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REVIEW

Can injured adult CNS axons regenerate by recapitulating development?

Brett J. Hilton* and Frank Bradke*

ABSTRACT

In the adult mammalian central nervous system (CNS), neurons typically fail to regenerate their axons after injury. During development, by contrast, neurons extend axons effectively. A variety of intracellular mechanisms mediate this difference, including changes in gene expression, the ability to form a growth cone, differences in mitochondrial function/axonal transport and the efficacy of synaptic transmission. In turn, these intracellular processes are linked to extracellular differences between the developing and adult CNS. During development, the extracellular environment directs axon growth and circuit formation. In adulthood, by contrast, extracellular factors, such as myelin and the extracellular matrix, restrict axon growth. Here, we discuss whether the reactivation of developmental processes can elicit axon regeneration in the injured CNS.

KEY WORDS: Axon regeneration, Central nervous system, Development, Spinal cord, Spinal cord injury

Introduction

Neurons transmit signals to their targets through specialized cellular processes called axons. These processes extend to other neurons, muscles, sensory organs and glands, conducting electrical impulses across long distances. The axons of most neurons grow towards their targets during embryonic development. The neuron differentiates and elaborates processes. It specifies one axon and several dendrites, which are shorter signal-receiving processes (Witte and Bradke, 2008). The axon elongates towards its target by sensing guidance molecules (O'Donnell et al., 2009). After reaching its target, the axon forms a specialized connection there called a synapse (Shen and Scheiffele, 2010). The coordinated growth and connectivity of axons during development is an impressive feat. In mammals, billions or trillions of synapses are formed between neurons and targets that are often long distances away from each other (Kasthuri et al., 2015; Sporns et al., 2005). As such, developmental axon growth is an intricate process. It relies on an axonal environment that enables directed elongation (Seiradake et al., 2016) and on a variety of intracellular mechanisms that allow the developing neuron to grow its axon long and rapidly (O'Donnell et al., 2009).

In contrast to the situation in development, and except in a few unusual cases (Fenrich and Rose, 2009; Jin et al., 2016; Omura et al., 2015), the axon of an adult mammalian neuron cannot grow back to its target in the central nervous system (CNS) following injury; the membrane of the axon reseals to re-establish ionic homeostasis (Bradke et al., 2012) but axon growth is typically limited. Most adult neurons fail to express the genes that orchestrate developmental axon elongation and instead express genes that restrict growth (He and Jin, 2016). Most adult neurons may also lack the energy necessary for axon regeneration (Zhou et al., 2016). The environmental cues that attract the axon to its target during development are not appropriately expressed in the adult system (Giger et al., 2010). Instead, the adult environment responds to injury by expressing factors that impede growth (Cregg et al., 2014; Schwab and Strittmatter, 2014). In some cases, such as in the descending corticospinal and rubrospinal systems, and in the primary sensory neurons that ascend to the brain in the direct dorsal column pathway, adult neurons survive axonal injury but remain disconnected from their targets (Kwon et al., 2002; Nielson et al., 2010; Ylera et al., 2009). In other cases, such as in most subtypes of retinal ganglion cells (RGCs), injured neurons respond to axon severance (axotomy) by eventually dying (Duan et al., 2015). Axon regeneration failure can permanently disrupt CNS connectivity and can lead to substantial dysfunction in cases of trauma, stroke or neurodegenerative disease.

A tantalizing strategy for enhancing axon regeneration is to recapitulate the processes that underlie developmental axon growth within adult neurons. By examining the processes that mediate axon outgrowth during development, it may be possible to reactivate the developmental growth program within the adult neuron and to unleash robust axon regeneration. Indeed, many experimental therapeutic strategies to enhance CNS regeneration that manipulate the extracellular environment or intrinsic growth capacity of a neuron converge on processes underlying developmental axon growth (He and Jin, 2016; Schwab and Strittmatter, 2014). Still, not every process that mediates axon regeneration is active during embryonic development, and there are clear differences between these forms of axonal elongation.

In this Review, we highlight the cellular and molecular mechanisms that orchestrate axon growth during development in various mammalian species. We then examine how these are impeded or fail to be activated following adult injury. We describe divergences between developmental and regenerative axon growth, and discuss recent work showing that it is possible to promote regeneration by reactivating developmental growth processes in adult neurons following CNS injury. Ultimately, a further understanding of how axons extend and form functional circuits during development may allow us to recapitulate these processes in adult neurons and to restore connectivity after disease or injury.

Axon regenerative capacity in the mammalian CNS: a developmental decline

Although axon regeneration is limited in the adult mammalian CNS, this is not the case when the CNS is immature. For example, opossums (such as *Monodelphis domestica*) are born with an

Laboratory for Axon Growth and Regeneration, German Centre for Neurodegenerative Diseases (DZNE), Sigmund-Freud-Strasse 27, 53127, Bonn, Germany.

*Author for correspondence (brett.hilton@dzne.de; frank.bradke@dzne.de)

B.J.H., 0000-0003-2813-2294

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immature CNS, including a two-layer cortex and a rudimentary cerebellum (Kraus and Fadem, 1987). Axons injured early after birth regenerate and form functional circuits (Saunders et al., 1995). However, the CNS restricts axon growth after a developmental transition. For example, retinal ganglion cells (RGCs) in the opossum regenerate if their axons are injured before postnatal day 12 but not afterwards (MacLaren and Taylor, 1997). Although the timeframe varies among organisms and pathways, this transition is virtually ubiquitous in mammals (Nicholls and Saunders, 1996). It has been referred to as a critical period of CNS axon regeneration, and understanding its mechanistic basis has been a focus of regeneration research for many decades.

Classically, it has been thought that extracellular molecules expressed in adulthood after injury but not during development underlie this transition. Oligodendrocytes start myelinating axons at around the same time as the transition and their myelin inhibits axon growth (Schwab and Strittmatter, 2014). Similarly, astrocytes respond to developmental and adult lesions differently (Cregg et al., 2014). However, when neural progenitor cells (NPCs) are transplanted into the injured adult mammalian spinal cord and provided with trophic factors and a fibrin matrix, they differentiate into neurons with a virtually limitless capacity for axon growth (Lu et al., 2012). These results demonstrate that, when properly stimulated and in the right environment, newly differentiated neurons can grow axons that are very long after adult injury. There are many mechanisms that contribute to the growth capacity of immature neurons. In the first part of this Review, we focus on intracellular mechanisms underlying this higher growth capacity (Fig. 1). In the second part, we discuss how the extracellular environment changes as the CNS matures in a way that contributes to regeneration failure. Importantly, intracellular and extracellular factors are not separate: there is a complex interplay between them, as the intrinsic growth capacity of a neuron is at least partially instructed by extracellular interactions (Burnside and Bradbury, 2014; Geoffroy and Zheng, 2014; Tedeschi and Bradke, 2017).

The developing and adult CNS: intracellular factors involved in regeneration

Gene expression

The difference in gene expression between immature and mature mammalian neurons is a primary factor that underlies their differential capacity for axon growth. The capacity of a neuron to regenerate is defined in part by its expression of regeneration-associated genes (Tetzlaff et al., 1991). The expression of these genes is activated in developing neurons and is restricted in adult neurons (Puttagunta et al., 2014; Tedeschi and Bradke, 2017). For example, developing mammalian neurons express Krüppel-like factor 7 (KLF7) and Sox11, but downregulate these transcription factors as they mature (Blackmore et al., 2012; Moore et al., 2009; Wang et al., 2015). KLF7 or Sox11 activation in the adult neuron promotes axon regeneration (Blackmore et al., 2012; Wang et al., 2015). Similarly, the α9 integrin receptor is downregulated as primary sensory neurons mature, and the overexpression of α9 promotes axon regeneration (Andrews et al., 2009). In most cases, transcription factors have been tested singularly for roles in regeneration. However, bioinformatic approaches have recently determined functional interactions between pathways involved in axonal outgrowth and may reveal transcription factor combinations that can optimize regeneration (Belin et al., 2015; Chandran et al., 2016; Venkatesh and Blackmore, 2016).

