

## Review

# Central nervous system regeneration

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## SUMMARY

Neurons of the mammalian central nervous system fail to regenerate. Substantial progress has been made toward identifying the cellular and molecular mechanisms that underlie regenerative failure and how altering those pathways can promote cell survival and/or axon regeneration. Here, we summarize those findings while comparing the regenerative process in the central versus the peripheral nervous system. We also highlight studies that advance our understanding of the mechanisms underlying neural degeneration in response to injury, as many of these mechanisms represent primary targets for restoring functional neural circuits.

## INTRODUCTION

Physical injuries and neurodegenerative diseases often bring about irreversible damage and loss of function to the central nervous system (CNS). In mammals, such loss of function is due to the inability of adult mammalian CNS neurons to regenerate. A limited degree of CNS self-repair exists early in development; however, the ability to spontaneously regenerate is dramatically reduced after parturition.

Tremendous effort has been devoted to characterizing the cellular and molecular origins of CNS regenerative failure, yet a complete understanding of these factors is still lacking. Efforts to promote CNS regeneration, particularly by removing factors known to restrict CNS regeneration such as myelin-associated proteins, have been met with mixed success. In some instances, increased neuronal survival and a small amount of axon re-extension was observed, but in every case, these approaches have failed to restore complete, or even near-normal, circuit function. To date, only a handful of experimental approaches have yielded CNS regeneration to a degree that inspired human clinical trials. No treatments yet exist for successfully stimulating regeneration of CNS neurons in humans. Nevertheless, a number of key mechanistic discoveries have been made that point to new directions for the field. We posit that, to achieve this goal of re-establishing functional connectivity following CNS damage in humans, multi-faceted strategies will be required that promote (1) neuronal survival, (2) axon re-extension, (3) synapse re-formation, (4) myelination, and (5) experience-dependent refinement of newly formed circuits.

Here, we summarize our current understanding of mammalian neuronal responses to injury and highlight key advances directed toward overcoming CNS regenerative failure. We provide a brief overview of the model systems that are most commonly used to study CNS injuries in mammals to highlight their relative advantages. We consider both the extrinsic and intrinsic mechanisms

activated by injury, with a focus on events that trigger axon degeneration, since these events constrain the viability of pro-regenerative approaches.

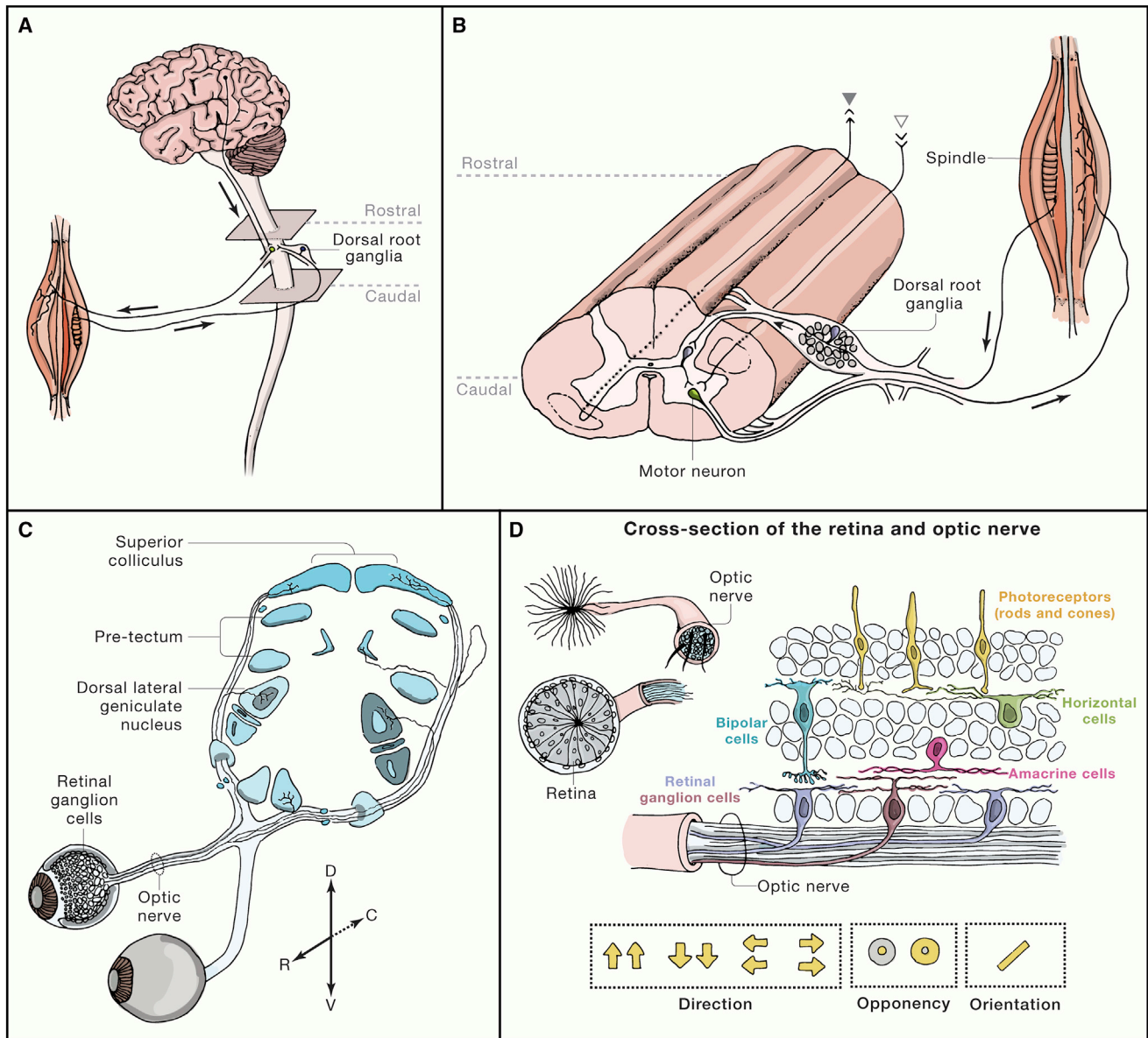
## Model species and pathways for exploring CNS regeneration

Many key discoveries that have advanced our understanding of the molecular and cellular mechanisms involved in axon regeneration and degeneration, including the identification of signaling pathways that regulate regeneration in mammals, were originally made in lower vertebrates (frogs, fish, and salamanders) and invertebrates (worms and flies).

Two valuable models for studying mammalian CNS regeneration are the spinal cord system and the visual system. Both carry out vital sensory-motor functions and have well-defined anatomical elements whose physiologies and relation to behavior are known. The spinal system includes descending projections from the brain to the spinal cord that make up the corticospinal tract (CST), ascending projections from peripheral sensory neurons that compose the spinothalamic tract, and intra-spinal connections (Figures 1A and 1B). Together, these pathways offer the opportunity to explore diverse features of neural circuits. Further, sensory neurons allow for unique insight into spinal circuit regeneration since dorsal root ganglia (DRG) neurons have both a centrally extending axon that cannot regenerate and a peripherally extending branch that readily regenerates following injury (Figure 1B). This highlights the differential ability of CNS and peripheral nervous system (PNS) axons to regenerate and emphasizes the importance of interactions with the environment (CNS versus PNS) and their subsequent intracellular responses on regeneration.

For somewhat distinct reasons, the visual system is also an attractive model system to study regeneration. Eye-to-brain circuits, collectively referred to as the retinofugal system, consist of retinal ganglion cells (RGCs), the output neurons of the eye, and





**Figure 1. Model systems for studying CNS regeneration: Spinal system and visual system**

(A) The brain and the spinal cord exchange information via descending projections from the brain to the spinal cord (arrow) and ascending projections. The spinal cord receives sensory input from external stimuli via the DRG neurons. Output signals from the brain and the spinal cord are relayed to the muscles via motor neurons and their peripheral projections.

(B) A longitudinal view of the spinal cord showing sensory inputs, interneurons, and motor outputs. The spinothalamic tract is comprised of ascending projections from interneurons (gray, closed arrowhead), whereas the CST consists of descending projections from the brain to the spinal cord (gray, open arrowhead). The DRG neurons have central and peripheral axonal branches.

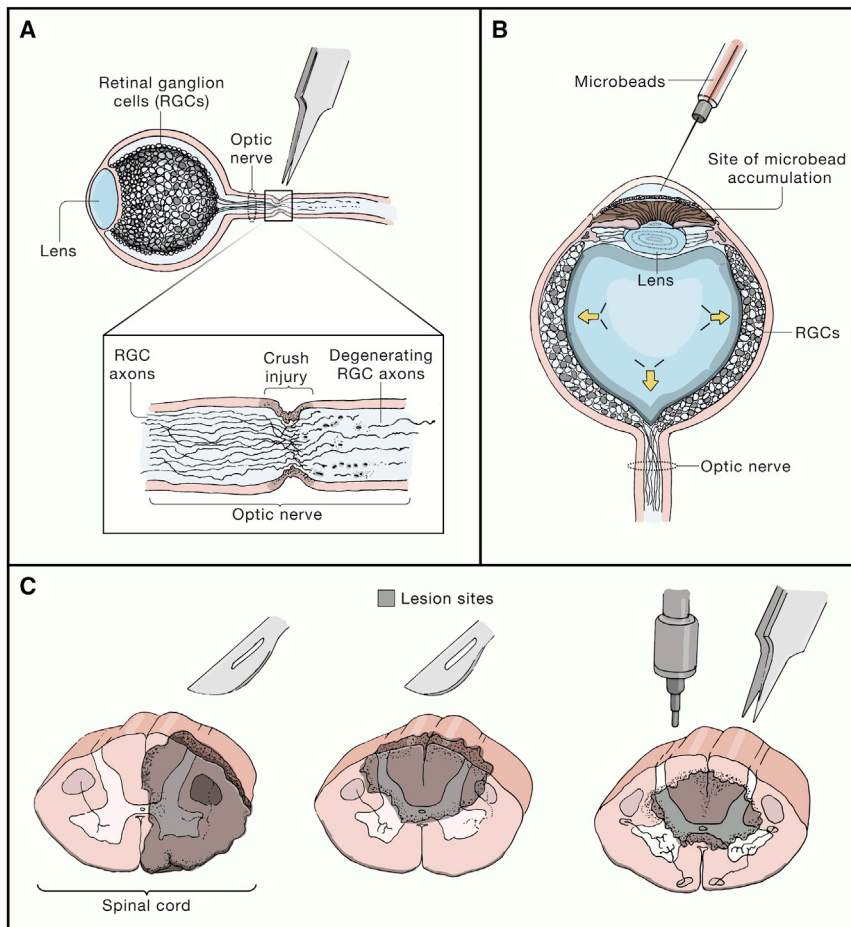
(C) The visual system includes RGCs, the neurons that relay sensory input from the eye, their axons that form the optic nerve, and their projections to target regions within the brain, including the dorsal lateral geniculate nucleus, pre-tectum, and the superior colliculus (blue).

