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# Non-mammalian model systems for studying neuro-immune interactions after spinal cord injury.

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## Abstract

Mammals exhibit poor recovery after injury to the spinal cord, where the loss of neurons and neuronal connections can be functionally devastating. In contrast, it has long been appreciated that many non-mammalian vertebrate species exhibit significant spontaneous functional recovery after spinal cord injury (SCI). Identifying the biological responses that support an organism's inability or ability to recover function after SCI is an important scientific and medical question. While recent advances have been made in understanding the responses to SCI in mammals, we remain without an effective clinical therapy for SCI. A comparative biological approach to understanding responses to SCI in non-mammalian vertebrates will yield important insights into mechanisms that promote recovery after SCI. Presently, mechanistic studies aimed at elucidating responses, both intrinsic and extrinsic to neurons, that result in different regenerative capacities after SCI across vertebrates are just in their early stages. There are several inhibitory mechanisms proposed to impede recovery from SCI in mammals, including reactive gliosis and scarring, myelin associated proteins, and a suboptimal immune response. One hypothesis to explain the robust regenerative capacity of several non-mammalian vertebrates is a lack of some or all of these inhibitory signals. This review presents the current knowledge of immune responses to SCI in several non-mammalian species that achieve anatomical and functional recovery after SCI. This subject is of growing interest, as studies increasingly show both beneficial and detrimental roles of the immune response following SCI in mammals. A long-term goal of biomedical research in all experimental models of SCI is to understand how to promote functional recovery after SCI in humans. Therefore, understanding immune responses to SCI in non-mammalian vertebrates that achieve functional recovery spontaneously may identify novel strategies to modulate immune responses in less regenerative species and promote recovery after SCI.

### Keywords

spinal cord injury; regeneration; immune system; inflammation



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## INTRODUCTION

SCI was once thought to be incurable, because of a lack of plasticity and regeneration in the adult mammalian central nervous system (CNS). However, studies performed over the past two decades have demonstrated both neurogenesis and considerable plasticity of the adult vertebrate CNS, stimulating studies of biological responses to SCI (Thuret et al., 2006). Based on the premise that neuronal function relies on fundamental pathways conserved across species, neurobiology has a long tradition of addressing basic biological questions by exploiting the advantageous experimental or natural traits of a variety of organisms including sea slug, squid, frog, chicken, and songbirds (Alvarez-Buylla and Nottebohm, 1988; Castellucci et al., 1980; Cohen et al., 1954; Fatt and Katz, 1951; Hodgkin and Huxley, 1939; Kupfermann and Kandel, 1969; Marder, 2002). Similarly, the ability to promote successful recovery after SCI will likely derive from identification of biological processes that determine both success and failure to regenerate axons, in species where regenerative capacity ranges from limited to robust.

Studies in mammals, which exhibit poor spontaneous recovery after SCI, have focused primarily on identifying factors both intrinsic and extrinsic to neurons that inhibit axon regeneration, *e.g.* (Buchli and Schwab, 2005; Giger et al., 2010; Gonzenbach and Schwab, 2008). These inhibitory mechanisms include reactive gliosis and a glial scar that contains chondroitin sulfate proteoglycans (CSPGs) and the presence of myelin and myelin-associated proteins (Filbin, 2003; Pernet and Schwab, 2012; Silver and Miller, 2004). Another extrinsic factor studied in the context of mammalian SCI is the immune response, as inflammation has been widely shown to exacerbate neuronal loss and induce secondary damage acutely after SCI (Bartholdi and Schwab, 1997; Klusman and Schwab, 1997; Schnell et al., 1997; Streit et al., 1998). One of the long-considered hypotheses to explain the differential regenerative capacity among vertebrates is that species able to accomplish spontaneous recovery after SCI have less inhibitory extrinsic factors present near the injury zone, including an acute immune response that is weaker or distinct from that observed in mammals (Tanaka and Ferretti, 2009). Increasingly, elements of the immune response are also recognized to benefit axonal regeneration after SCI in mammals, reviewed in (Benowitz and Popovich, 2011; Gensel et al., 2012). Therefore, studies of immune responses to SCI in regenerating vertebrates will enhance the ability to promote an optimal immune response in mammals, in order to prevent loss of neurons and promote recovery. This approach is clinically relevant, as the immune response is routinely manipulated in a variety of clinical settings. For example, FDA-approved antibodies or drugs that target pro-inflammatory cytokines are routinely used in patients with rheumatoid arthritis, Crohn's disease, diabetes (types I and II) and multiple sclerosis (Dinarello et al., 2012). In mouse models of SCI, treatment with three different FDA-approved TNF inhibitors improved biochemical, histological and functional outcomes after SCI (Genovese et al., 2006; Genovese et al., 2008a; Genovese et al., 2008b). Based on the clinical success of monoclonal antibodies and other biologic therapies, a pipeline of therapeutic small molecules targeting pro-inflammatory mediators is in development (Dinarello et al., 2012; Kopf et al., 2010). Therefore, understanding beneficial and detrimental aspects of the immune response to SCI across a variety of species may offer clinically relevant insights. Here, I review data from a

growing number of studies that investigated immune responses to SCI in non-mammalian model organisms including the jawless (lamprey) and jawed vertebrates, including teleosts (zebrafish), amphibians (salamander and frog), and reptiles (turtle) (Figure 1).