Instead of expressing regeneration-associated genes, mammalian adult neurons express genes that restrict axon growth. In a screen of genes differentially expressed by RGCs during the developmental transition to a restricted capacity to regenerate, Moore and colleagues identified KLF4 as a transcriptional repressor of regeneration (Moore et al., 2009). RGCs do not express KLF4 during development but upregulate it upon maturation, when it restricts growth. Why adult neurons express genes that prohibit regeneration is unclear. One possibility is that the expression of these genes is an evolutionarily conserved mechanism to prevent ectopic axon growth and aberrant synapse formation (Tedeschi and Bradke, 2017). Indeed, synaptogenesis instructs the expression of genes that prevent axon outgrowth (Tedeschi et al., 2016), as we discuss later.

At the translation level, developing neurons may be able to more effectively generate proteins involved in axonal elongation than adult neurons (Shigeoka et al., 2016), and signalling via the mechanistic target of rapamycin (mTOR) pathway may be involved in this difference (Park et al., 2008). Developing neurons have high levels of mTOR signalling but this decreases as they mature (Liu et al., 2010; Park et al., 2008). Enhancing mTOR signalling by knocking out phosphatase and tensin homolog (*Pten*) boosts axon regeneration (Geoffroy et al., 2016; Liu et al., 2010; Park et al., 2008). The mTOR pathway is pivotal for regeneration following *Pten* knockout (Park et al., 2008). Although mTOR regulates many intracellular events (Laplante and Sabatini, 2012), its activation of protein translation is particularly important for regeneration (Yang et al., 2014). To stimulate translation, mTOR activates S6 kinase 1 (S6K1) to generate new ribosomes (Chauvin et al., 2014), and S6K1 activation enhances axon regeneration in the mouse optic nerve (Yang et al., 2014). In addition to S6K1, mTOR activates eukaryotic initiation factor 4E (eIF4E) to initiate cap-dependent protein translation (Brunn et al., 1997). Accordingly, it has been shown that EIF4E is necessary for regeneration following *Pten* knockout in adult mice (Yang et al., 2014). Together, these results support a model in which developing neurons signal through mTOR to translate proteins more effectively than adult neurons do, and suggest that enhancing mTOR-mediated protein translation in adulthood can boost axon regeneration. However, it has been reported that S6K1 restricts mouse corticospinal axon regeneration (Al-Ali et al., 2017). These results highlight the complexity of mTOR signalling in regeneration and suggest that more research is needed to understand how different effectors of the PI3K/Akt/mTOR pathway influence axon growth.

Growth cone formation

Developmental axon growth relies on the formation of a subcellular structure at the distal tip of the axon called the growth cone (Fig. 2A). The growth cone comprises an actin-rich peripheral domain and a microtubule-rich central domain, separated by a transition zone in which dynamic interactions between these cytoskeletal elements take place (Coles and Bradke, 2015). It serves vital functions both in the guidance of the axon to its target and in the process of axonal outgrowth itself (Dent et al., 2011). At its distal tips, finger-like filopodia and sheet-like lamellipodia rapidly extend and retract as they sense the axonal microenvironment. During outgrowth, filamentous actin (F-actin) polymerizes, driving the extension of new membrane at the distal edges of the growth cone (Goldberg and Burmeister, 1986). The proximal growth cone then stabilizes into the axonal shaft, elongating the axon (Dent et al., 2011; Goldberg and Burmeister, 1986). After synaptogenesis, axon growth continues to occur, corresponding to the size of the growing organism, but it does so via stretch-based mechanisms (Smith, 2009).

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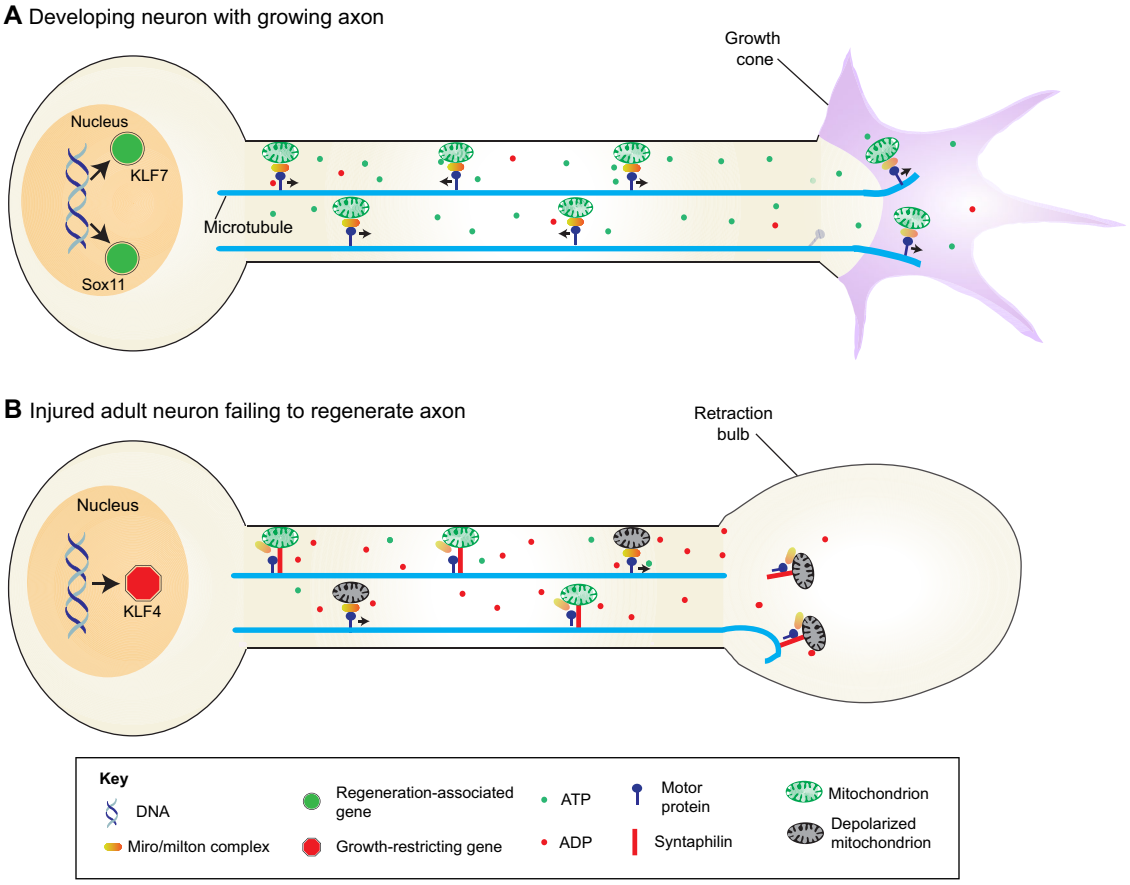

Fig. 1. Intracellular processes in a developing neuron and its growing axon, and in an adult neuron failing to regenerate. (A) A developing neuron with a growing axon. Developing neurons express regeneration-associated genes, such as *Klf7* and *Sox11*, that facilitate axon growth. Mitochondria are highly motile in the axon, are associated with Miro/milton complexes and are transported by motor proteins (kinesin and dynein) along microtubules. ATP is at a level sufficient to supply the energy required for axon growth. The axon has a growth cone at its tip (purple) that senses guidance molecules (not shown) and mediates axonal outgrowth. (B) A schematic of an injured adult neuron failing to regenerate. Adult injured neurons express genes that restrict axon outgrowth, such as *Klf4*. Mitochondria are depolarized after injury and are less motile in the adult axon due to the expression of the anchor protein syntaphilin. ATP levels are lower and ADP levels are higher, such that the axon does not have enough energy to grow. The axon has a retraction bulb with disorganized and disassembled microtubules at its tip.