(D) Cross-section of the retina and the optic nerve. The retina consists of five types of neural cells: photoreceptors (yellow), bipolar cells (blue), horizontal cells (green), amacrine cells (pink), and ~40 different types of RGCs (brown, purple). RGCs are the only neurons that send projections from the retina into the optic nerve and have various receptive field properties, as indicated by the yellow arrows and circles.

their connections with central target areas (Figure 1C). In many species, RGCs are easily accessible for drug treatments, viral infection, or anatomical labeling via injections into the eye. Moreover, RGCs and many of their central targets have well-defined physiological receptive field properties (Figure 1D), allowing for

precise assessments of functional recovery. Despite being located outside the cranial vault, RGCs are bona fide CNS neurons.

These two model systems, the spinal and visual systems, together offer comprehensive platforms for investigating neural



**Figure 2. CNS injury models**

(A) ONC injury showing axon degeneration distal to the injury site.

(B) Bead-induced mouse model of glaucoma, wherein microbeads injected into the eye increase intraocular pressure (arrows) and mimic the degenerative effects of glaucoma.

(C) Coronal views of the spinal cord depicting lesion sites (gray) following unilateral transection (left), dorsal bilateral hemisection (middle), and a contusion or crush injury (right).

ventions aimed at sparing and/or regenerating injured RGCs. Nonetheless, some RGCs die more quickly, and others resist injury-induced-death until the second half of the 8-day window. Still others resist injury-induced death entirely. Interestingly, these differences systematically relate to RGC subtype identity.

RGCs are divided into ~40 subtypes, each responding best to particular qualities of visual information. To date, studies of mammalian optic nerve repair have generally addressed regrowth (or lack thereof) following either (1) optic nerve transection or (2) optic nerve crush (ONC) (Figure 2A). Exploration of varying degrees of pressure applied during ONC is rare due to lack of instrumentation to accurately assess crush pressures. Alpha RGCs ( $\alpha$ RGCs), among the largest of the RGC subtypes, are motion-selective (but not direction-selective [DS]) and survive for

several months following optic nerve transection (Duan et al., 2015b; Holländer et al., 1985). Intrinsically photosensitive RGCs (ipRGCs), which respond directly to light due to the presence of melanopsin protein and set the central circadian clock (Hattar et al., 2002), are also resilient to ONC (Pérez de Sevilla Müller et al., 2014). Moreover, they resist the onset of death following other types of insults, including elevated intraocular pressure (a model of induced glaucoma; Figure 2B) and N-methyl-D-aspartate-receptor-mediated excitotoxicity (Cui et al., 2015). Recent work also suggests that a population of DS retinal ganglion cells (On-DSGCs) can survive up to 1 month following ONC, although anatomical changes suggest these cells would be actively undergoing apoptosis (Lilley et al., 2019). Other unidentified subtypes are resilient to cell death following ONC, but these make up a very small fraction of surviving RGCs (Tran et al., 2019).

## NEURONAL SURVIVAL

Dead neurons cannot regenerate. Thus, the goal of re-establishing functional neural circuits following CNS injury is first contingent upon maintaining the survival of damaged neurons. Some CNS neuronal subtypes appear less susceptible to damage-induced death than others. Identifying the molecular programs that render select neuron subtypes more resilient or particularly susceptible to the effects of neuronal insults has revealed both pro-survival and pro-death signaling proteins expressed to varying degrees following injury. These represent attractive entry points for the overall goal of enhancing CNS regeneration.

### Subtype-specific susceptibility

In the mammalian retina, very few RGCs die in the first 3 days after damage to the optic nerve (the dense axon bundle that exits each eye to innervate the brain) (Tran et al., 2019). Eight days after injury to the optic nerve, however, up to 70% of RGCs are dead and cleared away by glial phagocytosis (Tran et al., 2019). This provides a very narrow therapeutic window for inter-

Why are some RGC subtypes less susceptible to injury than others? One possibility is that endogenous levels of mammalian target of rapamycin (mTOR), a factor that promotes RGC axonal outgrowth during development, are higher in  $\alpha$ RGCs as compared to other RGC subtypes and thereby endow these cells with increased damage resilience (Duan et al., 2015b). This can be attributed to select expression of osteopontin, an mTOR activator, in these cells (Duan et al., 2015b). In addition, phosphoinositide 3-kinase (PI3K)-Akt activation that accompanies

melanopsin signaling in ipRGCs underlies the resilience of these cells following injury (Li et al., 2008; Pérez de Sevilla Müller et al., 2014). Furthermore, transcriptional activation of signal transducers and activators of transcription-3 (STAT3) in RGCs following injury bestows neuroprotective effects via stimulation of inflammatory signaling events, thereby increasing RGC survival (Leibinger et al., 2013); however, the extent to which individual subtypes of RGCs are affected by STAT3 activation requires further investigation.

While it is likely that subtype-specific neuronal survival following spinal cord injury is regulated by similar mechanisms as RGCs, the degree to which neurons survive following injury is also highly dependent on the type and location of the insult (Figure 2C). The most commonly used methods to produce a spinal cord injury either lesion a large number of axons in a specific spinal segment, such as the contusion model and spinal cord crush, or lesion specific ascending or descending tracts, such as unilateral or bilateral spinal hemisections (Figure 2C; reviewed in Steward and Willenberg, 2017). Upper motor neurons (UMNs), whose axons comprise the CST, are highly sensitive to axonal transection, with nearly 40% of these neurons undergoing apoptosis within 1 week of lesion (Hains et al., 2003). By contrast, neurons that elaborate descending projections of the rubrospinal and vestibulospinal tracts appear less vulnerable than UMNs to contusion injury, but this is highly dependent on the severity of the injury (Hassannejad et al., 2018).

Within the spinal cord, short thoracic propriospinal (STP) neurons are more susceptible to cell death in both contusion and transection injuries than long descending propriospinal tract (LDPT) neurons (Hassannejad et al., 2018). Interestingly, approximately 5% of LDPT projections remain intact 2 weeks post-contusion injury, whereas STP axons are completely lost (Conta and Stelzner, 2004). Despite the loss of projections, numerous STP somas are still present for at least 2 weeks following severe contusion, offering a therapeutic window to promote regeneration of this cell type.

A complete understanding of differential neuronal survival in both the retina and the spinal cord following injury remains to be unveiled. Meanwhile, insights into the transcriptional programs of resilient neurons are starting to reveal not only the kinetics of apoptosis, but also the specific pro-survival genes and signaling pathways that serve to stabilize all cell types following injury (Duan et al., 2015a; Tran et al., 2019).

### Reprogramming

Across development, multiple pro-growth signaling pathways that regulate cell size and neurite extension become gradually suppressed, preventing aberrant growth in adulthood. It was once thought that only PNS neurons re-activate developmental pro-growth signaling pathways to support axon regeneration following injury. However, recent work suggests that corticospinal neurons transiently reprogram themselves to an embryonic, pro-growth state following CST transection both in the presence and absence of neural progenitor cell grafts (Poplawski et al., 2020). However, this regenerative transcriptome is quickly downregulated in the absence of grafted neural progenitor cells at the injury site, suggesting that a pro-regenerative extrinsic environment can retrogradely influence sustained gene expres-

sion. Transcriptional profiling of RGCs following ONC suggests that RGCs do not share this reprogramming property with lesioned corticospinal neurons (Tran et al., 2019). Nevertheless, the possibility that other injured CNS neurons spontaneously reprogram, albeit transiently, has therapeutic implications.

Many groups have sought to exogenously reprogram injured CNS neurons to a development-like state in order to sustain pro-growth signaling in these cells. One way to achieve this is to manipulate the balance of oncogenes and tumor suppressors, including Lin28 and Let7, respectively, to favor activation of growth-promoting pathways. Initially described as a key regulator of developmental timing in *C. elegans* (Ambros and Horvitz, 1984), Lin28 can also reprogram differentiated cells to a stem cell-like state in multiple contexts (Li et al., 2017a). In mammals, Lin28 expression increases in injured DRGs and regulates the activity of several growth-promoting pathways, including PI3K-Akt-mTOR and GSK3 $\beta$ , to sustain axon regeneration (Wang et al., 2018; Zhang et al., 2019). Further, increased expression of the Let-7 target genes *c-myc* and *Tet3* following injury suggests that Lin28 acts as a reprogramming factor in injured DRGs.

In the CNS, where Lin28 levels remain low following injury, Lin28 overexpression mediated by adeno-associated viral delivery either before or after lesion in both RGCs and CST neurons promotes long-distance axon regeneration (Nathan et al., 2020; Wang et al., 2018). Manipulating Lin28 activity in neurons following injury to promote regeneration suggests potential benefits in treating human CNS injuries. However, like mTOR, increasing Lin28 in humans may carry additional concerns related to oncogenesis.