## Immune responses to SCI in Mammals

In mammals, immune responses are thought to contribute to deleterious outcomes, including neuronal death, inhibition of axon regeneration, and poor functional recovery of motor and sensory systems. Microglia, the resident immune cells of the CNS, are among the first cells to respond in mammalian experimental models of SCI (Adrian et al., 1978; David and Kroner, 2011; Popovich et al., 1993; Spitzbarth et al., 2011). Within minutes to days, microglia, together with neutrophils, macrophages and lymphocytes recruited from the periphery, are activated and accumulate at the lesion site (Carlson et al., 1998; Detloff et al., 2008; Fitch et al., 1999; Fitch and Silver, 1997; Gensel et al., 2011; Horn et al., 2008; Kigerl and Popovich, 2009; Popovich et al., 2002; Popovich and Hickey, 2001; Popovich et al., 1993; Popovich et al., 2003; Popovich et al., 1997; Schnell et al., 1997). Resident and invading immune cells release pro-inflammatory mediators, including cytokines that rapidly amplify the local immune response (Alexander and Popovich, 2009; Bartholdi and Schwab, 1995, 1997; Bethea et al., 1998; Brambilla et al., 2005; Fitch et al., 1999; Herbomel et al., 2001; Kigerl and Popovich, 2009; Klusman and Schwab, 1997; Schnell et al., 1999; Streit et al., 1998). Astrocytes promote scarring at the lesion site and synthesize CSPGs, which are inhibitory to neuronal regeneration (Bradbury and Carter, 2010; Bradbury et al., 2002; Garcia-Alias et al., 2009; Rudge and Silver, 1990; Rudge et al., 1989; Silver and Miller, 2004; Smith et al., 1986; Smith et al., 1990; Snow et al., 1990). At the lesion site, the molecular interactions between astrocytes and infiltrating immune cells are not well understood. After SCI in mice, reactive astrocytes expressing the transcription factor STAT3+ confined inflammatory cells at the lesion epicenter, while deletion of STAT3 from astrocytes was pro-inflammatory, resulting in a broader distribution of inflammatory cells around the injury site and decreased neuronal viability (Herrmann et al., 2008; Okada et al., 2006; Wanner et al., 2013). *In vitro* co-culturing of macrophages with resting STAT3+ astrocytes activated the astrocytes to reorient their processes and surround the inflammatory cells (Wanner et al., 2013). A subpopulation of neural stem cell-derived astrocytes recruited to the injury was shown to restrict the size of the lesion, by being neuroprotective (Goritz et al., 2011; Wanner et al., 2013). Surprisingly, removal of these ependymal-derived astrocytes decreased the numbers of immune cells in the injured spinal cord (Sabelstrom et al., 2013). These studies suggest complex and heterogeneous interactions between neuronal, immune and other non-neuronal cells at the lesion site, that together contribute to recovery after SCI in mammals.

Additional interactions of the nervous and immune systems may also be relevant in mammalian SCI. For example, the vagus nerve, which is part of the autonomic nervous system, has efferent connections to immune organs, which regulate immune functions, such as cytokine production (Olofsson et al., 2012). Autonomic dysreflexia, which occurs when the autonomic nervous system is interrupted and can be life-threatening in SCI patients, caused immune depression in mice and in a human SCI subject (Zhang et al., 2013b) (and see Zhang et al, this issue). At the molecular level, classic immune molecules, such as major

histocompatibility complex class I (MHCI), are now known to play a role in normal CNS synapse remodeling and plasticity (Corriveau et al., 1998), while the complement cascade participates in synaptic pruning (Stephan et al., 2012; Stevens et al., 2007) (see also Peterson and Anderson, this issue). Studies in mammals are just beginning to examine if these pathways are activated after CNS injury and if they influence outcomes after injury (Elmer and McAllister, 2012). Whether these systems are equally present and participate in regeneration in non-mammalian vertebrates is unknown, but the complement cascade has been implicated in limb regeneration in salamanders and is upregulated after tail amputation in frog tadpole (*Xenopus laevis*) (Kimura et al., 2003; Tazaki et al., 2005).

Intraspinal inflammation in humans can persist for years, where it has been proposed to inhibit functional recovery and contribute to the complications of chronic SCI, *e.g.* (Bao et al., 2010; Davies et al., 2007; Fleming et al., 2006; Hayes et al., 2002; Kanyilmaz et al., 2012; Kwon et al., 2010; Stein et al., 2013). Therefore, inflammation has long been considered a therapeutic target in SCI (Gensel et al., 2011). The 2013 Clinical Practice Guideline for Early Acute Management of SCI in Adults discourages the use of anti-inflammatory steroids or any other pharmacological agent, based on controversial findings from three national clinical trials of methylprednisolone (Medicine, 2013). Thus, there is a need to identify new anti-inflammatory and/or pro-regenerative therapies for SCI. Many molecular aspects of innate immunity are conserved across species, *e.g.* sea urchin, flies and human (Becker and Becker, 2007; Murphy et al., 2008), and understanding elements of the immune response that coexist with successful functional recovery from SCI in non-mammalian vertebrates may shed light on desired aspects of the immune response in mammals.

## Lamprey

Lamprey is the most extant basal vertebrate and to date, is the only experimental model that has been demonstrated to satisfy all NIH criteria for functional regeneration after complete spinal cord transection (Cohen et al., 1988; Guth et al., 1980; Smith et al., 2011). In most studies of SCI in lamprey, the injury model is a complete transection. Full functional recovery is achieved over a stereotypical time course of 12 weeks, which corresponds to regeneration of reticulospinal axons through the lesion site (Cohen et al., 1989; Cohen et al., 1986; Rovainen, 1967, 1976; Selzer, 1978; Wood and Cohen, 1979, 1981; Yin and Selzer, 1984). The lamprey spinal cord is non-myelinated and contains descending axons from reticulospinal neurons, as well as intraspinal motor neurons, sensory neurons and interneurons (Grillner and Jessell, 2009). Interestingly, regeneration does not occur equally in all reticulospinal axons, but instead, is accomplished by a predictable subset of descending giant reticulospinal neurons whose cell bodies are located in stereotypical locations in the brain (Busch and Morgan, 2012; Jacobs et al., 1997; Shifman et al., 2008). As in mammals, the regenerative process after SCI in lamprey is influenced by age, conditioning lesion, and cAMP, indicating that at least some pathways relevant to regeneration are shared across vertebrates with different spontaneous regeneration capacities (Cohen et al., 1989; Cohen et al., 1999; Jin et al., 2009; Zhang et al., 2004). For an illustration of lamprey axons regenerating across the lesion site, see Figure 2.