After injury, the formation of a growth cone or growth cone-like structure is vital for axon regeneration (Bradke et al., 2012). Injury exposes the interior of the axon to calcium, triggering membrane sealing and growth cone formation. Peripheral nervous system (PNS) axons form new growth cones after injury in order to regenerate (Ertürk et al., 2007). However, CNS axons fail to form growth cones (Bradke et al., 2012; Ertürk et al., 2007). Instead, they are tipped with dystrophic retraction bulbs; these structures accumulate anterogradely transported vesicles and mitochondria, and show microtubule disassembly (Fig. 2A-B). In the human spinal cord, retraction bulbs have been observed more than four decades after injury (Ruschel et al., 2015), highlighting their role in regeneration failure.

Growth cones contain organized microtubules that form tight bundles parallel to the axonal axis, whereas retraction bulbs have highly dispersed and disorganized microtubules (Ertürk et al., 2007). Application of the microtubule-destabilizing agent nocodazole transforms growth cones into retraction bulb-like structures *in vivo*, resulting in halted axon growth (Ertürk et al., 2007). Conversely, microtubule stabilization through the administration of taxol interferes with retraction bulb formation and facilitates growth cone formation *in vivo* (Fig. 2B,C). When applied *in vivo* after CNS injury, taxol administration boosts optic nerve axon regeneration (Sengottuvel et al., 2011) and serotonergic axon regeneration (Hellal et al., 2011). Microtubule stabilization also promotes locomotor recovery in rodent models of traumatic brain injury (Cross et al., 2015) and spinal cord injury (Hellal et al., 2011). Since microtubule stabilization drives axon outgrowth during development (Gomis-Rüth et al., 2008; Witte et al., 2008), these studies highlight how regeneration can be achieved by activating developmental processes.

Importantly, microtubule stabilization can be stimulated noninvasively through systemic administration of epothilone B (Ruschel et al., 2015). This drug binds to the αβ-tubulin heterodimer subunit of microtubules, decreasing its rate of dissociation and thus stabilizing the microtubule (Goodin et al., 2004). *In vitro*, epothilone B encourages growth cone formation (Ruschel et al., 2015), while in the adult rat, epothilone B administration improves skilled locomotion and enhances serotonergic axon growth following spinal cord injury (Ruschel et al., 2015). These functional improvements are abrogated by pharmacological ablation of serotonergic innervation, suggesting that the serotonergic axon growth is necessary for recovery. In addition to influencing growth cone formation, microtubule stabilization reduces fibrotic scarring by mitigating fibroblast polarization and migration (Hellal et al., 2011; Ruschel et al., 2015) (Fig. 2C,D). Thus, microtubule stabilization may be a viable strategy for promoting axon regeneration and functional improvements following CNS injury.

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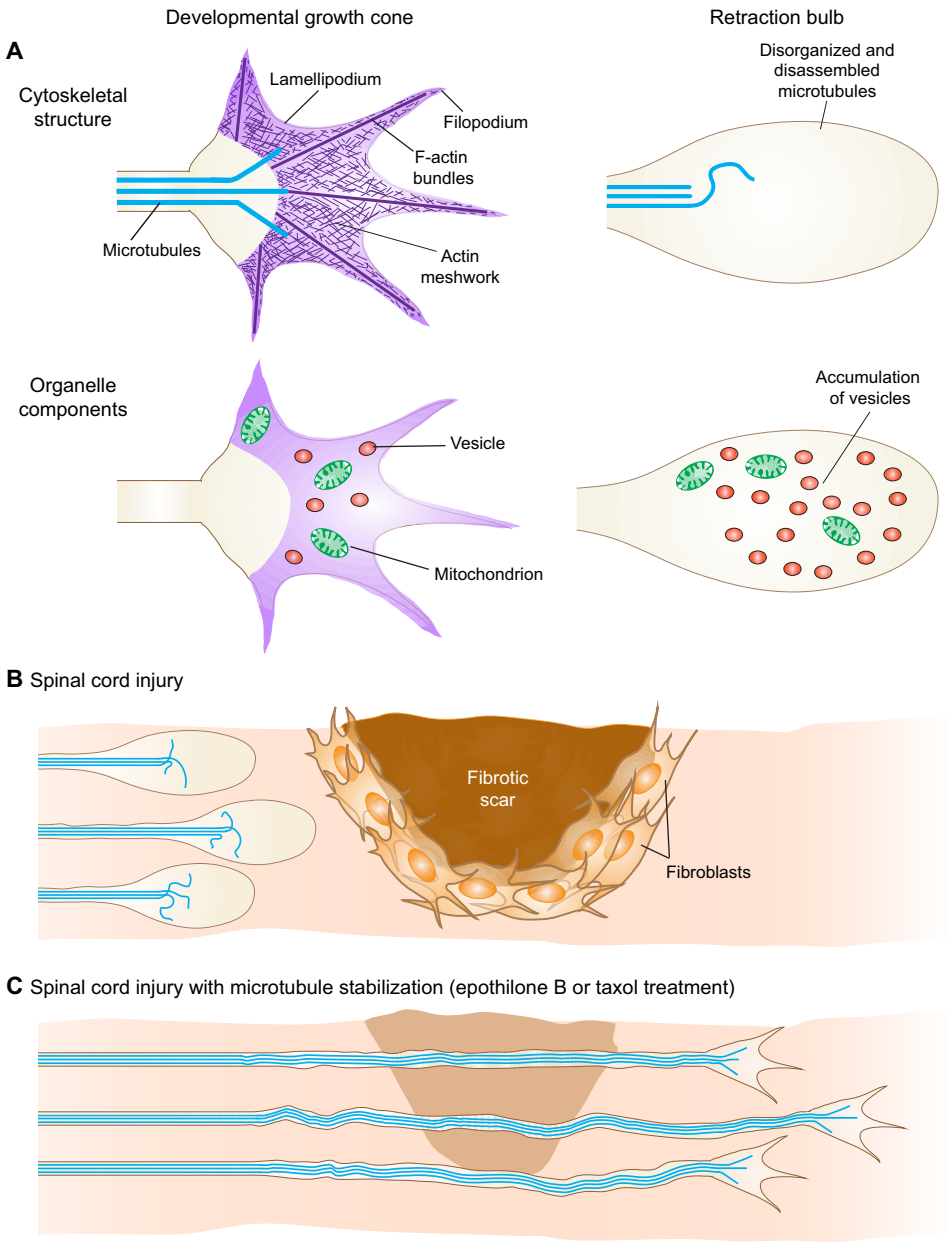

Fig. 2. The growth cone orchestrates developmental axon growth and is a target for CNS regeneration. (A) Cytoskeletal structure and organelle components of a developmental growth cone and a retraction bulb. In the growth cone, microtubules are oriented with the axonal axis. The distal aspect of the growth cone is actin rich (purple) and contains F-actin bundles, filopodia and lamellipodia. In the retraction bulb, microtubules are disorganized. Its actin structure is poorly understood. Mitochondria and vesicles are found at a higher concentration in the growth cone than in the axon shaft. In the retraction bulb, vesicles and mitochondria accumulate distally. (B) After spinal cord injury, sensory axons have retraction bulbs at their tips and axon growth is restricted by fibroblasts that form scar tissue at the injury site. (C) Microtubule stabilization (using epothilone B or taxol treatment) mitigates fibrotic scarring and enhances the formation of growth-cone-like structures at the distal tips of injured sensory axons, enabling regeneration.