An alternative approach is to initiate a global reprogramming of neurons to a development-like state by altering their epigenetic landscape following injury. Ten-eleven translocation methylcytosine dioxygenases (Tets) are a family of enzymes that promote the demethylation of CpG sites throughout the genome. Interestingly, sciatic nerve injury (SNI) leads to increased expression of Tet3 in DRG neurons, subsequently activating the expression of known regeneration-associated genes such as STAT3, *c-myc*, and ATF3 (Weng et al., 2017). Tet3 knockdown and a related Tet family member, Tet1, are required to promote spontaneous axon regeneration and for the pro-regenerative effects of mTOR upregulation in lesioned RGCs. Further evidence supporting a role for Tet family members in initiating a global regenerative program comes from the finding that the pro-regenerative effects garnered by reprogramming injured RGCs using Oct4, Sox2, and Klf4 overexpression are Tet1- and Tet2-dependent (Lu et al., 2020). This treatment can recover some visual behaviors in a glaucoma mouse model and can also stimulate robust RGC axon regeneration in aged mice, supporting the idea that Tet-dependent epigenetic reprogramming is a master switch to allow long-distance axon regrowth following injury. Although no tumor growth in Oct4-, Sox2-, and Klf4-expressing eyes (Lu et al., 2020), it remains important to determine whether such broad genomic manipulations are deleterious to proper cellular function. For instance, how do these manipulations impact neuronal properties such as subtype identity and presynaptic connectivity? Answers to these questions will help determine the clinical potential for exogenous reprogramming of neurons to restore function following injury.

## AXON REGENERATION

Axon regeneration is defined as axon regrowth and the subsequent innervation of target regions following injury, resulting in recovery of neuronal function and behavior. While many studies document robust CNS axon regrowth following modulation of pro-growth signaling pathways in injured neurons or alteration of extrinsic factors that inhibit axon re-extension, none have achieved complete regeneration of function. This is due to the long trajectories that mammalian projection neurons often must navigate to reach their developmental target regions and the presence of inhibitory factors encountered by these re-extending axons. This is especially true in the visual system since the optic nerve consists of RGC axons and glial cells and no classical synapses, thereby necessitating complete regrowth of injured axons into the brain to restore electrical connectivity. Although the optic nerve serves as a conduit for RGC axons, as is the case in development, regenerating axons must traverse the optic chiasm before ascending into the brain. To navigate the optic chiasm, an important choice point, developing RGC axons interact with a combination of attractive and repulsive guidance cues that ultimately mediate axon decussation (crossing) or non-decussation at the CNS midline (Petros et al., 2008). Although a few regenerating axons have been shown to cross the chiasm following growth stimulating treatments (Lim et al., 2016; de Lima et al., 2012), whether the midline glial cells and the guidance cues they express are present post-injury in adults remains unknown. Transcriptomic analyses of midline glial cells in uninjured and injured conditions may reveal molecular targets that can be manipulated to encourage extension of regenerating axons into and beyond the chiasm to achieve robust long-distance regeneration.

The spinal system includes neuronal cell bodies throughout the entire length of the spinal cord, allowing relay circuits to bypass the injury site and achieve connectivity with distal targets. Even if we are far from having a complete understanding of the events required to re-establish functional neuronal circuits, there are now several promising strategies for promoting long-distance regrowth of severed axons. Further, since PNS neurons can regenerate, there is much to learn about key features that bestow this property on these neurons. Therefore, we next discuss current findings on regeneration and the re-establishment of connectivity following injury by describing factors that promote PNS regeneration, as well as intrinsic and extrinsic hurdles that constrain CNS regeneration.

### PNS neurons regenerate following injury

The PNS has a remarkable capacity to regenerate following injury. A striking example can be found in reptiles and amphibia such as lizards and salamanders, where a damaged or even completely amputated tail or limb fully regenerates to restore normal movement. In mammals, PNS neurons regenerate less quickly and extensively than in reptiles or amphibians but more readily than CNS neurons. For example, spinal motor neurons that project in peripheral nerves regenerate axons and reinnervate muscle targets following injury even in adulthood (Kang and Lichtman, 2013). A primary distinction between the CNS and PNS lies in each system's response to injury. Following injury in the PNS, myelinating glia called Schwann cells undergo dedif-

ferentiation and subsequent trans-differentiation, allowing them to carry out various functions that facilitate debris clearance (Jessen and Mirsky, 2016). Further, PNS neurons have cell-intrinsic responses to injury that greatly increase their capacity to regenerate as compared to CNS neurons (Hoffman, 2010).

What is known about the molecular properties that bestow the capacity to regenerate on PNS neurons? Recent evidence implicates injury-induced reprogramming within peripheral sensory neurons that aids in regeneration following injury (Chandran et al., 2016; Renthall et al., 2020). This feature is perhaps best illustrated by DRG neurons, which are bipolar in shape; their cell body resides adjacent to the spinal cord, and their axons have two branches—one that projects into the CNS and another that extends into the PNS. Following damage to a DRG neuron, the peripheral branch readily regenerates at a rate of ~1 mm per day, whereas the central branch fails to regenerate (Schwab and Bartholdi, 1996). In the PNS, transcriptional activation of *Atf3* in injured DRG neurons within 3 days of injury suppresses cell identity and boosts regeneration (Renthall et al., 2020). Additionally, local translation of mTOR in axons following SNI upregulates not only mTOR, but also other proteins important for retrograde injury signaling, such as STAT3, that promote regeneration of the peripheral branch (Terenzio et al., 2018). In the CNS, the absence of such a robust injury-induced increase in growth-promoting proteins along with the absence of protein trafficking in axons—particularly alpha9 integrin, which belongs to a class of receptors that promote neurite outgrowth during development—considerably limit regeneration of the central branch (Andrews et al., 2009).

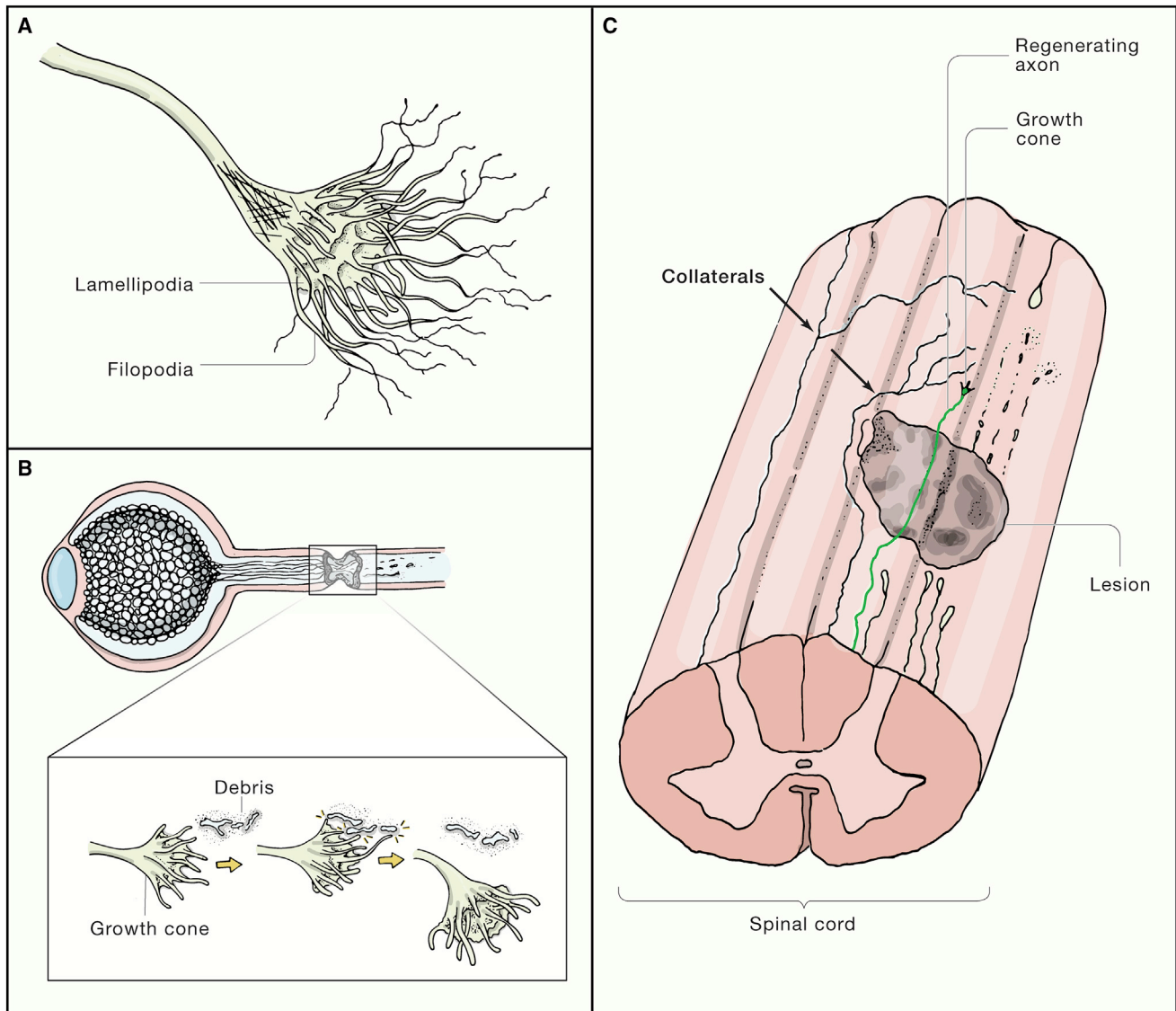
These findings indicate that both the environment encountered by regrowing axons following injury and the internal state of PNS neurons are key features for supporting PNS axon regeneration. Classic studies showed that peripheral nerve grafts used as physical bridges promote regeneration of central axons in the spinal cord and from the retina to the brain (David and Aguayo, 1981). Similarly, a conditioning PNS lesion, or introduction of a cAMP analog, alters the intrinsic state of DRG neurons, supporting central axon regrowth past the injury site through an otherwise inhibitory environment; this suggests that CNS neurons may retain the potential to regenerate axons (Neumann and Woolf, 1999; Neumann et al., 2002). Thus, the two main categories of factors that influence mammalian CNS regeneration following injury are (1) cell-intrinsic factors including transcriptional programs and growth-promoting signaling pathways and (2) extrinsic factors such as the molecules and cells in the environment that damaged axons encounter (reviewed in Crair and Mason, 2016).