A series of recent, elegant studies from the laboratory of Max Cooper (Emory University) and others, demonstrated that lampreys have both innate and adaptive immune systems (Guo et al., 2009; Khalturin et al., 2004; Sato et al., 2003; Uinuk-Ool et al., 2002). Similar to mammals, cellular elements of the lamprey immune system include neutrophils, microglia, B cells and at least two distinct T cell populations (Alder et al., 2008; Alder et al., 2005; Cannon et al., 2005; Das et al., 2013; Guo et al., 2009; Hirano et al., 2013; Kasamatsu et al., 2010; Khalturin et al., 2004; Mayer et al., 2002; Pancer et al., 2004; Sato et al., 2003; Slack et al., 2008; Uinuk-Ool et al., 2002; Uinuk-ool et al., 2003). Despite differences in the molecular mechanisms of antigen presentation and the structure of antigen receptors, general strategies of adaptive immunity appear to be similar between lamprey and jawed vertebrates (Boehm, 2011). Lamprey hematopoetic tissues include the typhlosole, a tissue that is analogous to bone marrow, as well as the gills, which are thymus-like (Bajoghli et al 2011). The lamprey CNS contains macrophages/microglia, as defined by their morphology and binding to isolectin-B4 (Shifman and Selzer, 2007; Shifman et al., 2009). While the source of lamprey microglia in the injured spinal cord is not well understood, there is evidence consistent with a peripheral origin (Laramore et al., 2011). In mammals, resident microglia appear to derive from cells that migrate to the brain early in development, which are distinct from monocyte-derived macrophages that infiltrate the spinal cord from the periphery after injury (Ginhoux et al., 2013; Shechter and Schwartz, 2013). Evidence suggests that ratio and function of these distinct cell types may influence outcomes in mouse model of SCI, including regeneration of damaged neurons (Donnelly et al., 2011).

Immediately after lamprey spinal cord transection, the central canal enlarges at the lesion, a blood clot forms, blood cells accumulate, ependymal cells from the central canal proliferate across the lesion, and a glial-ependymal bridge forms (Lurie et al., 1994; Lurie and Selzer, 1991a, b; Rovainen, 1976; Selzer, 1978). Starting at 2-4 weeks post-injury, regenerating axons grow across the lesion site, even when allowed access to uninjured tissue, as in a hemisection injury (Lurie and Selzer, 1991b; Wood and Cohen, 1981). It appears that glial cells, and not just their secreted products, are necessary for axonal regeneration in lamprey, since a reduction in glial cell number by freezing reduced axon regeneration (Lurie and Selzer, 1991a). Unlike mammalian astrocytes, after SCI in lampreys, glial processes reorient and extend across the lesion site, parallel to the normal axonal trajectory and form a bridge (Boehm, 2011; Lau et al., 2013; Lurie et al., 1994). A glial bridge is also observed in most of the other species discussed here, although it is unclear what aspects of glial responses to SCI are conserved across species. For example, it is unclear if lampreys have reactive astrocytes, as they do not appear to accumulate at the scar and a common marker of astrocytes, GFAP, has not yet been identified in the lamprey genome (Lurie et al., 1994; Smith et al., 2013). Recently, extensive gliogenesis, as well as limited neurogenesis, both mostly associated with the ependymal region, was reported (Zhang et al., 2013a). Interestingly, as mentioned above, in mammals, GFAP- astrocytes, derived from ependymal cells, play a positive role in limiting spinal cord damage after SCI (Sabelstrom et al., 2013). Glial organization in lamprey can be modulated, as cAMP promotes a greater and more rapid organization of glial fibers after SCI, which are arranged longitudinally through the lesion site (Lau et al., 2013). Molecular constituents of the scar that are shared with or distinct from mammals are largely unknown in lamprey, although preliminary evidence

suggests that CSPG does accumulate at the lesion, where they are detrimental to axon regeneration (Hu et al., 2013). As in mammals, lamprey microglia/macrophages are increased in number after SCI and subsets of microglia have been shown to express both axon guidance and repulsion molecules, weeks to months after injury (Laramore et al., 2011; Lau et al.; Shifman and Selzer, 2007; Shifman et al., 2009). Application of cAMP to the spinal cord at the time of transection increased the numbers of microglia one-month post injury (Lau et al., 2013).

In contrast to the detailed knowledge of molecular pathways induced after SCI in mammals, genomic resources in lamprey have been lacking and only a few cytokines have been identified conclusively thus far (Guo et al., 2009; Najakshin et al., 1999; Sato et al., 2003; Tsutsui et al., 2007). Similarly to what is known about the conservation of inflammatory responses across species, lamprey lymphocytes stimulated with classic, non-traumatic inflammatory stimuli upregulate expression of macrophage migration inhibitory factor (MIF), interleukin (IL)-8 and IL-17 (Alder et al., 2008; Tsutsui et al., 2007). Whether these and other pro-inflammatory mediators are induced by SCI in lamprey is currently unknown. Recent publication of the lamprey genome has increased the potential to pursue such studies (Smith et al., 2013) and gene expression profiling of immune-related transcripts upregulated by SCI in lamprey are currently being pursued (Bloom O, Brown CT, Buxbaum J, Li W, Morgan JR, Smith J, unpublished studies). Therefore, while the effects of immune responses on recovery from SCI in lamprey are largely unexplored, molecular tools are increasingly available to address this question. The first question to address is whether the immune response to SCI in lamprey supports or impedes regeneration.

## Zebrafish

Due to the general experimental advantages of zebrafish and their capacity to regenerate spontaneously after CNS injury, they are rapidly emerging as an attractive model organism in which to study immune responses to SCI (Becker and Becker, 2008; Lieschke and Currie, 2007). In adult zebrafish, it is possible to perform assays of anatomical recovery, cellular activity, and behavioral responses, in intact animals in real time. Imaging *in vivo* immune responses to SCI should be a major advantage in the zebrafish, as an increasing number of studies have demonstrated cellular immune responses in other pathological settings (Renshaw and Trede, 2012), and transgenic lines selectively labeling a variety of central and peripheral immune cell types relevant to SCI including T and B cells, macrophage/microglia, and neutrophils are available (Ellett et al., 2010; Page et al., 2013; Renshaw et al., 2006). Providing an opportunity to couple *in vivo* with *in vitro* studies, a method to culture primary zebrafish spinal cord neurons was recently described (Chen et al., 2013). Astrocyte biology and its relation to radial glial cells and neurogenesis is a rapidly growing area of research in zebrafish, although it is mostly studied in the context of brain and not spinal cord injury (Kyritsis et al., 2013).