Actin dynamics are also key to growth cone formation. Following axotomy, F-actin destabilizes at the injured stump, contributing to retraction bulb formation (Nawabi et al., 2015). Overexpression of doublecortin like kinase 2 (DCLK2) can prevent this destabilization and promotes both growth cone formation and axon regeneration (Nawabi et al., 2015). DCLK2 is enriched in the transition zone of the growth cone, at the interface between the actin-rich peripheral domain and microtubule-rich central domain, where it is thought to help coordinate microtubule/actin dynamics (Bielas et al., 2007). Interestingly, although overexpression of either the microtubule-binding domain or actin-regulatory domain of DLK2 promotes axon regeneration, simultaneous overexpression of both does not (Nawabi et al., 2015). These results highlight the complexity of molecular interactions within the distal tip of the axon following injury. Although DCLK2 encourages growth cone formation by stabilizing F-actin, some actin destabilization may be beneficial as it permits microtubules to protrude into distal areas of the growth cone as the axon shaft is consolidated (Bradke and Dotti, 1999; Flynn et al., 2012). Understanding the role of actin in different compartments of the growth cone is thus essential for understanding how it influences axon regeneration. Moreover, how the neuron coordinates actin/microtubule dynamics to effectively elongate its axon remains an important avenue for further research (Coles and Bradke, 2015).

Mitochondrial function and axonal transport

Axon growth is an energetically intensive process that requires mitochondrial biogenesis and adenosine triphosphate (ATP) production (Vaarmann et al., 2016). Although ATP diffuses freely in the cytosol, neurons, like many other cells, traffic mitochondria to regions of high ATP consumption (Schwarz, 2013). During axon specification and outgrowth in development, the growth cone contains a particularly high concentration of mitochondria (Bradke and Dotti, 1997; Morris and Hollenbeck, 1993). Axons transport mitochondria both in the anterograde and retrograde direction along

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microtubules by using a motor/adaptor complex that contains kinesin and dynein in addition to the proteins Miro (RhoT1/2) and milton (Trak1/2) (Schwarz, 2013). The motor adaptor Trak1 is required for axonal mitochondria transport (van Spronsen et al., 2013) and its depletion inhibits axon outgrowth (van Spronsen et al., 2013). Thus, mitochondria and their axonal transport are essential for sustained axon growth during development.

Axotomy depolarizes mitochondria and depletes ATP in injured axons (Cavallucci et al., 2014; O'Donnell et al., 2013; Zhou et al., 2016). When axons do not have mitochondria, they rapidly degenerate (Rawson et al., 2014). Conversely, boosting mitochondrial ATP production facilitates axon regeneration. Cytokines stimulate axon regeneration in part by inducing signal transducer and activator of transcription 3 (STAT3) to translocate to the inner membrane of the mitochondria (Luo et al., 2016). There, STAT3 enhances ATP production by optimizing the function of the electron transport chain (Luo et al., 2016; Wegrzyn et al., 2009). In cytochrome c oxidase-deficient mice, which have defective mitochondrial ATP production, cytokine-stimulated RGC regeneration is reduced (Luo et al., 2016). Thus, although the cell can produce ATP via glycolysis in the cytoplasm, ATP generated by the electron transport chain in mitochondria is important for axon regeneration, and the efficiency of this process is a target for repair.

Enhancing axonal transport of mitochondria also promotes regeneration. Adult mammalian neurons transport mitochondria in their axons less than do developing neurons because they express syntaphilin (SNPH), a protein that anchors axonal mitochondria to the axon (Kang et al., 2008). In adult mouse RGCs that have a high regenerative ability due to dual genetic knockout of *Pten* and suppressor of cytokine signalling 3 (*Socs3*), armadillo repeat containing X-linked 1 (*Armcx1*) is highly expressed (Sun et al., 2011). *Armcx1* encodes a mitochondrial protein and belongs to a family of genes unique to placental mammals (López-Doménech et al., 2012). The protein contains a putative outer mitochondrial membrane-targeting sequence, flanking a transmembrane domain necessary for its mitochondrial localization (Cartoni et al., 2016). Overexpression of *Armcx1* in adult mouse increases mitochondrial motility and enhances RGC survival and axon regeneration (Cartoni et al., 2016). In addition, upon simultaneous *Pten* and *Socs3* knockout, which greatly enhances neuronal intrinsic growth capacity (Sun et al., 2011), *Armcx1* mediates neuron survival and axon regeneration (Cartoni et al., 2016). Similarly, a high mitochondrial density is crucial for axon regeneration in the nematode, *C. elegans* (Han et al., 2016). However, it is unclear how the axonal transport of mitochondria influences regeneration. While enhancing ATP production in the axon is likely to be one factor, mitochondrial density might also influence regeneration by altering signalling through changes in metabolite production or intracellular calcium levels (Chandel, 2014; Williams et al., 2013). It is also known that mitochondria can regulate axon branching by determining sites of axonal protein synthesis (Spillane et al., 2013) and that mitochondrial deficiency can activate signalling cascades that result in dendritic branching (Gioran et al., 2014). The relationship between the mitochondria in the signal transduction cascades that mediate axonal outgrowth is an important issue for future research.

Synaptic transmission

During embryonic mammalian development, axons are connected to far more targets than those in the adult (Luo and O'Leary, 2005). This exuberance is mediated in part by neurons extending linearly and rapidly past their target cells (Stanfield et al., 1982). Following this, side branches form on the elongated axon and grow slowly into the target area. The overshooting axon degenerates, and terminations are refined during a protracted period of synaptogenesis and synapse elimination (Hua and Smith, 2004; Low and Cheng, 2006; Purves and Lichtman, 1980). Together, exuberant outgrowth and pruning during development generate precise connectivity in the adult (Luo and O'Leary, 2005).

After adult connectivity is established, neurons employ multiple strategies to avoid ectopic axon growth and to maintain appropriate synaptic connectivity (Shen and Scheiffele, 2010). These same strategies might restrict axon regeneration and rely on synaptic transmission. In an elegant series of experiments, Lorenzana and colleagues used *in vivo* two-photon imaging of adult mouse spinal dorsal column sensory axons to assess whether a surviving intact branch influences the ability of the neuron to regenerate (Lorenzana et al., 2015). These axons present a particularly good model for this because they bifurcate into an ascending branch, which extends rostrally towards the brain, and a descending branch, which courses down the spinal cord. Eliminating either of these branches is followed by a poor regenerative response but eliminating both increases regeneration. Thus, an intact axonal process suppresses regeneration. Although the neurons projecting these central axonal processes do not effectively regenerate them, they can regenerate their axonal processes in the PNS. One signal underlying this difference is electrical activity (Enes et al., 2010). After peripheral nerve injury, electrical activity is lost, which may signal regeneration to initiate. In contrast, electrical activity is maintained after central axon injury and may suppress growth by triggering an increase in intracellular calcium concentration (Enes et al., 2010). Thus, an intact axonal process can discourage regeneration and may do so by maintaining electrical activity. This exciting possibility needs to be directly tested.

The synaptic-based suppression of axon growth is something that can be targeted therapeutically. *Cacna2d2*, the gene encoding the α2δ2 subunit of voltage-gated calcium channels, limits sensory axon regeneration (Tedeschi et al., 2016). α2δ2 subunits promote synapse formation and enhance the probability of synaptic transmission (Eroglu et al., 2009). Thus, they may act as a molecular switch to synaptically suppress axonal outgrowth. Gabapentinoids, including pregabalin and gabapentin, are clinically approved drugs used to treat epilepsy, neuropathic pain and fibromyalgia, which bind with high affinity and selectivity to α2δ1/2 subunits (Gee et al., 1996). Systemic gabapentinoid administration induces adult mouse sensory axon regeneration following spinal cord injury (Tedeschi et al., 2016). A meta-analysis found that spinal cord-injured individuals that received these drugs have enhanced motor recovery if they are administered in the first month after injury (Warner et al., 2017). Together, these promising results pave the way for a detailed exploration of gabapentinoid use to treat spinal cord injury and other neurological disorders characterized by paralysis.