### Intrinsic factors that regulate CNS regeneration

Neuron-intrinsic factors play a critical role in determining CNS regenerative capability. Though our knowledge of the genes expressed in mature CNS neurons that limit regeneration has increased in recent years (Bray et al., 2019; Tran et al., 2019), we are far from a complete understanding of the intrinsic responses that impact CNS axon regeneration.

### Initiating axon growth from lesioned CNS neurons

An important measure of whether a neuron can successfully regenerate is its ability to form an axonal growth cone



**Figure 3. Axon growth following injury**

(A) Growth cone at the leading edge of an axon. The fingerlike protrusions are filopodia, which are composed of actin filaments and are crucial for the growth cone's ability to grow toward or away from environmental cues.

(B) Axons regenerating following an ONC injury. Growth cones at the leading edge of regenerating axons grow past the lesion site (box, magnified), interact with microglia/axonal-debris/guidance cues, and alter their direction of growth accordingly.

(C) Longitudinal view of the spinal cord showing a lesion site (gray), distal processes of injured axons degenerating (dotted lines), a regenerating axon extending a growth cone distal to the lesion site (green), and two spared axons extending collaterals that circumvent the lesion site toward target neurons distal to the lesion.

(Figure 3A). Growth cones of regenerating axons sample their environment as they navigate toward their targets, similar to their activities during development (Figure 3B). What are the key influences that determine whether an injured axon forms an active growth cone or a retraction bulb—the characteristic post-injury axon tip that resembles a dystrophic growth cone? Selective transection of the CNS-projecting branch of DRG axons revealed injured axons regrowing either from the tip of the axon or sprouting from the proximal node of Ranvier within 24 h following a lesion (Kerschensteiner et al., 2005). A third of the injured axons began regenerating within 2 days of the injury at

approximately 4 microns/h, which is considerably slower than PNS axons (Kerschensteiner et al., 2005). These rapid, dynamic changes in the injured axon, proximal to the lesion site, suggest the presence of an initial, albeit short-lived, regenerative response in CNS axons. The majority of injured CNS axons, however, form retraction bulbs as they encounter proteoglycan substrates. They then protrude, undulate, and endocytose vesicles, ultimately remaining dystrophic without advancing further (Tom et al., 2004). Recent work demonstrates that destabilized and disorganized microtubules cause active growth cones to become dystrophic, an effect that can be overcome by

administering epothilone B, an FDA-approved drug, or by specifically deleting a small GTPase ras homolog gene family member A (RhoA) in neurons (Ruschel et al., 2015; Stern et al., 2021). In the visual system, other mechanisms have been shown to be effective in initiating a growth cone; for example, following ONC, overexpression of doublecortin-like kinases promotes growth cone initiation, resulting in more axons regenerating past the lesion site in the optic nerve (Nawabi et al., 2015). Ultimately, growth cone formation is an important aspect of axon regeneration involving a cascade of events, some of which overlap with mechanisms activated by axonal responses to injury (Bradke et al., 2012).

### **Transcriptional regulation of axon growth**

Several transcriptional programs active in developing neurons are downregulated postnatally and have the capacity to influence the regenerative potential of mature CNS neurons. For example, the cell-intrinsic signaling pathway that regulates mTOR to modulate cell survival, growth, metabolism, and protein synthesis is elevated in newborn CNS neurons; however, inhibition by phosphatase/tensin homolog (PTEN) causes mTOR signaling levels to rapidly taper down, becoming low-to-absent in most neurons by adulthood. Genetic deletion of PTEN, a negative regulator of mTOR, leads to robust regenerative responses by RGC axons following ONC (Park et al., 2008). Manipulating the mTOR pathway has proven to be a robust approach for promoting axon extension and cell survival after acute injury in both the visual and spinal systems (Duan et al., 2015b; Jin et al., 2015; de Lima et al., 2012; Sun et al., 2011). Further, suppression of the PTEN pathway, along with other neuron-intrinsic transcriptional programs that suppress cytokine signaling through SOCS3, synergistically induces robust regeneration of RGC axons and improves sprouting of corticospinal axons following injury (Jin et al., 2015; Leibinger et al., 2013; Luo et al., 2013; Sun et al., 2011).

Transcriptional programs can also be modulated to activate growth-promoting elements, as is the case with the transcription factor SRY-Box 11 (Sox11). Overexpression of Sox11 in RGCs increases axon growth following ONC (Norsworthy et al., 2017). Interestingly, Sox11 overexpression is deleterious to  $\alpha$ RGC cell survival and only promotes regeneration of non- $\alpha$ RGC axons, thus demonstrating neuronal subtype-specific effects (Chang et al., 2021; Norsworthy et al., 2017; Welsbie et al., 2017). Similarly, another family of transcription factors, the Kruppel-like proteins (KLFs), have distinct effects on the regulation of axon growth after injury in the visual and spinal systems; KLF-4 and -9 are up-regulated postnatally during development and suppress axon regeneration (Trakhtenberg et al., 2018), while KLF-6 and -7 are normally downregulated postnatally but, upon activation, support axon regrowth (Moore et al., 2009). Interestingly, Klf-6, but not Klf-7, expression is higher in ipRGCs following ONC (Bray et al., 2019), potentially increasing the resilience of these neurons after injury. Similarly, several transcriptional programs involving epigenetic control of injury-induced transcription and also signaling pathways that have pro-regenerative effects have been described (reviewed in Mahar and Cavalli, 2018).

### **Extrinsic factors regulating CNS regeneration**

Major extrinsic factors that regulate regeneration include myelin debris from injured axons and the astrocytes, fibroblasts, extra-

cellular matrix (ECM) components, microglia, and blood-borne immune cells that are present or infiltrate the lesion site to form a glial scar. In the PNS, Schwann cells facilitate axon regrowth by clearing axonal debris following peripheral injury. Upon physical injury or degeneration, Schwann cells downregulate their production of myelin and transdifferentiate into repair cells. With the assistance of resident macrophages and blood-borne leukocytes, Schwann cells initiate a rapid response that facilitates clearing of debris, upregulation of trophic factors, and regeneration of axonal components (Brosius Lutz et al., 2017; Kang et al., 2014). Additionally, Schwann cells also form bridges called “regeneration tracks” or “Bungner’s bands”—strings of Schwann cells along which injured axons navigate back to their targets (Jessen and Mirsky, 2016; Son and Thompson, 1995).

Injury to the CNS, by contrast, is met with significantly delayed clearance of CNS myelin. The myelin sheaths that encase neurons in the CNS are made up of oligodendrocytes. Oligodendrocytes, unlike Schwann cells, do not undergo injury-induced reprogramming and do not phagocytose myelin debris. Further, in the CNS, microglia themselves do not phagocytose myelin debris as efficiently as macrophages. As a result, myelin sheaths are not cleared away from injury sites, thereby leaving behind a “ghost” of the former axon trajectory. In some instances, myelin ghost sheaths remain years after the injury and are never cleared away (Vargas and Barres, 2007). This lack of myelin clearance is a barrier to axon regeneration through damaged territory, impeding circuit restoration (Filbin, 2003).

It is interesting to note that outside of the visual and spinal systems, long-range axon regrowth and functional recovery following CNS injury have been observed for both serotonergic and catecholaminergic neuromodulatory neurons (Dougherty et al., 2020; Jin et al., 2016). This extensive long-range axon regeneration may result from these neurons failing to receive inhibitory signals from the extracellular matrix that normally impede glutamatergic axon regeneration; however, it is also possible that volumetric synaptic transmission used by neuromodulatory neurons relieves precise innervation constraints, since conventional synaptogenesis is not required for these neurons to restore functional connectivity.

Another impediment to CNS regeneration was thought to be the glial scar formed around the lesion site. In the CNS, the glial scar is composed of fibroblasts, ECM components, and inflammatory immune cells at the lesion core, all surrounded by reactive astrocytes (Adams and Gallo, 2018). Although research into the glial scar has mainly centered on the inhibitory effects of cells surrounding the lesion and how they negatively affect axon regeneration, recent evidence questions this inhibitory role of the glial scar following injury.

### **Glial scar in axon regeneration**

Astrocyte activation at the site of CNS lesions results in the deposition of ECM molecules, such as chondroitin sulfate proteoglycans (CSPGs), and the formation of the glial scar, which, for many years, has been considered exclusively refractory to axon extension (Silver and Miller, 2004). In addition to CSPGs, inhibitory cues such as semaphorin 3A and ephrin B within the scar also contribute to regenerative failure (Bundesen et al., 2003; Pasterkamp et al., 2001). Other cues including semaphorin 5A and semaphorin 6D are also elevated at the injury site

following SCI, resulting in increased axonal dieback of CST neurons (Ueno et al., 2020). This axonal retraction can be suppressed by removal of these semaphorins or conditional deletion of their Neuropilin 1 or Plexin A1 holoreceptor complex components from CST neurons prior to injury (Ueno et al., 2020).

However, not all molecules found in the glial scar are inhibitory to axon growth, since some glycosaminoglycan side chains actually promote axon extension (Miller and Hsieh-Wilson, 2015). The CSPG side chain governs the response of interacting axon growth cones, so a potential therapeutic strategy investigated by many groups is the enzymatic degradation of inhibitory CSPG glycosaminoglycans using chondroitinase ABC (chABC). This strategy has resulted in increased axon growth and some recovery of motor function in rodents following lesion (Barritt et al., 2006; Moon et al., 2001). In addition, chABC, combined with cell transplants or optimization of the mode of chABC delivery, may substantially promote regeneration and improve functional recovery (Muir et al., 2019; Rosenzweig et al., 2019). Encouragingly, intraparenchymal chABC injections in rhesus monkeys following spinal cord hemisection also resulted in increased synapse formation in gray matter caudal to the injury site and in partial restoration of motor function (Rosenzweig et al., 2019). Administering chABC to treat human patients afflicted with traumatic nerve injury is promising, but additional studies will determine whether the beneficial effects of this treatment occur in other CNS injury models.