Adult zebrafish can regenerate axons after SCI and recover behaviors such as swimming and escape response (Becker et al., 2004; Becker et al., 1997). The time course to regain substantial behavioral recovery in adult zebrafish is approximately 1 month for either a crush or transection injury (Becker et al., 2004; Becker et al., 1997; Hui et al., 2010). After

spinal cord transection and treatment with cAMP in larval zebrafish, recovery of escape responses can be observed 3-5 days after injury (Becker et al., 2004; Becker et al., 1997; Bhatt et al., 2004; Bhatt et al., 2007). For an illustration of zebrafish axons regenerating across the lesion site, see Figure 2B. As in lamprey, not all zebrafish reticulospinal neurons have the capacity to regenerate equally, and poor regenerators, such as the Mauthner neurons, which also regenerate poorly in the lamprey, can be induced to regenerate by intracellular injection of cAMP (Becker et al., 1998; Becker et al., 1997; Bhatt et al., 2004). In the spinal cord, cervical transection is followed by neurogenesis that results in new motor neurons that are functionally integrated (Reimer et al., 2008). Behavioral recovery is achievable by a varying number of regenerating neurons (Reimer et al., 2009), suggesting the importance of network plasticity, as well as neuronal regeneration (Reimer et al., 2009). Regeneration of spinal motor neurons was recently shown to depend on brain-derived dopamine from descending dopaminergic axons, demonstrating the importance of interneuronal signaling in regeneration (Reimer et al., 2013).

Zebrafish possess all major immune cell types present in mammals, including neutrophils, macrophage/microglia, dendritic cells, B and T lymphocytes (Becker and Becker, 2007; Sunyer, 2013). Zebrafish microglia are present in the brain by day 3 of development, being derived from myeloid precursors, they resemble the morphologies of their mammalian counterparts and have the ability to phagocytose both pathogens and apoptotic neurons (Herbomel et al., 2001; Peri and Nusslein-Volhard, 2008; Svahn et al., 2012). At steady state, microglial activity in the brain has been linked to regulation of neuronal activity (Li et al., 2012). After targeted laser-induced brain injury, microglia accumulate at the lesion site, where their migration is influenced by calcium waves (Sieger et al., 2012). While understudied in SCI, a series of recent elegant experiments have tied inflammation to neurogenesis after traumatic brain injury (TBI). After stab lesion in the adult zebrafish brain, microglia proliferate, accumulate at the lesion site and neurogenesis is induced (Baumgart et al., 2011; Kyritsis et al., 2012; Marz et al., 2011). Acute inflammation drives neurogenesis and neuronal regeneration (Kizil et al., 2012a; Kizil et al., 2012b; Kyritsis et al., 2013; Kyritsis et al., 2012). The mechanism of reactive neurogenesis depends on the transcription factor gata-3 (Kizil et al., 2012a; Kyritsis et al., 2012). Immunosuppression, either by the steroid dexamethasone or by an antagonist of the leukotriene system (Pranlukast), reduced neurogenesis after stab injury or sterile inflammation (Kyritsis et al., 2012). This effect was also observed in 2 separate studies that showed regeneration of the fin after mechanical injury and lateral line regeneration, suggesting that acute inflammation may promote repair in multiple zebrafish tissues (Barros-Becker et al., 2012; Kyritsis et al., 2012).

It should be noted that in mammals, CNS regional differences in regenerative capacity exist, with robust neurogenesis in the lateral ventricles and dentate gyrus of the hippocampus and little appreciable neurogenesis in the spinal cord (Ihrie and Alvarez-Buylla, 2011; Thuret et al., 2006). So whether inflammation would promote neurogenesis in the zebrafish spinal cord, as it does in the brain, is an open question. After complete spinal cord transection of adult zebrafish, the number of microglia/macrophages increased significantly at 14 days post injury, caudal to the lesion site, where they were observed clearing myelin debris (Becker and Becker, 2001). After a crush induced injury to the adult zebrafish spinal cord, macrophages and other immune cells also increased in number at the injury site acutely,

where they were observed clearing myelin debris and dying neurons (Becker and Becker, 2002, 2007; Hui et al., 2010). A recent study demonstrated that acutely (<1 week) post SCI, GFAP+ glial cells and macrophages increased near the lesion site and at three weeks post SCI, astrocytes differentiated into presumptive radial glial (GFAP+nestin+) cells that extended processes across the lesion site (Goldshmit et al., 2012b). A bridge resulting from bipolar glial cells provided a permissive substrate for axons from newborn and established neurons to cross the lesion site, supporting functional recovery (Goldshmit et al., 2012b). By five weeks post SCI, the glial bridge disassembled, coinciding with reassembled spinal cord tissue (Goldshmit et al., 2012b).

Establishment of a regeneration-promoting glial cell bridge across the lesion site is regulated by fibroblast growth factor (FGF), which triggers glial cell proliferation and migration (Goldshmit et al., 2012b). Significantly, application of FGF to primate astrocytes in culture promotes changes in their morphology that resembled the zebrafish astrocytes, demonstrating the translational relevance of SCI studies in zebrafish. FGF also promotes recovery from SCI in *Xenopus laevis* tadpoles and adult rats (Lin et al., 2012b; Lu et al., 2012). Pro-inflammatory and anti-regenerative effects of the lysophosphatidic acid (LPA) on SCI have been shown in both zebrafish and mice (Goldshmit et al., 2012a). In contrast, HMGb1, which promotes developing neurite outgrowth and is also a pro-inflammatory cytokine, had pro-regenerative effects in zebrafish after SCI (Fang et al., 2013).

Thus, despite evidence of acute inflammation promoting regeneration in the brain and elsewhere, the effects of the immune response on regeneration in the zebrafish spinal cord are still not well understood. Unlike many other non-mammalian species discussed here, the zebrafish is an NIH-designated model organism and extensive molecular resources facilitating experimentation are available. In particular, several experimental tools exist to study immune responses to SCI, including the ability to manipulate and monitor gene expression via transgenic reporter lines, morpholinos, the Cre recombinase system, and photoconversion (Gemberling et al., 2013). In addition, the zebrafish research community has long history of sharing protocols, fish lines and reagents to support the use of zebrafish in biomedical research, e.g. (http://zfin.org/). A recent study of the zebrafish genome demonstrated that 82% of the genes listed in the Online Mendelian Inheritance in Man database had at least one zebrafish ortholog, further supporting the use of zebrafish as a translational model of SCI (Howe et al., 2013).