In summary, intracellular processes define the capacity of developing neurons, and the inability of adult neurons, to regenerate. In turn, these processes occur together with changes in the extracellular environment of the neuron that are also instructive to axon growth and restraint.

Developing and adult CNS: extracellular factors involved in regeneration

In addition to intracellular differences between developing and adult neurons, the extracellular environment of the neuron changes as the

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CNS matures in a way that restricts regeneration. However, it is becoming clear that extracellular factors that guide developmental axon growth can facilitate regeneration in the adult.

Axon guidance cues

As an axon extends during development, its growth cone senses an array of guidance molecules expressed by the environment that allow it to steer towards the correct target. Several phylogenetically conserved families of guidance cues have been discovered, including netrins, slits, semaphorins and ephrins (O'Donnell et al., 2009). The growth cone integrates these signals and transmits them into cytoskeletal changes that underlie steering decisions, such as attraction and repulsion (O'Donnell et al., 2009).

Some guidance molecules that function primarily in an inhibitory or repulsive role developmentally are expressed following adult injury and inhibit axon growth (Giger et al., 2010). For example, during development, Wnt proteins are expressed in spinal cord grey matter along the rostral/caudal axis in a concentration gradient that decreases caudally (Liu et al., 2005). Corticospinal axons express the repulsive Wnt receptor Ryk (related to receptor tyrosine kinase), which repels these axons to steer them away from the brain and towards their spinal cord targets (Li et al., 2009; Liu et al., 2005) (Fig. 3A,B). Wnt proteins are virtually undetectable in the adult spinal cord (Fig. 3C), but their expression is induced around the injury site following spinal cord injury, where they inhibit axon growth (Liu et al., 2008) (Fig. 3D). Reducing the expression or axonal detection of repulsive guidance cues can thus enhance axon growth. Blocking Wnt/Ryk signalling enhances corticospinal axon sprouting in adult mice after cervical spinal cord injury (Hollis et al., 2016; Liu et al., 2008) (Fig. 3E). The corticospinal tract mediates skilled forelimb motor control in rodents and primates (Lawrence and Kuypers, 1968; Starkey et al., 2005). Interestingly, although blocking Wnt-Ryk signalling can promote corticospinal sprouting, this is not sufficient to promote forelimb motor recovery after spinal cord injury (Hollis et al., 2016). Instead, recovery requires the combination of this blockade with a form of rehabilitative, task-specific training. If mice are coaxed to perform repeated reaching movements, Ryk inhibition promotes extensive forelimb motor recovery. Importantly, this recovery is associated with motor cortical reorganization. Motor cortical areas that drive hindlimb motor responses prior to injury acquire the capacity to activate forelimb muscle groups controlled by motor neurons located rostral to injury in Ryk-inhibited, rehabilitative trained mice. This provides strong evidence that releasing corticospinal axons from Wnt inhibition promotes circuit plasticity when combined with activity-based rehabilitative approaches (Dietz and Fouad, 2014). More generally, these results highlight the importance of developing approaches that direct growing axons into forming functional circuits and of strengthening connections.

In addition to blocking the action of repulsive or inhibitory guidance cues, an alternative strategy to promote regeneration is to provide injured axons with attractive signals that they receive developmentally (Fig. 4). Grafting NPCs into sites of spinal cord injury in the adult rat promotes robust corticospinal axon regeneration via contact-based mechanisms (Kadoya et al., 2016). However, NPCs derived from the rat spinal cord facilitate greater regeneration than do NPCs derived from the rat brain. Moreover, NPCs driven toward a spinal cord-like fate via treatment with retinoic acid, which caudalizes the cells in a concentration-dependent manner (Okada et al., 2004), also facilitate more corticospinal axon regeneration than do brain-derived NPCs. Interestingly, the maturity of rat NPCs following their engraftment is not a primary factor in the regenerative response: when NPCs are matured for 70 days prior to axonal injury, they support regeneration as well as do immature NPCs grafted the same day as the injury (Kadoya et al., 2016). These data support a model in which the regional similarity of transplanted cells to the original substrate of an axon is an essential factor in determining their capacity to support regeneration. Understanding the molecular factors underlying this effect may allow us to provide these signals to axons without the need for cell transplantation (Assinck et al., 2017).

Astrocytes and the lesion site scar

Cells of the astrocyte lineage play a vital role in axon pathfinding and circuit formation during development (Clarke and Barres, 2013; Fitch and Silver, 1997). They secrete extracellular matrix (ECM)

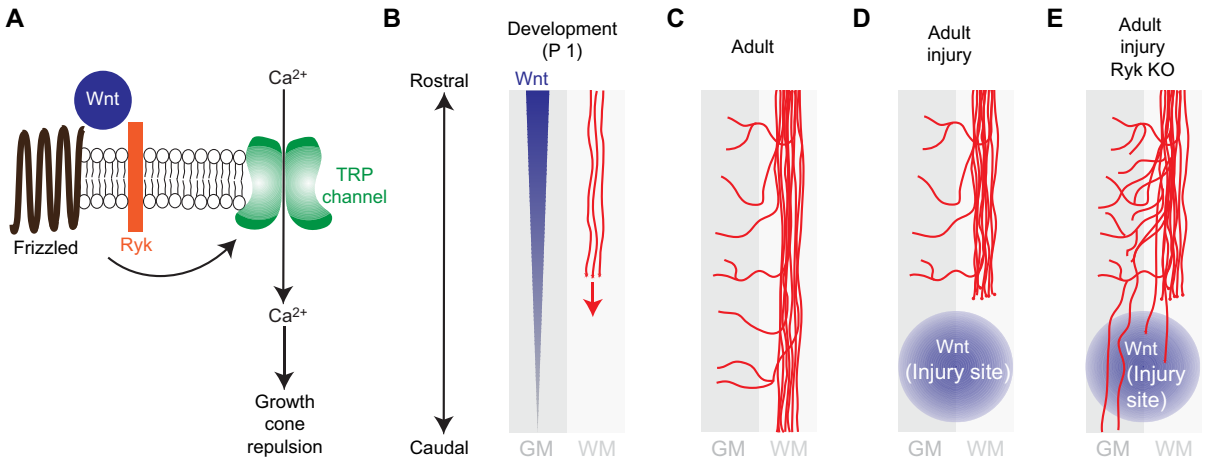

Fig. 3. Wnt signalling directs corticospinal axon guidance during development but restricts corticospinal sprouting after injury. (A) A Wnt/Ryk signalling pathway underlies corticospinal axon repulsion (Li et al., 2009). Wnt (blue) binds to the Ryk (orange) and Frizzled (black) receptors, leading to activation of TRP channels (green). Calcium influx through TRP channels leads to growth cone repulsion. (B) During development, pioneer corticospinal axons (red) steer away from the brain towards their spinal targets in white matter (WM) by sensing Wnt, a repulsive guidance cue expressed in grey matter (GM) that instructs the axons to grow caudally. (C) In adulthood, the corticospinal tract is fully mature, with axonal sprouts extending into the GM, and Wnt is not expressed. (D) After spinal cord injury, corticospinal axons are inhibited from sprouting by Wnt, which is expressed at the injury site. (E) Knocking out the repulsive Wnt receptor Ryk enables corticospinal axon sprouting around a site of spinal cord injury.