In addition to inhibitory factors, transcriptomic profiling analyses reveal that cells occupying the glial scar also express a host of cues that are permissive for axon regrowth (Anderson et al., 2016). Among these, reactive astrocytes and non-astrocytic cells, such as fibroblasts, upregulate CSPG subtypes known to support axon growth (Anderson et al., 2016). Interestingly, axons fail to regenerate within the CNS when the astrocytic scar is ablated following a conditioning peripheral lesion, suggesting that components of the glial scar can, in fact, promote axon regrowth. Further, axon guidance signaling components such as Plexin-B2 are upregulated in injury-activated microglia and macrophages and play a role in organizing the glial scar (Zhou et al., 2020). Investigation into the role of microglia in neonatal mice following SCI shows that microglia form transient ECM bridges and express peptidase inhibitors that promote axon regrowth and wound healing (Li et al., 2020). Therefore, a conundrum of using chABC and other approaches to ablate the glial scar is the degradation of attractive and repulsive cues expressed by its constituents that are essential for directing axon growth. One approach is to identify key elements within the scar that are refractory to axon growth and specifically manipulate those components, as shown by the role of astrocyte-specific activation of RhoA in regulating injury-induced scarring and CSPG production, which ultimately promotes axonal regeneration (Stern et al., 2021). In the visual system, glial scars may not be as refractory to axon regeneration as they are in the spinal cord, since manipulations of intrinsic growth mechanisms such as mTOR can trigger substantial regeneration without any need to suppress glial scarring (Park et al., 2008).

Taken together, the glial scar should not be viewed as a homogeneous inhibitory environment but, rather, as a complex milieu of both permissive and inhibitory signals through which extending axons must traverse to reach their targets (reviewed in Adams and Gallo, 2018; Tran et al., 2021).

### Axon guidance molecules in regeneration

Axon guidance molecules play a critical role in neural circuit formation during development by directing the growth of axons and influencing target selection (Chédotal, 2019). These molecules can elicit attractive or repulsive responses from growth cones (Bashaw and Klein, 2010; Tessier-Lavigne and Goodman, 1996). Expression of many axon guidance molecules persists into adulthood; however, the role of guidance cues in adulthood, and whether they provide the same attractive or repulsive forces as they do during neural development, varies from one cue to another (Giger et al., 2010). Consequently, the role of guidance cues in re-forming neural circuits after injury, though promising, is complex and requires thorough investigation in order to understand how guidance cues might be successfully applied to regenerating axons (Giger et al., 2010; Harel and Strittmatter, 2006).

Repulsive guidance molecule A (RGMA) is a glycosylphosphatidylinositol-linked membrane-associated protein that binds to its receptor, Neogenin (Neo1), to specify repulsive axon guidance. Following spinal cord injury, RGMA is upregulated in neurons and oligodendrocytes and is also expressed by astrocytes, activated microglia, and macrophages. Monoclonal antibodies that neutralize the inhibitory effects of RGMA by specifically binding to this ligand promote axon regeneration and improve functional recovery 6 weeks after injury (Mothe et al., 2017). RGMA-Neo1 signaling is also known to promote cell survival, but not axon regrowth following optic nerve transection in the adult rat retina (Koeberle et al., 2010). These observations provide another promising therapeutic avenue to neutralize anti-regenerative factors and thereby promote axon re-extension following injury.

The Wnt signaling pathway is also implicated in providing repulsive signaling via its Ryk receptor during CNS regeneration. Following cervical spinal cord injury, inhibition of Wnt-Ryk signaling either using conditional knockout (KO) of *Ryk* or function-blocking monoclonal antibodies resulted in enhanced sprouting of lesioned CST axons (Hollis et al., 2016). These studies also demonstrated that when mice received task-specific forelimb training, their cortical maps underwent significant reorganization, resulting in novel recruitment of cortical areas that normally only control the hindlimb. Importantly, this work suggests that the greatest axon collateral branch formation and behavioral recovery following injury can be attributed to the joint contributions of task-oriented learning and blockade of Ryk receptor function. Recent evidence in the visual system shows that Wnt5a, acting at the optic chiasm, promotes decussation of contralateral RGC axons and repels axons of ipsilateral RGCs via the EphB1 receptor (Morenilla-Palao et al., 2020). Whether such mechanisms will be automatically reinstated or whether they need to be exogenously introduced after injury remains unclear. In the mammalian retinocollicular system, RGC axon injury triggers upregulation of select guidance cues in the target as well as in astrocytes surrounding the lesion (Symonds et al., 2007), potentially confounding growth-promoting signaling from target neurons.

Injury models that spare some axons, though not ideal for understanding the total regenerative potential of a particular treatment, are meritorious since they are clinically relevant. Most injuries in humans caused by accidents or trauma likely



result in spared axons. Axon regeneration and sprouting refer to differences in the origin of a regenerating axon; a newly regenerating axon arises from the injured axon itself, whereas sprouting refers to collaterals extending from spared axons that were not severed by the injury (Figure 3C) (reviewed in Fischer et al., 2017; Steward and Willenberg, 2017). The formation of axon collaterals allows mostly uninjured axons (and a few injured ones) to elaborate new processes that bypass the lesion site and promote connection and subsequent synaptogenesis with neuronal processes distal to the lesion site. Thus, there is a pressing need to identify strategies that can preserve the remaining neurons and their processes and to maximize their potential to restore function.

A more typical approach for enhancing functional recovery after CNS injury is to use guidance cues to promote the formation of collateral branches from the axon shaft (Figure 3C). Ephrins, a family of canonical guidance cues, play major inhibitory roles in the post-injury environment by blocking axonal sprouting following both ONC and SCI (Duffy et al., 2012; Joly et al., 2014; Overman et al., 2012). These studies demonstrate that ephrin/Eph expression is increased post-injury, mainly by reactive astrocytes. However, the use of ephrin/Eph blockers to neutralize injury-induced increases in expression was not successful in improving regeneration by permitting axonal sprouting. Remarkably, a combination of behavioral activity, fostered by forced-use of the affected limb, along with an Ephrin-A5 blockade following a stroke, resulted in a small but significant increase in axonal sprouting compared to controls with no forced-use. Several additional studies show that axon collateral sprouting improves functional recovery of motor circuits following spinal cord injury (Fouad et al., 2001; Lee et al., 2010; Massey et al., 2006). A caveat is that aberrant or constitutive upregulation of growth-signaling factors and guidance cue signaling pathways can lead to profuse, but abnormal, growth that may not be conducive for the functional reconnection of neural circuits (Bray et al., 2017; Pernet and Schwab, 2014; Pernet et al., 2013). Identifying and combining key guidance molecules and growth factors necessary for encouraging growth post-injury may promote synaptogenesis of regenerating axons.

### RESTORING SYNAPTIC CONNECTIVITY IN DISTAL TARGET REGIONS

A central goal of CNS regeneration studies is to understand how to achieve long-distance axon regrowth and connectivity to distal targets to restore CNS function. However, can regenerating axons properly reinnervate their developmental targets without exogenous application of guidance cues? In the mammalian spinal cord, injured corticospinal motor axons can successfully navigate through a neural progenitor cell graft and reinnervate appropriate pre-motor interneurons distal to the lesion (Ceto et al., 2020; Kumamaru et al., 2019). *Trans*-synaptic labeling in these studies demonstrates that premotor, but not presensory, interneurons become synaptically connected to regenerating corticospinal motor axons, suggesting that successful reinnervation of distal targets in the mammalian spinal cord is indeed possible independent of exogenous guidance cues.

Likewise, in the mammalian visual system, regenerating RGC axons can reinnervate retinorecipient targets following ONC, re-

