNS (Marquart et al., 2015; Otsuna et al., 2015) is increasing the toolkit for visualizing and manipulating specific cell types. The ability to directly image regeneration of axons and the behavior of surrounding cells in real time, in live adult animals, with specific genetic manipulations, would provide a level of clarity not possible in any other vertebrate model of neural repair.

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