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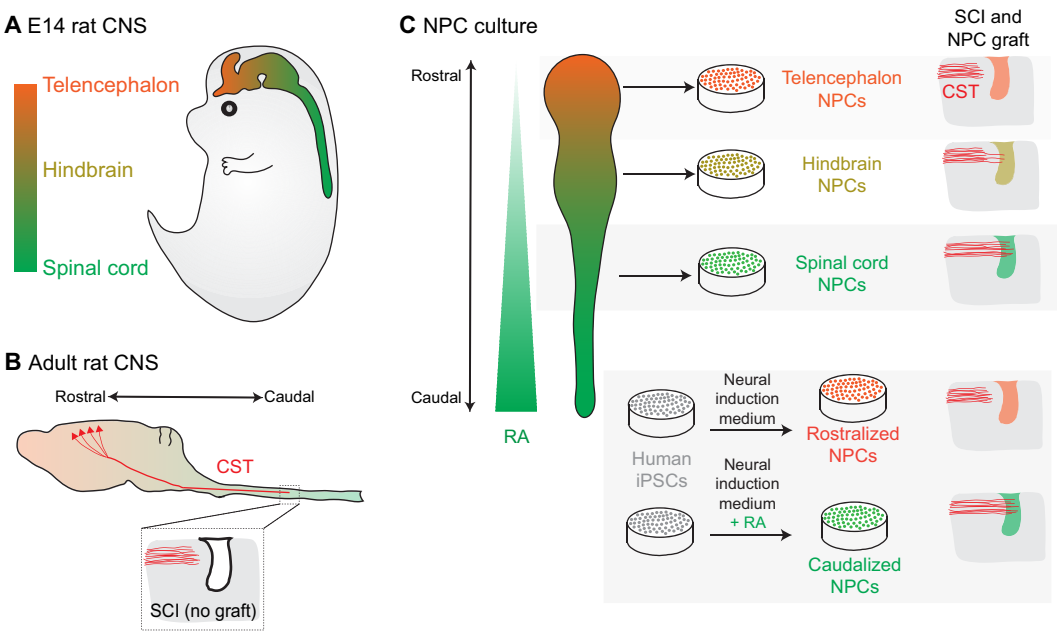

Fig. 4. The capability of neural progenitor cells to support regeneration is influenced by their site of origin. (A) A schematic of an embryonic day (E) 14 rat embryo showing its developing CNS. During embryonic development, the rostrocaudal axis of the CNS is specified by morphogens such as retinoic acid (RA), which caudalizes tissue in a concentration-dependent manner (green). This process distinguishes the spinal cord (green) from the more rostral hindbrain (khaki) and telencephalon (orange). (B) In the adult, axons in the corticospinal tract (CST) project from the cortex to the spinal cord and fail to regenerate after spinal cord injury (SCI). (C) Neural progenitor cells (NPCs) can be dissociated and cultured from an E14 rat CNS, embedded in a fibrin matrix with a cocktail of trophic factors, and grafted into sites of spinal cord injury. NPCs derived from the telencephalon do not support CST regeneration into the graft (orange), whereas spinal cord graft NPCs do (green). Hindbrain-derived NPCs (khaki) support some CST regeneration (but less than that produced by spinal-cord NPCs). In addition, human induced-pluripotent stem cells (iPSCs) driven towards a rostralized NPC fate (orange) do not support CST regeneration but iPSCs caudalized by treatment with RA (green) do.

molecules, such as chondroitin sulphate proteoglycans (CSPGs), that form repulsive barriers to developmental axon growth (Brittis et al., 1992). After adult injury, astrocytes become reactive and direct the formation of a scar at the injury site by walling off a core of cells, including fibroblasts, macrophages, NG2 glia, pericytes and ependymal cells (Cregg et al., 2014). This response to injury serves an important purpose: in directing scar formation, astrocytes mitigate inflammatory damage and are thus neuroprotective (Faulkner et al., 2004; Hilton et al., 2016b). However, many scar cells inhibit axon growth, typically by physically interacting with the distal tips of axons (Filous et al., 2014) or by secreting ECM molecules such as CSPGs (Burnside and Bradbury, 2014; Cregg et al., 2014; Tan et al., 2011). When single mouse sensory axons are axotomized by a laser with minimal scarring, the axons regenerate robustly a few days after receiving a peripheral conditioning lesion that boosts their intrinsic growth potential (Ylera et al., 2009). In contrast, sensory neurons that are growth competent due to a peripheral conditioning lesion cannot regenerate axons through mature scar territory (Ylera et al., 2009).

Therapeutic strategies that target these extracellular impediments to axon regeneration can reactivate developmental growth processes. For example, during development, visual circuits are plastic during a well-defined critical period that ends as the ECM matures (Hubel and Wiesel, 1970; Pizzorusso et al., 2002). Digesting CSPGs via treatment with chondroitinase-ABC reactivates this developmental plasticity (Pizzorusso et al., 2002), while chondroitinase-ABC treatment enhances axon growth in part by facilitating expression of the same regeneration-associated genes that mediate developmental outgrowth (Bradbury et al., 2002). Interestingly, chondroitinase-ABC decreases the amplitude and charge of excitatory postsynaptic currents *in vitro*, suggesting that its influence on axon growth may relate to its modulation of synaptic activity (Pyka et al., 2011). Of note, treatment with the microtubule-stabilizing drugs taxol and epothilone B reduce CSPG deposition and fibrotic scarring after spinal cord injury (Hellal et al., 2011; Ruschel et al., 2015).

Immature astrocytes can support the regeneration of adult central axons (Filous et al., 2010; Reier et al., 1986; Smith et al., 1986) and synthesize less CSPGs than adult astrocytes do (Dow et al., 1994). This is similar to glial cells in lower vertebrate species, which form multicellular structures (‘bridges’) that provide a substrate onto which axons regenerate and form new connections after injury (Butler and Ward, 1967; Zukor et al., 2011). In zebrafish, this bridging is directed by connective tissue growth factor a (CTGFa) signalling (Mokalled et al., 2016). Interestingly, some adult astrocytes retain a capacity to form bridges that facilitate regeneration following *Pten* knockout in adult mouse (Liu et al., 2010). It is tempting to speculate that axon growth could be promoted by manipulating astrocytes into forming regenerative bridges. However, it would be important to promote bridge formation in a way that does not stop astrocytes from preventing inflammatory cell loss.

Recent work has suggested that astrocytic scar formation aids CNS axon regeneration (Anderson et al., 2016). Sensory axons regenerate after spinal cord injury when they are administered a peripheral conditioning lesion, trophic factors and hydrogels. However, axons do not regenerate after this treatment if lesion site astrocytes are depleted by selectively killing proliferating astrocytes or preventing astrocytic STAT3 signalling (Anderson et al., 2016). As reactive astrocytes sequester inflammatory cells to protect nervous tissue following injury (Faulkner et al., 2004), their ablation likely enhances inflammatory cell damage (Sofroniew, 2015),

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precluding the possibility of regeneration (Silver, 2016). Indeed, astrocytic scar formation is driven by type 1 collagen, which instructs astrocytes to adhere to one another by activating the integrin/N-Cadherin signalling pathway (Hara et al., 2017). Blockade of collagen 1 signalling in reactive astrocytes, which prevents scar formation without depleting reactive astrocytes from the lesion site, enhances axon regeneration (Hara et al., 2017). Thus, although astrocyte depletion does not promote regeneration, some astrocyte phenotypes produced in response to spinal cord injury inhibit axon growth.

Intriguingly, recent work in mouse and rat has highlighted the heterogeneity of reactive astrocytes following CNS disease and injury by providing evidence that classically activated microglia induce astrocytes to become neurotoxic (Liddelow et al., 2017). Microglia secrete interleukin 1α (IL1α), tumour necrosis factor (TNF) and complement component 1, subcomponent q (C1q), inducing the formation of neurotoxic 'A1' astrocytes that kill RGCs after axotomy (Liddelow et al., 2017). In contrast, trophic-factor producing 'A2' astrocytes form in response to ischemia and may be neuroprotective. The relationship of this type of astrocyte heterogeneity to the bridge-forming or scar-forming characteristics of some astrocytes is unknown. Nonetheless, blocking the neurotoxic activity of A1 reactive astrocytes or directing A2 astrocyte formation may promote neuron survival and regeneration following disease or injury.