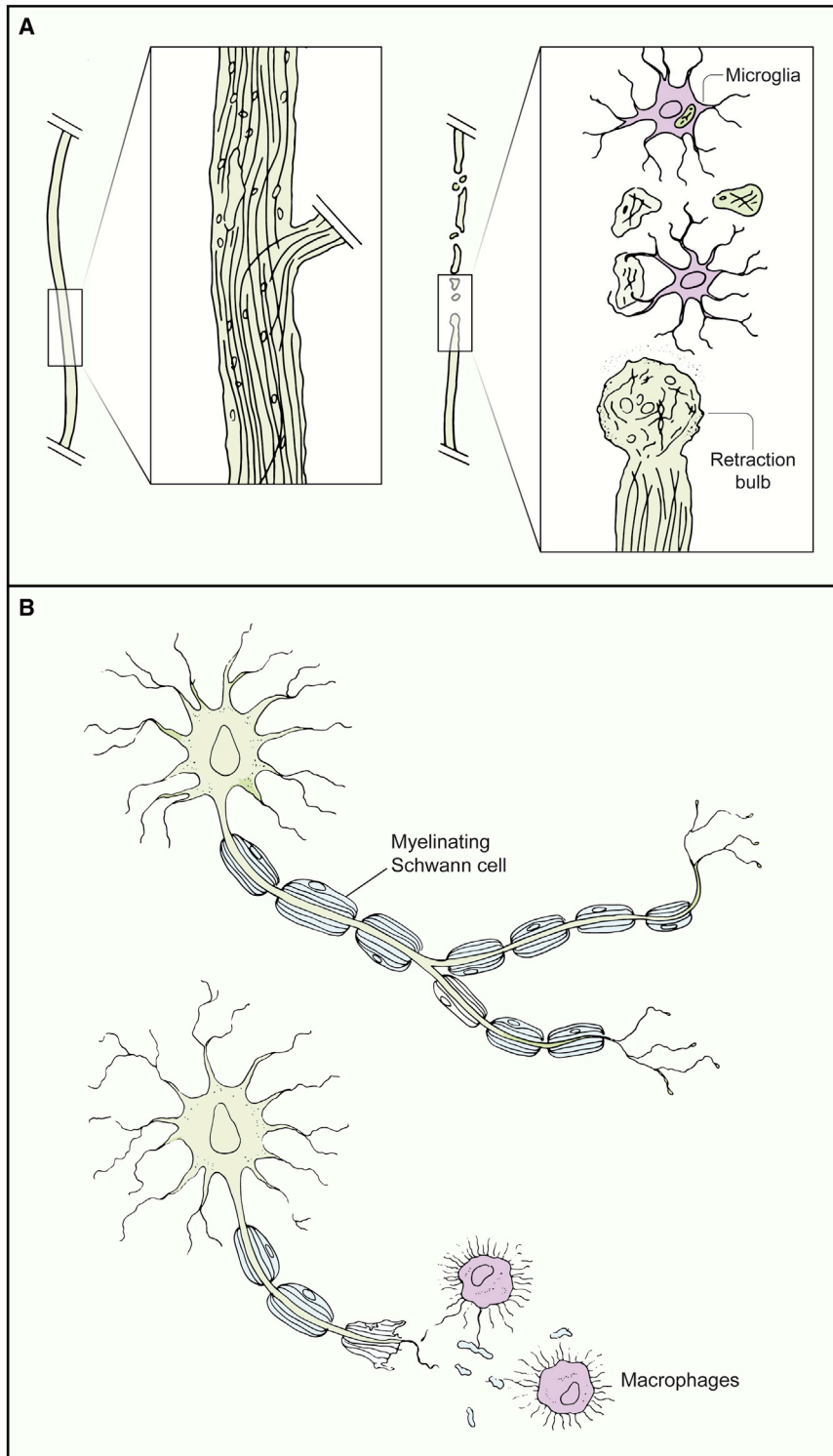
sulting in partial recovery of visual function. Intraocular injections of zymosan and a cAMP analog elicits an inflammatory response in RGCs that, when combined with mTOR upregulation, leads to long-distance axon regeneration into retinorecipient centers (de Lima et al., 2012). Though it is unclear whether target-specific regeneration occurs following this treatment, the partial recovery of visual behaviors, including the optomotor response and circadian photoentrainment, suggests this is the case.

Another strategy to promote long-distance RGC axon regeneration involves combining mTOR activation with biased visual stimulation to stimulate retinal activity only in the injured eye. A small subset of regenerating RGC axons navigate through the optic chiasm and reinnervate retinorecipient centers following this approach (Lim et al., 2016). The mechanistic basis at play here is unknown, yet during development, neural activity is known to promote RGC survival and axon outgrowth (Goldberg et al., 2002)—processes that may involve activity-dependent delivery of Tropomyosin receptor kinase B (TrkB) and other trophic receptors to the RGC cell surface (Meyer-Franke et al., 1998). To address the issue of target-specific reinnervation, the Cochlin-GFP (CoCH-GFP) mouse line, in which many labeled RGCs are  $\alpha$ -RGCs, was used (Lim et al., 2016). Regenerating CoCH-GFP-positive RGC axons re-extended into some of their normal developmental targets, including the ventral lateral geniculate nucleus and superior colliculus (SC), but avoided others, including the hypothalamic supra-chiasmatic nucleus and brainstem medial terminal nucleus.

However, aside from a few rare instances of long-distance axon regeneration, most studies have failed to achieve the degree of axon regrowth following ONC required to assess whether regenerated axons can indeed form functional synapses in retinorecipient targets. In order to address this issue, implementation of a distal injury model in which the optic tract is severed immediately proximal to the SC has been employed (Bei et al., 2016). Co-deletion of PTEN and SOCS3 leads to target reinnervation and the formation of functional synapses following optic nerve lesion, but not restoration of visual behavior. The regenerated axons in this injury model are demyelinated, however, voltage-gated potassium channel blockers can enhance conductivity, ultimately leading to recovery of visual function. These experiments provide hope that, given the proper stimulation paradigm, regrowing axons can properly reinnervate their developmental targets and restore function following CNS injury.

### AXON DEGENERATION

The sequelae of events leading to axon degeneration are well known. Axon degeneration is a multi-stage process that occurs following a traumatic injury to the nervous system. An injury can present in the form of a contusion and does not have to breach the skull or spinal cord to cause degeneration. Degeneration can also originate from neurons themselves without any overt physical insult, as in amyotrophic lateral sclerosis, Alzheimer's, or Parkinson's disease, wherein genetic predispositions, age-related triggers, or environmental stressors can affect neuronal survival and cause progressive degeneration and dysfunction of neuronal circuits (Hou et al., 2019). Regardless of the initial cause, a key issue is whether the degeneration occurs in the CNS or in the PNS, since these two divisions of the nervous



**Figure 4. Axon degeneration and immune response to injury**

(A) Degenerative mechanisms following injury: an intact axon before injury (left) and an injured axon undergoing WD (right) distal to the lesion site. Boxed regions of the intact and injured axons are shown magnified: retraction bulb sealing the axolemma at the proximal end of an injured axon and microglia (pink) clearing axonal debris.

(B) Injury in the PNS: Schwann cells in the PNS myelinate regenerating axons (top), and macrophages phagocytose axonal debris (bottom, pink).

disintegrates in a fragmented manner by a process called Wallerian degeneration (WD) (Figure 4A). Disconnection from target cells and the inability of the parent neuron to receive target-derived trophic support may cause the proximal stump of the axon to degenerate further, although not as dramatically as the distal axon. Axon degeneration, therefore, proceeds in both directions from the injury site, with distal axons completely disappearing over time while the proximal axons “die back” and may then begin to regenerate. Despite recent progress in achieving long-distance axon regrowth following CNS injury, no pro-regenerative strategies have completely restored neural circuit function. One possible impediment to functional regeneration that remains to be investigated is whether the delayed clearing of degenerating neuronal elements further curbs the growth of regenerating axons.

The degeneration process has a deleterious component—the damage and loss of neurons—but also an adaptive component—the clearance of debris from the site of injury. Indeed, the latter is a distinguishing factor that enhances the regenerative capacity of PNS neurons and circuits (Vargas and Barres, 2007). Conversely, lack of clearance of injury-induced debris, in particular, the persistence of myelin, is thought to be a major contributor to the lack of regeneration observed in the mature CNS.

#### Underlying molecular pathways

To achieve robust CNS regenerative outcomes, it is imperative to not only understand the events that unfold after an injury

system diverge sharply in their capacity to support regeneration and replenish damaged neurons.

When axons are crushed or severed, the portion of the axon that is distal to the injury, and therefore disconnected from the soma,

but to also take into account how degeneration impacts the regeneration of surviving neurons. We consider here observations that provide insight into the underlying signaling pathways that mediate degeneration.

A genome-wide screen using small interference RNAs identified DLK as an important regulator of cell death (Watkins et al., 2013; Welsbie et al., 2013, 2017). DLK, a mitogen-activated protein kinase present in both the CNS and PNS, regulates apoptosis, axon growth, and degeneration during development with a conserved function in many organisms including *C. elegans* and *Drosophila* (He and Jin, 2016). DLK activates c-Jun N-terminal kinase (JNK) signaling, and it induces both apoptotic and regeneration-associated gene expression (Watkins et al., 2013; Welsbie et al., 2013). In the PNS, mTOR and DLK are required to transport phosphorylated-STAT3 mediated injury signals from peripheral axons to the cell body to initiate pro-regenerative pathways, and they are robustly upregulated in peripheral axons following conditioning lesions (Shin et al., 2012; Terenzio et al., 2018). Similarly, in the CNS, DLK protein levels first increase in axons and later in cell bodies following ONC (Watkins et al., 2013; Welsbie et al., 2013). Remarkably, DLK loss-of-function in an ONC model promotes RGC survival but results in very little axon regeneration. These findings suggest DLK is a signal conveyed by the injured axon to its soma that can ultimately mediate cell death in injured neurons.

### Calcium

One of the main events leading to degeneration is the influx of extracellular  $\text{Ca}^{2+}$  to the axon through injury-induced plasma membrane holes called mechanopores (Williams et al., 2014). Elevated intra-axonal  $\text{Ca}^{2+}$  initiates degenerative mechanisms, including calpain activation and autophagy, that break down cytoskeletal components in the injured axon (Knöferle et al., 2010; Ma, 2013). However, not all injured axons undergo these events, since the severity of axonal injury likely influences the degree to which axons are able to evade  $\text{Ca}^{2+}$ -dependent degeneration. For example, a small percentage of axons recover from a spinal cord contusion through spontaneous closure of mechanopores and restoration of pre-injury intra-axonal calcium levels (Williams et al., 2014).

To overcome the deleterious effects of increased intracellular calcium following severe axonal injury, various strategies have been employed that block autophagy or inhibit calcium signaling using calcium-channel blockers or chelating agents (Knöferle et al., 2010; Ribas et al., 2017). Calpain inhibitors can also limit axon degeneration, but they appear to preferentially spare neurofilaments over microtubules (Park et al., 2013). Intriguingly, a recent investigation of the role of  $\text{Ca}^{2+}$  signaling in RGC survival showed that reactivation of Calmodulin-dependent protein kinase II (CaMKII), a key regulator of  $\text{Ca}^{2+}$  homeostasis, provides neuroprotective effects on RGC survival and promotes RGC axon regeneration following injury (Guo et al., 2021). Further, CaMKII reactivation acts as a positive regulator of RGC survival and axon regeneration in models of excitotoxicity and glaucoma, slowing disease progression. Therefore, increased  $\text{Ca}^{2+}$  levels, and possibly a dysregulation of calcium homeostasis following injury, negatively affect cell survival. Regardless of the intervention, efforts to restore intracellular calcium levels to basal conditions after injury may preserve distal axon integrity and increase the likelihood of functional recovery.