## Salamanders and Frogs

Immune responses have been observed to correlate with regenerative capacity after SCI in amphibians (Harty et al., 2003; Mescher and Neff, 2005, 2006; Mescher et al., 2007; Tanaka and Ferretti, 2009). Experimental paradigms used to study responses to SCI in salamanders (axolotls and newts) are tail amputation and spinal cord transection. For a review of the responses to SCI during tail regeneration, see (Tanaka and Ferretti, 2009). Spinal cord transection is followed by axon regeneration, neurogenesis and recovery of almost normal swimming ability over 2-3 months, which depends on regeneration of descending neurons (Davis et al., 1990; Hui et al., 2013). Newts have microglia with morphologies similar to those in other species, while the presence of astrocytes is still an open question (Kirkham et

al., 2011). Ablation of tyrosine hydroxylase-containing neurons in the brain induced microglial activation and proliferation (Kirkham et al., 2011). Inhibition of inflammation via the introduction of dexamethasone acutely after neuronal ablation decreased the number of microglia and increased neuronal regeneration, consistent with an inhibitory effect of inflammation on recovery (Kirkham et al., 2011). Interestingly, a recent study of limb regeneration in axolotl concluded that an early macrophage response, which included upregulation of both inflammatory and anti-inflammatory cytokines, is necessary for successful regeneration (Godwin et al., 2013).

Immune responses to SCI in salamander is largely unexplored. In the axolotl, a crush model of spinal cord injury induced accumulation of macrophage/microglial cells at the injury site and peripheral to the spinal cord (Zammit et al., 1993). In the newt, complete transection of the spinal cord induced a cellular inflammatory response, including an influx of lymphocytes and phagoyctic macrophage-like cells, as indicated by hematoxylin/eosin staining (Zukor et al., 2011). In the newt, as in lampreys and zebrafish, GFAP+ astrocytes do not appear to form a dense scar at the lesion site. In fact, astrocytes and ependymal-derived glial cells appear to extend processes across the lesion, parallel to the direction of regenerating axons forming a bridge (Zukor et al., 2011). For an illustration of newt axons regenerating across the lesion site and the glial cell bridge, see Figure 2C (Godwin et al., 2013).

At the molecular level, gene expression profiling after tail amputation in the axolotl demonstrated immune and injury response genes upregulated during the first week after injury (Monaghan et al., 2007). Genome and transcriptome resources are available and growing in axolotl (www.ambystoma.org). A reference transcriptome has also just been reported for the newt (Abdullayev et al., 2013). A recent RNA-Seq analysis of the developing blastema formed after forelimb amputation in axolotl demonstrated that immune genes were upregulated in the initial 24h after amputation (Stewart et al., 2013). Proteomics are also becoming available as a resource in axolotl (Rao et al., 2009).

## Frogs

Historically, tadpoles have been a classic model used to delineate cellular and molecular mechanisms governing vertebrate development and are being increasingly used to understand questions in regeneration (Slack et al., 2008). The injury model most commonly used to study SCI in tadpoles is tail amputation and signals are required from the spinal cord to accomplish tail tissue regeneration (Lin et al., 2012a). Recovery of swimming after spinal cord hemisection depends on regeneration of descending axons (Gibbs and Szaro, 2006). Until relatively recently, tadpoles were considered to be able to regenerate their spinal cord after injury throughout their development and unable to do so after metamorphosis. However, Slack and colleagues discovered a “refractory period,” at 4-6 days of development, during which time tadpoles *(X. laevis*) transiently lose their ability to regenerate muscle, spinal cord and notochord (Beck et al., 2003). The Szaro laboratory conducted a study of cellular and molecular processes differentially expressed before and after metamorphosis, corresponding to conditions when tadpoles are able or unable to regenerate (Gibbs et al., 2011). Importantly, results of this investigation demonstrated a link

between regenerative capacity and activation of the immune system. Thus, when tadpoles were kept in prolonged larval state by pharmacological blockade of thyroid hormone with methimazole, wound healing was less complete than in untreated animals at 3 weeks post injury; this treatment led to upregulation in MHC class II and T cell receptor (TCR) components, which are critical for function of the adaptive immune system, supporting the important role of immune system activation for successful spinal cord repair (Gibbs et al., 2011). Gene expression profiling after tail amputation of tadpoles in another frog species also showed early inflammatory/wound healing responses (Tazaki et al., 2005). Surprisingly, at the cellular level, few studies have been performed in tadpoles to investigate their immune response to SCI. After optic nerve crush, an extensive macrophage/microglia response was observed, which peaked at 5 days post injury and declined by about a month after injury (Goodbrand and Gaze, 1991; Wilson et al., 1992). Since the microglial response was initiated acutely and its peak coincided with axon regeneration, the authors proposed a beneficial role of microglia on axon regeneration (Goodbrand and Gaze, 1991). Reflecting both potential positive and negative influences of the immune response on regeneration, immune genes induced in a model of tail amputation were either delayed or prolonged during the refractory period, when tadpoles cannot regenerate (Fukazawa et al., 2009). For example, expression of the chemokine CXCR2 was upregulated during the post-refractory regeneration period, which the authors suggest may induce invasion of peripheral leukocytes (Fukazawa et al., 2009). Perhaps such cells represent anti-inflammatory cell populations that promote remodeling of the tissue and removal of debris. On the contrary, MHCII, which is expressed on antigen presenting cells such as monocytes and macrophages/microglia, was upregulated during the refractory period. Perhaps these cells represent inflammatory macrophages/microglia that are deleterious to recovery. Treatment of tadpoles with immunosuppressive drugs or immune cell depletion during the refractory period restored regenerative capacity. Notably, the authors suggested that regulatory T cells in particular, whose development coincides with the post-refractory period, may dampen immune signals that inhibit regeneration (Fukazawa et al., 2009). Cellular and molecular inflammatory responses in *X. tropicalis* were initiated within 6 hours of amputation and sustained for several days, with gene ontology terms “immune/defense response” enriched among transcripts upregulated at 6h post amputation (Love et al., 2011). The role of reactive oxygen species (ROS), which are produced by inflammatory cells in many injury models across species, is currently unclear in frog after SCI. ROS were shown to increase dramatically during the first three days following tail amputation in *X. laevis*, where they promoted regeneration (Love et al., 2013). However, while inflammatory cells were also increased in number after tail amputation, they did not appear to be the source of ROS (Love et al., 2013).