Oligodendrocytes and myelin

Myelin insulates axons and accelerates the conduction of electrical impulses (Fancy et al., 2011). Most myelin-forming oligodendrocytes are born in the early postnatal period at approximately the same time as the developmental transition occurs that restricts axon regeneration (Fancy et al., 2011; Keirstead et al., 1992). As such, they have long been considered potential mediators of this transition (Schwab et al., 1993). Suppressing the onset of myelination can extend the permissive period of functional regeneration in the chick (Keirstead et al., 1992). Various myelin-associated molecules inhibit axon outgrowth, including Nogo, myelin-associated glycoprotein (MAG) and oligodendrocyte-myelin glycoprotein (OMgp) (Schwab and Strittmatter, 2014). Nogo blockade boosts central axon growth and circuit plasticity following a stroke in an adult rat if followed by rehabilitation training to consolidate newly grown axons into mature circuits (Wahl et al., 2014).

Although oligodendrocytes restrict axon growth, they may play a vital role after injury by myelinating newly regenerated axons (Fig. 5). If the axon has regenerated, it may require new myelin for efficient conduction and to form a functional circuit, particularly if that axon was well myelinated prior to injury. In adult mice, knockout of *Pten* and *Socs3* or overexpression of osteopontin (OPN), insulin-like growth factor 1 (IGF1) and ciliary neurotrophic factor (CNTF) induces RGC regeneration (Bei et al., 2016). After regenerating, some axons form functional synapses with their presumptive targets in the superior colliculus. However, these axons lack myelin, fail to conduct action potentials and do not restore visual function. Administration of the voltage-gated potassium channel blocker 4-aminopyridine (4-AP) improves visual function by promoting conduction in regenerated axons (Bei et al., 2016). Neurotrophin 3 expression guides sensory axon regeneration and synapse formation but the axons remain unmyelinated (Alto et al., 2009). In contrast, after *Pten* knockout, cAMP administration and inflammatory stimulation, regenerating RGC axons are myelinated and assemble nodes of Ranvier (Marin et al., 2016). After NPCs are transplanted into the injured spinal cord and differentiate into neurons that extend axons long distances, about a quarter of their axons are myelinated by host oligodendrocytes (Hunt et al., 2017). As such, myelination of newly regenerated axons is not an automatic process and varies based on the treatment that induces regeneration to begin with. Similar to analyses of axon regeneration, assessment of myelination in regeneration studies must be carefully interpreted because these results can be confounded by the presence of myelin on uninjured axons (Tuszynski and Steward, 2012).

The signals that direct myelination of regenerating axons are unclear. Following focal chemical demyelination, oligodendrocytes effectively regenerate myelin on denuded axons in a process that recapitulates aspects of developmental myelination (Fancy et al., 2011). Both oligodendrocytes and Schwann cells are capable of regenerating myelin (Plemel et al., 2017), with oligodendrocyte remyelination relying on myelin regulatory factor signalling (Duncan et al., 2017) and Schwann cell remyelination requiring neuregulin 1 signalling (Bartus et al., 2016). One possibility is that denuded axons contain signals necessary for myelination that regenerated axons may lack. In this regard, one potential signal is electrical activity (Fields, 2015). Another possibility is that myelination of regenerated axons requires inflammatory stimulation, given that macrophages drive oligodendrocyte differentiation during remyelination of denuded axons (Miron et al., 2013). Understanding the signals that regulate the myelination of regenerated axons is a new area of research that will yield valuable insight into developing effective repair strategies.

Do adult axons continue to diminish in their regenerative capacity with age?

Although the CNS diminishes in regenerative capacity early after birth, one issue of substantial therapeutic relevance is whether it continues to decline as it ages. In other words, do mid-adult-aged neurons have even less capacity to regenerate relative to their young adult counterparts? Age is an important risk factor in cases of stroke and glaucoma (Leske et al., 1995; Sacco et al., 1997), both of which would benefit from axon regeneration therapies. Additionally, the average age of incidence of spinal cord injury has increased since the 1970s and now occurs in the late 30s or early 40s (DeVivo and Chen, 2011). However, assessing the role of aging in axon regeneration has been a challenge because young adult mammals already have an extremely low capacity to regenerate to begin with.

A number of key insights into neuronal aging have come from studies of *C. elegans*. After *C. elegans* reach adulthood, aging diminishes their ability to generate growth cones and to extend axons after injury (Byrne et al., 2014). This restricted axon regeneration is not merely a consequence of organismal aging. Instead, it is an active intracellular process: in aged *C. elegans*, insulin signalling via the insulin growth factor 1 (IGF1) receptor inhibits forkhead transcription factor *daf-16*/FOXO activity, suppressing regeneration (Byrne et al., 2014). In the mammalian peripheral nervous system, axon regenerative capacity declines with age because Schwann cells are impaired in their ability to dedifferentiate and clear myelin debris (Painter et al., 2014). Mammalian central neurons also decline in their regenerative capacity as they age from young adulthood to mid-adulthood (Geoffroy et al., 2016). After *Pten* knockout in 4-week-old mice, corticospinal and rubrospinal neurons regenerate axons past sites of spinal cord injury (Geoffroy et al., 2016; Liu et al., 2010). However, when *Pten* is knocked out at older ages (10 weeks or older), axons fail to navigate past the injury site by 8 weeks post-injury (Geoffroy et al., 2016). Importantly, *Pten* knockout boosts axon sprouting above the lesion in older animals,

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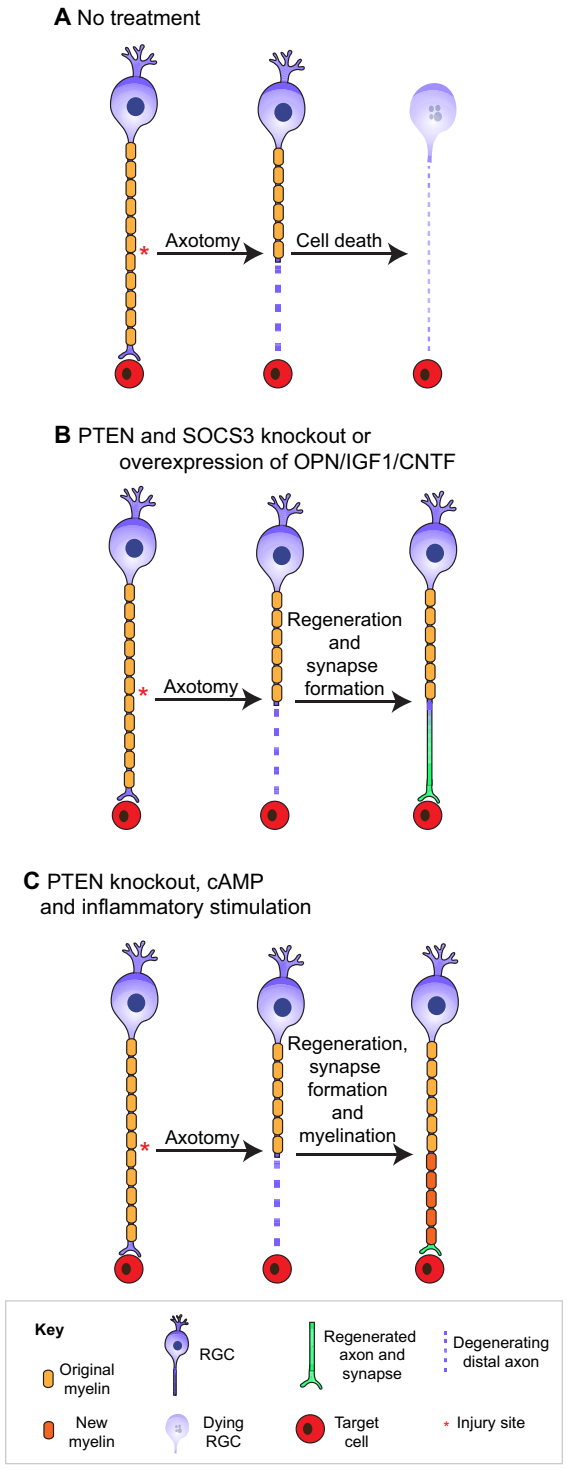