### Acute axon degeneration

There are at least two distinct phases during which axon degeneration occurs: an initial acute axon degeneration (AAD) and subsequent WD phase. As the name suggests, AAD is the response that takes place within the first few minutes following an injury. Though the time kinetics may vary among different systems, resulting in distinct phases of degeneration, AAD in general results in a rapid degeneration of both the proximal and distal stumps of the axon over a few hundred microns. For example, following a spinal cord lesion in which labeled central projections of DRG axons are selectively transected and then imaged *in vivo*, AAD starts within the first 24 h and lasts less than 5 min, causing both proximal and distal stumps to retract ~300–400 microns (Kerschensteiner et al., 2005).

In an ONC model, AAD initiates a similar retraction of the proximal stump by ~400 microns; however, in this scenario, AAD does not occur as a distinct phase and is instead interspersed with the formation of retraction bulbs, perhaps due to the structural simplicity of the optic nerve (Knöferle et al., 2010). Regardless, considering the range over which AAD affects injured axons, the therapeutic potential of addressing the molecular mechanisms underlying AAD may prove to be limited in promoting long-distance regeneration. What, then, might be the purpose of an immediate rapid degenerative response as compared to the slower WD response? The proximal axon degeneration that is characteristic of AAD is thought to be a necessary step for subsequent initiation of glia-mediated events surrounding the injury. However, experiments that specifically inhibit this initial response while preserving WD are necessary to fully understand the functional implications of AAD.

Following AAD, retraction bulbs form on the axon stumps (Figure 4A). Both the proximal and distal stumps of injured axons close their axolemma to contain the anterograde and retrograde transport of proteins and other molecules. During this phase, retraction bulbs form at the ends of CNS axons within the first 30 min after ONC or within 30 h after SCI. Retraction bulbs remain more or less stable unless the axon establishes contact with a macrophage, which triggers phagocytosis within an hour (Evans et al., 2014).

### Wallerian degeneration

The WD phase involves the well-studied, evolutionarily conserved cell-autonomous program that neurons undergo following an injury or insult and has been best investigated in the context of the sciatic nerve and DRG neurons in the PNS (Vargas and Barres, 2007). It is generally thought that all CNS neurons undergo Wallerian-type degeneration after injury, although that has not been systematically explored. During WD, distal segments of injured axons form connected beadlike swellings that gradually separate and then, more rapidly, fragment and disappear. Notably, classical work showed that a spontaneous autosomal dominant mutation in the *Wld<sup>s</sup>* gene conferred neuroprotective effects and a delay in the degradation of the injured PNS axon (Perry et al., 1990). This mutation results in the mobilization of the enzyme nicotinamide mononucleotide adenylyltransferase 1 (NMNAT1) from the nucleus to the axoplasm, leading to an increase in nicotinamide adenine dinucleotide ( $\text{NAD}^+$ ) levels (Sasaki et al., 2016; Wang et al., 2015).

Importantly, a local increase of NAD<sup>+</sup> in cultured embryonic DRG neurons within 4 h after injury is sufficient to protect injured axons, similar to what is provided by the *Wld<sup>s</sup>* mutation (Wang et al., 2015). These results point toward a promising therapeutic window during which axonal degeneration might be prevented (Wang et al., 2015).

Similar to *Wld<sup>s</sup>*, the NAD<sup>+</sup>-cleaving enzyme sterile alpha and TIR motif containing 1 (Sarm1) regulates axon degeneration (Gerdt et al., 2015; Osterloh et al., 2012). Axon injury causes NMNAT2 degradation and an increase in axonal nicotinamide mononucleotide (NMN) levels (Gilley et al., 2017). Normally, both NMN and NAD<sup>+</sup> compete for binding to the ARM domain of Sarm1. However, increased NMN levels and subsequent binding to Sarm1 induces a conformational change that activates the NADase activity of Sarm1, initiating an axon destruction program; therefore, Sarm1 is a major regulator of axonal degeneration and is an attractive candidate for strategies designed to promote axon regeneration (Figley et al., 2021; Gilley et al., 2017; Tian et al., 2020).

Although WD was initially described as a self-destruction program that axons undergo upon trophic deprivation (TD) or injury, recent *in vitro* studies in embryonic sensory neurons support the idea that WD is an active process regulated by the soma (Vargas and Barres, 2007). Normally, the neurotrophin nerve growth factor (NGF) binds to its receptor TrkA at the distal end of the axon and initiates several signaling events, including Akt signaling. However, TD results in the phosphorylation of Akt, subsequently activating JNK/DLK and leading to a cascade of signaling events that ultimately engages Puma, a pro-apoptotic protein and a key regulator of degeneration. Upon TD, transcriptional regulation of Puma in the cell body sends pro-apoptotic caspase-dependent signals back to the distal axon (Simon et al., 2016). Interestingly, further investigation shows that the anterograde signaling that activates axonal-caspase is also mediated by the tumor suppressor p53 and is likely distinct from the somatic and axonal caspase activation induced by Puma (Simon et al., 2021). A screen for cleavage targets enriched by TD identified the neuronally enriched protein RUN-and-FYVE-domain-containing protein 3 (Rufy3), which is cleaved downstream of caspase3 and is required for WD, independent of Puma (Hertz et al., 2019). These findings highlight the presence of a quiescent apoptotic machinery that is already present in the axon, one that is readily activated by the cell body upon TD. Future work examining these signaling pathways in the CNS of adults may prove valuable in understanding the mechanistic differences that determine how cells in the CNS versus PNS respond to degeneration in their respective environments.

Trophic factor deprivation-induced cell death can also occur at the dendrites of a neuron. Reduced responsiveness of RGCs to insulin-like growth factor-1 (IGF-1) leads to activation of the Akt pathway, followed by a TD response (Ambacher et al., 2012). IGF-1 receptors normally accumulate in RGC primary cilia; however, they are dramatically reduced within a week of ONC as a result of strong inhibition from amacrine cell hyperactivity (Zhang et al., 2019). Remarkably, overexpression of Lin28 in amacrine cells robustly promotes RGC cell survival and axon regeneration, which is further potentiated by overexpression of IGF-1 (Zhang et al., 2019). Similarly, exogenously

supplied IGF-1 promotes survival of corticospinal neurons in a proximal injury model (Hollis et al., 2009). Thus, in addition to the role of trophic factors in injury-induced degeneration, trophic factor responsiveness by the cell body and axon may underlie diverse regenerative responses in different neuronal subpopulations.

In light of the crosstalk between axonal and somal signaling pathways in response to an injury, it is understandable that there is overlap among apoptotic signaling pathways and those responsible for axonal degeneration. Various kinases, including DLK, JNK, and MAPK, have been implicated in the regulation of cell death and axon degeneration, albeit to varying degrees in each context (Shin et al., 2012; Watkins et al., 2013). It is critical to identify downstream effectors of these signaling pathways and to determine how they differentially trigger degeneration in the soma and the axon. For example, deletion of Sarm1 does not impede activation of DLK/JNK in RGC somas after ONC and hence fails to prevent RGC death, even though it reduces axon degeneration (Fernandes et al., 2018). With a better understanding of the downstream signaling, these overlapping pathways may offer opportunities to simultaneously alleviate cell death and slow axon degeneration, thus providing attractive therapeutic strategies.

### Immune mechanisms of axon degeneration

In the weeks following injury, the immune system is tasked with the removal of damaged axons (Figure 4). Following injury in the PNS, severed axons and dying cells are phagocytosed, myelin debris is cleared, and macrophages are swiftly recruited for further containment of the lesion (Figure 4B) (Vargas and Barres, 2007). However, in the CNS, this relatively simple process becomes maladaptive, leading to an exaggerated immune response that inhibits axon re-extension. The earliest response to an injury is initiated by glial subtypes, including microglia and reactive astrocytes in the CNS and by Schwann cells in the PNS (Beck et al., 2010; Greenhalgh et al., 2020). Interactions between immune cells and glia can exert a significant influence on the regenerative capacity of the nervous system and must be considered carefully when identifying new therapeutic approaches (reviewed in Greenhalgh et al., 2020).

Studies on microglia offer further insight into the role of glia and the immune system in the injured environment since microglia can be classified as both immune cells and as a glial cell type (Greenhalgh et al., 2020). Microglia are an important component of the glial scar that forms after an injury, as they proliferate and populate the overall lesion site. This component of the glial scar forms within 7 days of an injury and is sandwiched between the fibrotic and astrocytic scars (Beck et al., 2010; Bellver-Landete et al., 2019). Depleting microglia after SCI reduces IGF-1 expression, leading to a disorganized astrocytic scar and impaired locomotor recovery; this demonstrates an important pro-regenerative role for microglia in inducing the astrocytic response via IGF-1 (Bellver-Landete et al., 2019). Microglia also exert an anti-regenerative influence by inducing the production of reactive astrocytes via cytokine secretion, including interleukin 1 $\alpha$ , tumor necrosis factor, and the complement component 1. Together, these three cytokines secreted by microglia induce one of two types of reactive astrocytes that are cytotoxic and

are responsible for killing RGCs in an ONC or glaucoma model. Blocking all three cytokines prevents the formation of cytotoxic astrocytes and increases RGC survival, highlighting axotomy-induced cytokine secretion as the cause of RGC death (Liddelow et al., 2017). Interestingly, injury or cytokine toxins alone are insufficient to cause RGC death. A triple KO mouse line that prevents astrocytes from becoming activated preserves RGCs after injury, while the toxins injected into a healthy adult mouse do not affect RGC viability (Guttenplan et al., 2020).