As with the other model organisms described above, the experimental tools available to investigate the role of the immune response in SCI in frog are growing. Tadpoles have many of the experimental advantages of zebrafish and many of the features that made it a classic model organism to study nervous system development also make it attractive to study nervous system regeneration, including their small size, affordability, availability of transgenic lines, transparency of CNS tissues and the ability to assay simple regeneration-dependent behaviors, such as swimming, (Cline and Kelly, 2012; Pratt and Khakhalin,

2013). *X. tropicalis* is an NIH-designated model organism whose genome is publicly available. This species can also regenerate its spinal cord after tail amputation (Love et al., 2011). To support the use of *Xenopus* in biomedical research, the National Xenopus Resource Center was recently established at the Marine Biological Laboratory (http://www.mbl.edu/xenopus/) and the *Xenopus laevis* Research Resource for Immunobiology promotes the study of immunological questions by providing a variety of experimental resources (http://www.urmc.rochester.edu/mbi/resources/Xenopus/index.cfm). In Europe, the European Xenopus Resource Centre is located at the University of Portsmouth (http://www.port.ac.uk/research/exrc/) and Xenbase offers a variety of genomic and other resources to the research community (http://www.xenbase.org/common/). Together, these organizations offer reagents to target specific immune cell types, including transgenic stocks, genetic manipulations of gene expression and other resources, which should enable future studies of the immune response to SCI in frogs.

### Turtle

A few recent studies have also demonstrated axon regeneration after complete spinal cord transection in the turtle (Garcia et al., 2012; Rehermann et al., 2009; Rehermann et al., 2011). After injury, axon regeneration, primarily of propriospinal and sensory neurons, is followed by partial restoration of locomotion in ~50% of spinalized turtles (Rehermann et al., 2009; Rehermann et al., 2011). In this model, histological assays demonstrated that a blood clot filled with red and white blood cells formed immediately after SCI. As in the other regenerative non-mammalian species discussed above, after SCI, glial cell processes grew parallel to the long axis of the spinal cord. At the same time, phagocytic macrophages were observed at the lesion (Rehermann et al., 2009). Studies are just beginning to explore cellular aspects of neurogenesis and molecular programs influencing regeneration in this organism (Garcia et al., 2012; Rehermann et al., 2011). For an illustration of turtle axons regenerating across the lesion site, see Figure 2D.

## Advantages and Caveats

The major and significant advantage to studying immune (or any type of) responses to SCI in the non-mammalian vertebrates described here is that they have a high rate of success in recovery of function (Figure 2). Therefore, in these models, the emphasis is on understanding mechanisms promoting, rather than inhibiting, the desired outcome. There are also a range of experimental advantages, including: small organism size, low cost, availability of transgenic models, ability to do forward and reverse genetics, visibility of relevant tissues and cells *in vivo*, identifiable cell types with predictable regenerative responses, simple behavioral assays that depend directly on axon regeneration, and the relative simplicity of CNS circuits.

Of course, as with any model system, there are several caveats in interpreting insights gained from studying immune responses to SCI in non-mammalian model organisms. For example, in several of the species described above, only subpopulations of neurons regenerate and yet, functional recovery is still achieved. How much regeneration is needed in order to promote functional recovery from SCI in mammals? Historically, the biggest obstacle to studying regeneration in non-mammalian species was a lack of molecular

resources to investigate such questions, but with next generation sequencing technologies, that is changing rapidly. Proteomic resources are still lagging behind those available in mammals, which impacts the availability of antibodies for immunohistochemistry and flow cytometry. This is less of a challenge in the CNS and innate immune system, where proteins are often highly conserved, than in the adaptive immune system, where molecular strategies are more diverse across species. Because inflammation is widely conserved across species and induced by a variety of traumatic and non-traumatic neurodegenerative settings in mammals, it is a reasonable candidate pathway to study in any vertebrate after SCI. The increasing number of available molecular resources should encourage the development of translational studies in these animals.

### Challenges and Opportunities

Despite such caveats, the potential opportunity to translate knowledge gained from studying cellular and molecular responses to SCI in non-mammalian model organisms to mammals is growing. The underlying rationale for studying SCI-triggered responses in a variety of organisms is based on the hypothesis that neuronal regeneration, like neuronal function, relies on fundamental pathways conserved across species. It would follow that regenerative pathways activated in non-mammalian vertebrates after SCI could be activated in mammals, leading to better outcomes. While it was once challenging to investigate the mechanisms leading to functional recovery after SCI in non-mammalian vertebrates, next generation sequencing technologies have enabled the analysis of molecular pathways modulated by SCI in these species (Smith et al., 2011). Molecular data from transcriptome and genome studies in these organisms is leading to the generation or identification of reagents that can mark specific cell types and molecules. These resources will then enable more mechanistic studies. At the cellular level, resources accumulating in these species also enable critical questions that are unapproachable in mammalian systems. For example, what intrinsic signals distinguish typically selectively vulnerable or regenerating neurons in zebrafish or lamprey? At the tissue level, comparing molecular data across multiple non-mammalian species should reveal fundamental and basic aspects of pro-regenerative responses that are conserved throughout evolution (Figure 3). By contrasting those responses with data similarly collected in mammalian models of SCI, it will also be possible to reveal anti-regenerative responses that are specific to mammals or shared across vertebrates. For example, identifying beneficial aspects of the immune response to SCI that are shared across non-mammalian vertebrates may identify aspects of the immune response predicted to promote recovery from SCI in mammals. Future studies are necessary to test these predictions and identify new targets for intervention in mammalian models of SCI.