Fig. 5. Regenerated axon myelination is not an automatic process and may involve inflammatory signalling. (A) A mouse retinal ganglion cell (RGC) projects its axon via the optic nerve to a target cell in the superior colliculus. The axon is myelinated by oligodendrocytes. After axotomy, the RGC axon distal to the injury site degenerates and the RGC eventually dies. (B) The combined knockout of PTEN and SOCS3 or the overexpression of OPN, IGF1 and CNTF stimulates an axotomized RGC to survive and to regenerate its axon back to, and form a synapse with, its putative target. However, the regenerated axon lacks new myelin, leading to poor action potential conduction, which prevents functional recovery. (C) PTEN knockout, cAMP and inflammatory (zymosan) stimulation also prompts an axotomized RGC to regenerate its axon back to its target, where it forms a synapse. With this treatment, the regenerated axon is myelinated by oligodendrocytes and forms new nodes of Ranvier.

demonstrating that it remains effective in enhancing axon growth. *Pten* restricts axon regeneration in *C. elegans* independently of age (Byrne et al., 2014). Hence, *Pten* is likely an evolutionarily conserved inhibitor of regeneration. After knockout of the myelin associated inhibitor *Nogo*, the regeneration of corticospinal and serotonergic axons is much less after spinal cord injury in 14-week-old mice relative to 8-week-old mice (Cafferty et al., 2007).

How mammalian neurons continue to decline in their regenerative capacity as they age is unknown. As in *C. elegans*, specific signalling pathways might be activated in older mammalian neurons that further restrict their regeneration, but this is unclear. Another possibility is that older neurons have the capacity to regenerate following treatment but their growth through scar territory is much slower. When *Pten* is knocked out in corticospinal neurons a full year after spinal cord injury, axon regeneration is observed 7, but not 4, months later (Du et al., 2015). This is a much longer timeframe for regeneration than in studies in young adult mice, where knockout is induced prior to injury (Geoffroy et al., 2016; Liu et al., 2010). In those cases, axon regeneration is observed 6-12 weeks post-injury. One major difference between these scenarios is that the scar at the injury site is significantly denser and more mature at 1 year post-injury (Cregg et al., 2014). When the mid-adult mouse spinal cord is injured, it has enhanced macrophage density and astrogliosis relative to an injured spinal cord from a young adult (Geoffroy et al., 2016). Moreover, in the adult rat, aging increases the density of CSPGs sulphated at position 6, which cause more inhibition of axon growth, and decreases CSPGs sulphated at position 4, which are more growth permissive (Foscarin et al., 2017). As such, aging might accelerate the formation or maturity of inhibitory scar territory, making it harder for axons to regenerate. Understanding the mechanistic basis of this decline in central axon regeneration from young adulthood to mid-adulthood is an exciting avenue of research with important implications for CNS repair.

Divergences between developmental and regenerative axon growth

Although recapitulating aspects of developmental growth enhances axon regeneration, it is instructive to understand how these two processes differ. In zebrafish, axon regeneration is mediated by activating regeneration-associated gene expression using promoter elements that are not active during embryonic development (Udvadia et al., 2001). Similarly, mouse DRG neurons that regenerate because of a conditioning lesion rely on signalling pathways that are partially distinct from those mediating their outgrowth during development (Liu and Snider, 2001). Indeed, the global patterns of gene expression that are active during peripheral axon regeneration only partially overlap with those that are active during developmental axon growth (Chandran et al., 2016; Tedeschi et al., 2016). Thus, the gene expression programs underlying regeneration in non-mammalian vertebrates and in the mammalian PNS are at least partially distinct from those underlying developmental axon growth.

Some intracellular processes key to axon outgrowth are either unique to or particularly important for regeneration. For example, in adulthood, the distance between the axon tip and cell body of most neurons is far longer than at any stage of embryonic development. In

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line with this, axonal transport is crucial for regeneration (He and Jin, 2016). In the retrograde direction, initiation of the regenerative program requires signalling from the injured axon to the cell body (Plunet et al., 2002). The upregulation of regeneration-associated genes in response to injury is less pronounced in the neuron when it is axotomized at a longer distance from its cell body compared with when it is axotomized closer to its cell body (Fernandes et al., 1999). STAT3 and dual leucine zipper kinase (DLK) are retrograde messengers key to injury signalling (Lee et al., 2004; Shin et al., 2012). In the anterograde direction, the building blocks of the axon, including membrane, proteins and organelles are transported towards the tip from the cell body to generate new axon. We surmise that the inability of long descending and ascending axons in the adult mammalian spinal cord to regenerate is due in part to the failure of these systems to adequately transport new materials.

Conclusions

Recent studies have demonstrated that recapitulating aspects of developmental axon growth can promote regeneration in the adult. However, many challenges remain. For example, it is unclear how regenerating axons form functional circuits. For many manipulations, whether regenerated axons find their targets, form synapses, are myelinated or become functional circuits following injury or disease is unknown. The development of circuit-specific genetic technologies to activate or silence neurons will allow researchers to assess functional connectivity following CNS injury (Hilton et al., 2016a; Kadoya et al., 2016). These technologies allow transient activation or silencing of neuronal activity in response to light (Deisseroth, 2011) or to designer drugs (Roth, 2016). For example, a dual-virus approach was recently used to express an engineered G-protein-coupled receptor, the designer receptor exclusively activated by designer drug hM4Di (DREADD), within dorsolaterally projecting mouse corticospinal neurons (Hilton et al., 2016a). hM4Di activation transiently hyperpolarizes the neuron and suppresses presynaptic glutamate release (Roth, 2016), thus allowing the role of the dorsolateral corticospinal pathway in mediating spontaneous recovery following cervical spinal cord injury to be determined (Hilton et al., 2016a). In principle, such strategies will allow researchers to assess whether a manipulation promotes synaptic transmission or to probe the necessity of specific neuron populations in function after injury (Hilton et al., 2016a; Jayaprakash et al., 2016; Kadoya et al., 2016).

The employment of novel rabies and adeno-associated viral strategies to dissect the anatomy and functionality of genetically defined circuits will also help the field of neuroregeneration (Azim et al., 2014; Esposito et al., 2014; Zampieri et al., 2014). Bioengineered rabies viruses permit the illumination and interrogation of synaptic partners of genetically and/or anatomically defined subpopulations of neurons, and were recently used to analyse synaptic connectivity between transplanted cells and host axons after spinal cord injury (Adler et al., 2017). With the recent development of self-inactivating rabies virus (Ciabatti et al., 2017), which expands the temporal window of the study of neural circuitry, it is possible to precisely determine the synaptic connectivity of regenerating axons.

Ultimately, our understanding of the relationship between axon regeneration and functional recovery remains rudimentary. In some cases, axon regeneration is associated with worse functional outcome (Takeoka et al., 2011; Wang et al., 2015), signifying the importance of understanding the relationship between these variables. In this regard, investigating synaptic connectivity during embryonic development may provide clues as to how regenerating axons can be directed into forming functional circuits. For example, classical studies in the visual system have demonstrated the influence of neuronal activity in synapse formation, elimination and re-arrangement (Katz and Shatz, 1996). Whether neuronal activity can influence synaptic connectivity of regenerating axons in a similar manner is unclear. At the same time, axotomized neurons can form new circuits, underlying recovery following injury in the absence of regeneration-promoting treatment (Bareyre et al., 2004). Thus, exploring how therapies that promote axon regeneration can build on, and not antagonize, endogenous repair mechanisms will be essential for their translation to the clinic.

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Competing interests

The authors declare no competing or financial interests.

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