One of the major tasks for the immune system after a nerve injury is clearance of injury-induced debris, including degenerating myelin sheaths and fragmented axons (Figure 4B). Unlike the CNS, in which axonal and myelin debris are cleared over a prolonged period of ~90 days or longer, WD in the PNS adopts a faster time frame with debris clearance completed by ~30 days, partly due to rapid recruitment of macrophages (George and Griffin, 1994). Further, within the first few days post-injury in the PNS, Schwann cells reprogram into repair cells that clear myelin debris (Vargas et al., 2010). A compression injury or nerve transection to the PNS triggers a rapid and well-orchestrated immune response, comprised of macrophages and blood-borne leukocytes in the injured region. Neutrophils also enter the injured nerve within hours, followed by monocytes that subsequently differentiate into macrophages. In addition to fiber debris removal, macrophages phagocytose apoptotic cell bodies in the injured nerve and thereby contribute to inflammation resolution (Kalinski et al., 2020). Repair Schwann cells and macrophages employ different mechanisms for myelin phagocytosis. The engulfment receptors MerTK and Axl are key players in repair Schwann cells (Brosius Lutz et al., 2017), while antibody-dependent opsonization of myelin debris is a primary mechanism for Fc receptor-mediated phagocytosis by macrophages (Kuhlmann et al., 2002).

### What are the consequences of preventing degeneration?

While we do not have a clear answer to this question, it is important to consider the negative consequences of delaying or preventing degeneration following injury. The pathological changes that take place following spinal cord injury in *Wld<sup>s</sup>* mice reveal that, apart from a slower degenerative response, scar formation is also considerably slower in these mutant mice (Zhang et al., 1996). Delayed scar formation in the *Wld<sup>s</sup>* mice also results in delayed disappearance of the lesion site and leads to incomplete healing. More recently, it was shown that delayed degeneration in *Wld<sup>s</sup>* mice results in a subsequent delay in collateral sprouting from spared axons, leading to delayed functional recovery (Collyer et al., 2014). Therefore, degeneration and the inflammatory responses to injury may be as important as the regenerative mechanisms critical for mediating functional nervous system repair.

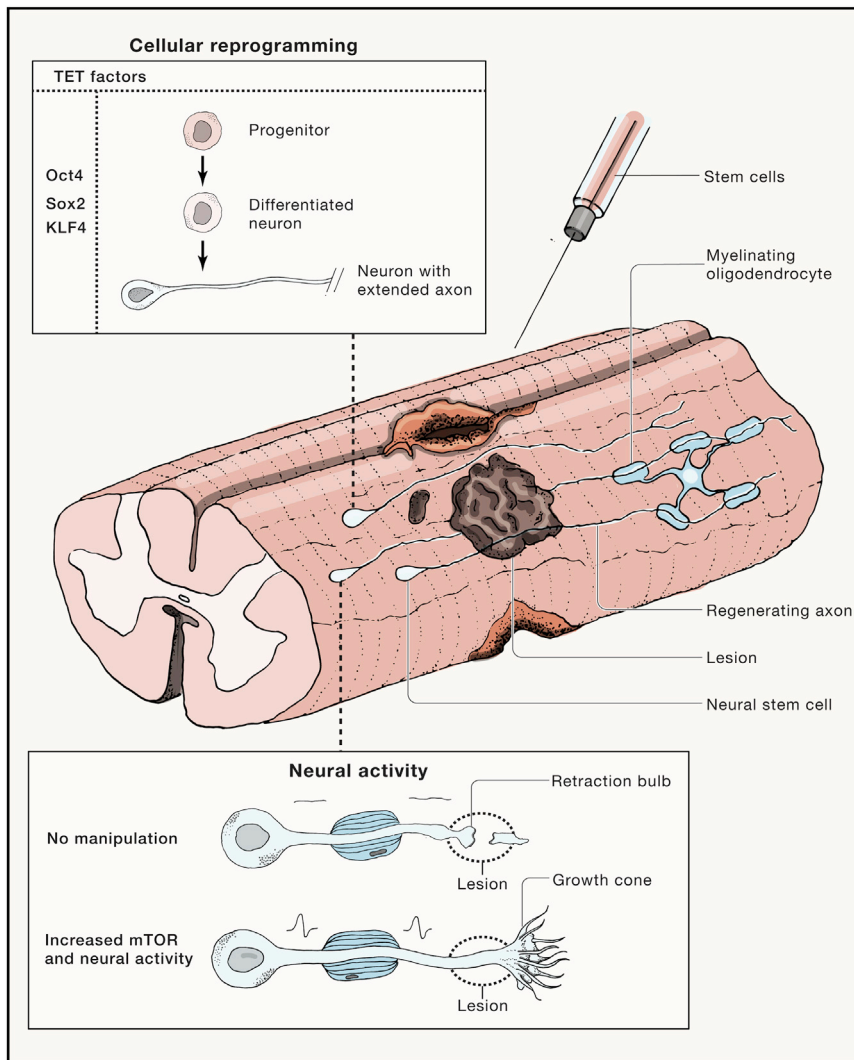
In the PNS, delayed WD significantly affects the recruitment of macrophages, regeneration of sensory and motor axons as assessed by reinnervation of muscle targets, and the restoration of action potentials (Bisby and Chen, 1990; Brown et al., 1991). Interestingly, the regenerative ability of motor axons in *Wld<sup>s</sup>* mice was comparable to that observed in control mice, albeit with smaller action potentials, while sensory axons suffered in their ability to reinnervate muscles, an effect attributed to the

intact injury environment. Degeneration, therefore, is necessary to clear the distal stumps that can otherwise prevent regenerating axons from navigating past the lesion site and reinnervating targets. This highlights the need for further study to assess whether clearance of debris accelerates overall regeneration repair mechanisms and whether promoting rapid clearing of degenerating axons should be included in strategies designed to enhance axon regeneration.

### CONCLUSION

Cell death and clearance of neuronal debris, decreased intrinsic growth ability, and the presence of inhibitory factors all contribute to CNS regenerative failure following trauma. We have considered recent progress in efforts to stimulate axon regeneration which, if leveraged with approaches that accelerate distal axon regeneration, represent rational strategies for restoring CNS function following injury. Though some studies have demonstrated long-distance axon regeneration following ONC (Lim et al., 2016; de Lima et al., 2012), the manipulations required were multifaceted, and only a very small number of axons reached central targets. Nevertheless, these results suggest that, given the proper treatment, regrowing axons can navigate long distances and reinnervate target regions.

Although many of the strategies for promoting neuronal regeneration described thus far facilitate some degree of regeneration and even functional recovery, the prophylactic nature of many approaches makes it difficult to extrapolate to the clinic. Nevertheless, a few promising studies demonstrate that provision of extrinsic and intrinsic factors after SCI promotes axon regeneration (Kadoya et al., 2009; Nathan et al., 2020). A combinatorial treatment administered up to 15 months post-spinal cord injury consisting of a conditioning lesion, a syngenic bone marrow stromal cell graft, and delivery of an NT-3 gradient is able to sustain axon regrowth through and past the lesion site (Kadoya et al., 2009). Additionally, overexpression of Lin28a within 5 days of either spinal cord injury or ONC supports robust axon regeneration of lesioned axons (Nathan et al., 2020). These positive outcomes serve as models for future work directed toward determining tractable approaches for stimulating robust axon regeneration in humans. Overall, combinatorial approaches such as increasing mTOR and neural activity (Lim et al., 2016), increasing growth factor signaling and improving conduction with potassium channel blockers (Bei et al., 2016), transplanting neurons or reprogramming cells to differentiate into neurons (Lu et al., 2020; Venugopalan et al., 2016), and many other approaches have led to significant advances in addressing neuronal survival, axon re-extension, and myelination following injury. Future work investigating mechanisms to promote dendritic regeneration, synapse formation, and experience-dependent plasticity of re-formed neural circuits will greatly increase our understanding of how to achieve meaningful functional regeneration. Additionally, though each approach uncovers new means to promote survival and regeneration, identifying the best, yet most tractable, combination of approaches to fully repair neural circuits is crucial, as is consideration of expanding the range of model organisms and anatomical pathways for testing regeneration-stimulating candidates.



**Figure 5. Clinically relevant therapeutic strategies**

The most promising therapeutic strategies to promote re-connectivity of neural circuits are illustrated.

Stem cells can be utilized to promote functional recovery following injury by neural stem cell grafts or by overexpressing TET factors (Oct4, Sox2, and KLF4) (Kumamaru et al., 2019; Lu et al., 2020) to reprogram cells to a development-like state that encourages axon regrowth. Upregulation of mTOR signaling and neuronal activity can promote axon regeneration past the lesion site (Lim et al., 2016), while voltage-gated potassium channel blockers can be used to promote myelin reformation in regenerating axons (Bei et al., 2016).

When considering approaches that might hold the most therapeutic potential for human treatment, we must remember that many of the strategies described in this review are limited by the possibility of oncogenic effects brought on by upregulating pro-growth pathways normally active early in development. For this reason, many of these studies serve as models for understanding mechanisms of axon regrowth and target reinnervation following injury, with the goal of transitioning these observations into safe regeneration-promoting strategies. For example, it is possible that combining a non-oncogenic, axon-growth stimulating approach such as zinc chelation with a strategy to promote rapid clearance of degenerating axons might yield a desirable clinical outcome while decreasing the risk of deleterious side effects (Li et al., 2017b).

Other strategies aimed at restoring function following CNS injury also deserve consideration. Grafts of neural stem cells and neural progenitor cells have shown promising results, supporting regeneration and promoting functional recovery after

SCI (Figure 5) (Cummings et al., 2005; Kadoya et al., 2016; Kumamaru et al., 2019). Transplanted RGCs that integrate and extend axons to visual targets in the brains of uninjured mice also provide a promising direction for replacing degenerating cells, thereby promoting RGC axon regeneration following injury (Venugopalan et al., 2016). These advances, along with the extensive work that demonstrates robust axon regeneration in a wide range of model systems, provide hope that restoration of CNS function following injury will be a reality for human patients in the near future.

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## DECLARATION OF INTERESTS

The authors declare no competing interests.

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