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## Highlights

- Many non-mammalian vertebrate species exhibit significant recovery after SCI.
- The immune response influences regenerative capacity across species.
- Understanding immune responses to SCI across vertebrates may promote recovery.

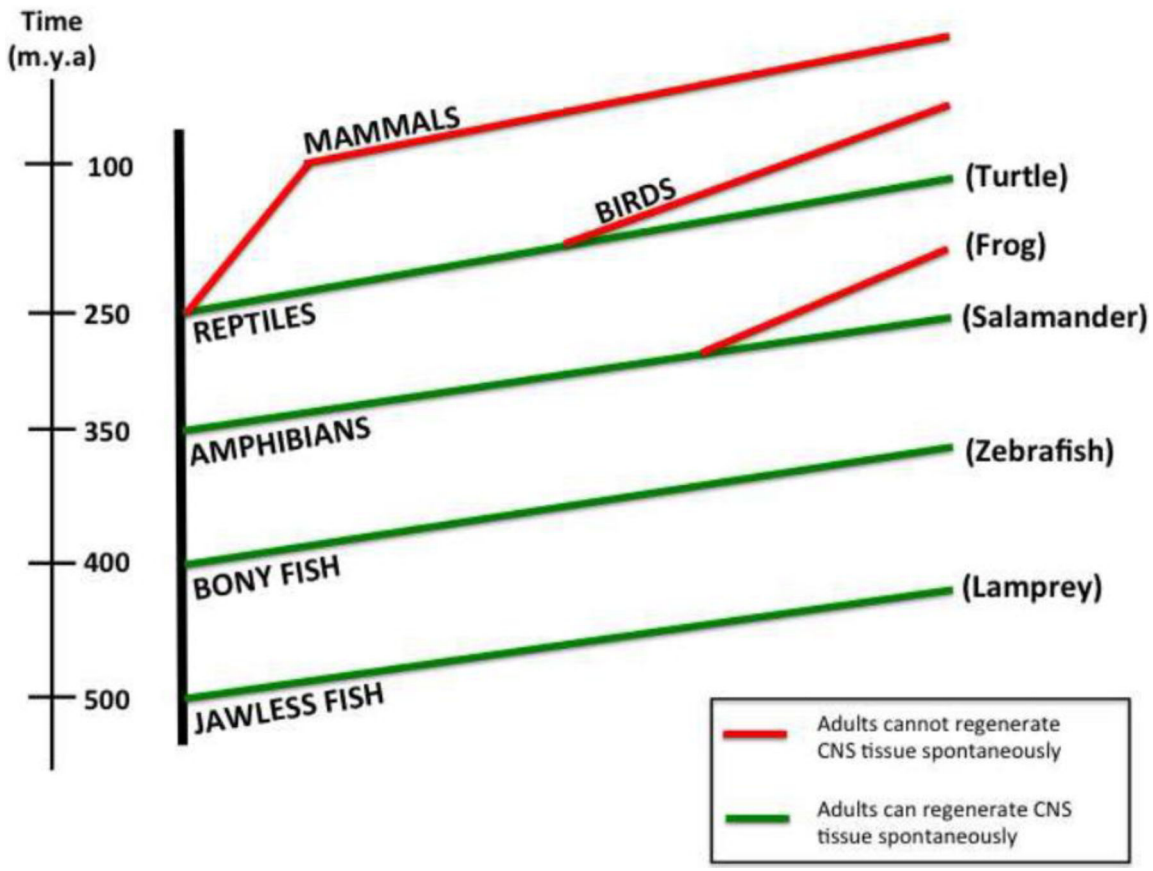


**Figure 1.**

Phylogenetic Tree illustrates evolutionary relationships of multiple vertebrate species and their differential capacity to regenerate CNS tissue after injury. The dimensions of the timeline are not drawn to scale.

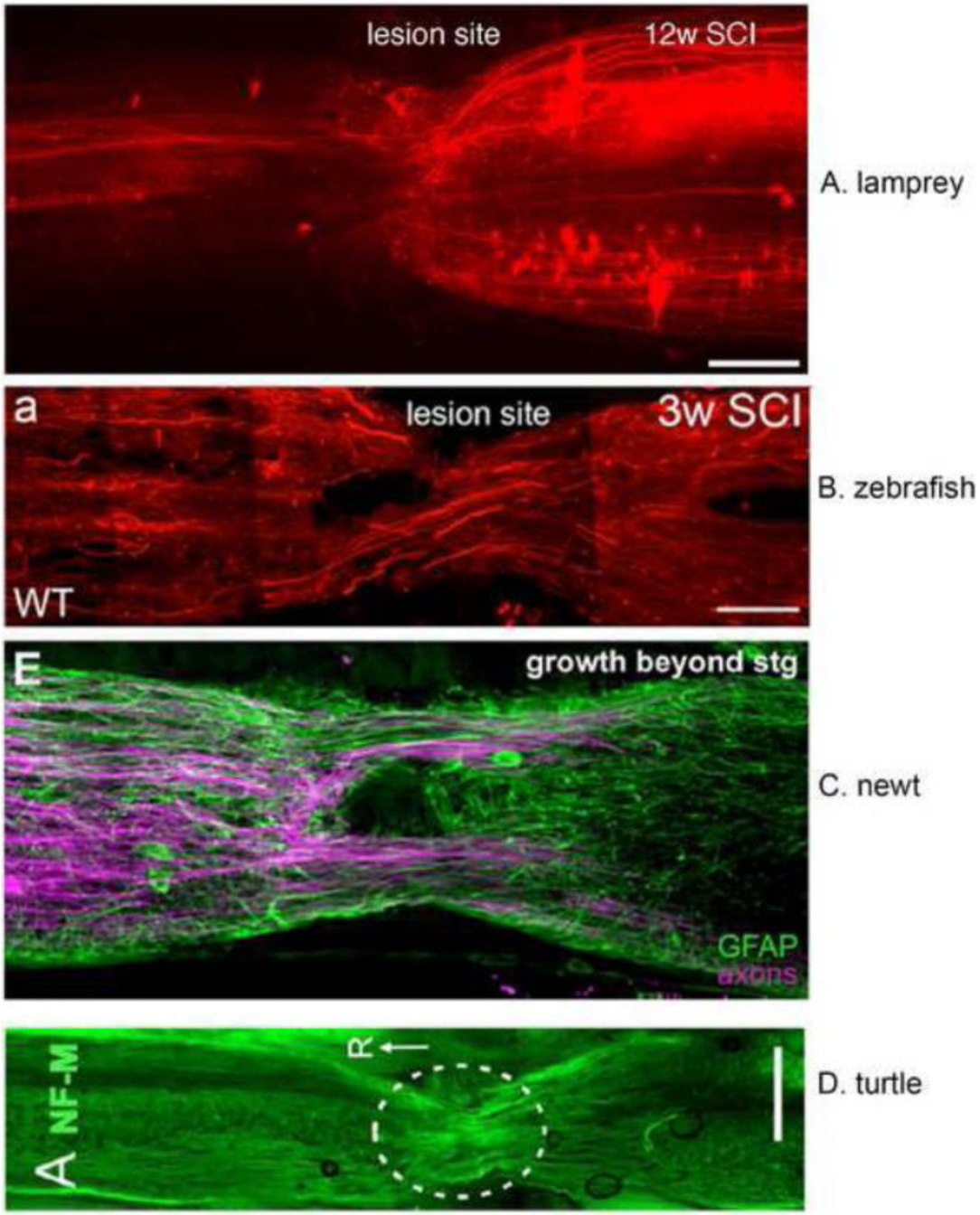


**Figure 2.**

Robust axonal regeneration after spinal cord injury occurs in multiple non-mammalian model organisms.

(A) Retrograde tracing with DTMR (red) was performed on lampreys twelve weeks after complete spinal cord transection, when behavioral recovery is maximal. DTMR was applied 1cm caudal to the original transection site. Arrowheads indicate three regenerated axons. Since this is a complete transection injury model, any axons crossing the original lesion site

have regenerated. Scale bar: 200μM. Image provided by Angelos Papatheodorou and Ona Bloom.

(B) Retrograde axonal tracing was performed on adult zebrafish three weeks after complete spinal cord transection, when swimming ability is almost normal. Experimental details and original image from: Figure 6A, Goldshmit, Sztal, Jusuf, Hall, Nguyen-Chi, Currie PD. Journal of Neuroscience, May 30, 2012, 32(22):7477-7492.

(C) Axonal tracing and anti-GFAP antibody labeling was performed on adult newt after complete spinal cord transection. Experimental details and original image from: Figure 6E, Zukor K, Kent D, Odelberg S. Neural Development, January 4 2011, 6:1-22.

(D) Regenerating axons visualized by neurofilament M antibody labeling in a turtle after complete spinal cord transection. Experimental details and original image from: Figure 10A, Rehermann M, Marichal N, Russo R, Trujillo-Cenoz O. The Journal of Comparative Neurology March 20, 2009, 515: 197-214.

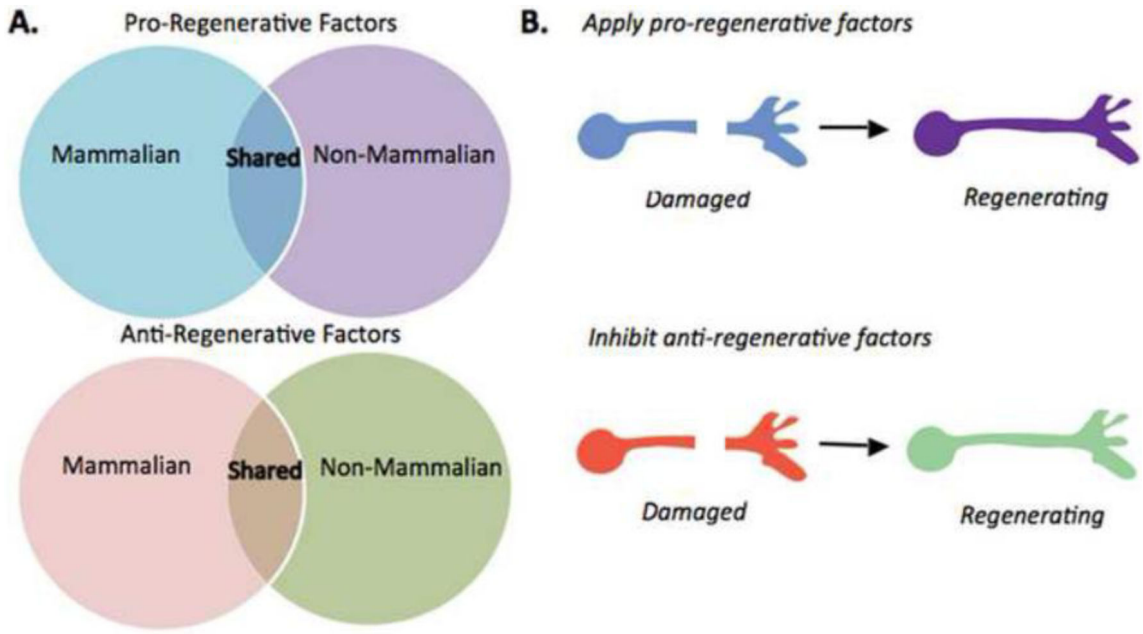


**Figure 3.**

Comparative approach to discovering biological responses that promote or inhibit regeneration after spinal cord injury.

(A) Gene expression profiling of responses to SCI in non-mammalian model organisms that recover function after SCI will provide a critical step towards understanding the responses able to regulate this process in mammals. By comparing responses shared across regeneration-competent species and contrasting with those in mammals, we can predict pro-regenerative factors that are evolutionarily conserved and those that are unique to non-mammalian vertebrates. It will also allow us to predict anti-regenerative factors regulated by SCI that are evolutionarily conserved and those that are unique to mammals. (B) Ongoing and future studies using tools of genetic and pharmacologic manipulation will functionally test these predictions by applying candidate pro-regenerative factors (upper) or inhibiting candidate anti-regenerative factors (lower). Together, such experiments will identify new targets for intervention in mammalian models of axonal injury and regeneration.