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Review article

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Regulation of axonal regeneration after mammalian spinal cord injury

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Abstract

One hundred years ago, Ramón y Cajal, considered by many as the founder of modern neuroscience, stated that neurons of the adult central nervous system (CNS) are incapable of regenerating. Yet, recent years have seen a tremendous expansion of knowledge in the molecular control of axon regeneration after CNS injury. We now understand that regeneration in the adult CNS is limited by (1) a failure to form cellular or molecular substrates for axon attachment and elongation through the lesion site; (2) environmental factors, including inhibitors of axon growth associated with myelin and the extracellular matrix; (3) astrocyte responses, which can both limit and support axon growth; and (4) intraneuronal mechanisms controlling the establishment of an active cellular growth programme. We discuss these topics together with newly emerging hypotheses, including the surprising finding from transcriptomic analyses of the corticospinal system in mice that neurons revert to an embryonic state after spinal cord injury, which can be sustained to promote regeneration with neural stem cell transplantation. These gains in knowledge are steadily advancing efforts to develop effective treatment strategies for spinal cord injury in humans.

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Introduction

During development, neurons extend axons and dendrites to form connections with other neurons. Compared with dendrites, axons often travel long distances to reach their innervation targets. Axonal growth occurs at growth cones, structures at the tip of growing axons that sense and interpret environmental cues to make appropriate growth choices along their trajectories. Following nervous system maturation, axonal projection and connectivity patterns remain for the most part fixed, providing the structural basis for a stable, functional nervous system[1].

In adult mammals, spinal cord injury is a devastating condition that frequently results in permanent muscle paralysis, sensory loss and autonomic dysfunction below the level of the injury. The permanence of functional impairments is largely attributable to the limited ability of injured neurons in the central nervous system (CNS) to regrow axons and re-establish functional connections. The unique structure of the spinal cord renders it akin to an information superhighway such that any severe damage at a particular location along the path may substantially block information flow in both directions. As such, spinal cord injury has long served as a fitting model to study how long-distance connections may be re-established after damage in any region of the CNS.

'Regeneration' is a broad term that has been subject to differing interpretations. In the case of the nervous system, regeneration is sometimes used to refer to any structural change that leads to functional improvement, including regrowth of axons or dendrites, reformation of synapses, and even responses of glial cells and their associated structures such as myelin. However, in the context of this Review, regeneration specifically refers to axonal growth arising from injured neurons (Fig. 1a). Following injury, the distal axonal segment degenerates completely over time; the proximal segment retracts for a relatively short distance, is still connected to — and is thus supported by — the neuronal cell body and can mount a regenerative response. In the peripheral nervous system (PNS), regeneration often succeeds with the newly growing cut axon reaching appropriate synaptic targets to support functional recovery. However, in the CNS, regeneration of a transected axon typically fails[1]. Therefore, a major goal of spinal cord injury research is to promote CNS axon regeneration.

Another form of axonal growth after injury is 'sprouting', defined as axonal growth from uninjured neurons (Fig. 1a). In response to an injury, an uninjured neuron may extend new branches from an existing axon into a denervated region. Compared with regeneration, sprouting can initiate distal to the injury site, and does not require growth through or around the injury site. Both regeneration and sprouting can contribute to functional recovery; whereas regeneration is more difficult to achieve in the CNS, sprouting readily occurs and can be experimentally augmented[2,3]. It should be noted that axonal growth or repair does not invariably lead to functional recovery, even though this is often implied in the literature. Indeed, pain syndromes sometimes occur after CNS injury as a maladaptive consequence of sprouting[4].

This article summarizes our current understanding of the molecular and cellular mechanisms contributing, negatively or positively, to axonal repair (Fig. 1b). There are three mutually non-exclusive theories to explain CNS regeneration failure: the extrinsic inhibitor theory, the neuron intrinsic theory and the growth factor theory (Box 1). The past decade has witnessed tremendous progress and major shifts in understanding their relative contributions and interplay. In particular, studies in recent years have highlighted the limitations of the once-dominant extrinsic inhibitor hypothesis along with the increasing importance of neuron-intrinsic control of axonal repair. The role of trophic factors in regeneration has also been expanded by neural stem cell (NSC) transplantation studies. In addition to worms, flies and mammalian models, studies in non-mammalian vertebrates, including zebrafish, lamprey and axolotls, have contributed new knowledge on axon regeneration[5]. Here, we focus on mammalian systems and discuss recent advances in the context of a historical perspective, emerging concepts and hypotheses, and paths for clinical translation to treat spinal cord injury.

Inhibition by the injured CNS niche

The inhibitory nature of the injured CNS niche has long been considered a major hurdle to regeneration. CNS myelin, the glial 'scar' and axon guidance molecules have all been shown to contribute to growth inhibition.

Myelin inhibitors

A seminal experiment in the early 1980s showed that implants of peripheral nerve bridges into the spinal cord resulted in central axon regeneration through the entire nerve; however, upon reaching the end of the bridge, regenerating central axons could not penetrate back into the spinal cord[6]. These findings prompted a search for axon growth inhibitors in the injured CNS niche[3]. CNS myelin was first shown to inhibit axon growth in vitro in the 1980s. An antibody, IN-1, was raised against inhibitory epitopes on myelin. Administration of IN-1 in vivo promoted regeneration of the corticospinal tract (CST) and improved functional outcomes after spinal cord injury[7,8]. The gene encoding the inhibitory protein was identified as *Nogo* (also known as *Rtn4*) but results of genetic studies in mice were mixed — deleting *Nogo* did not reproducibly enhance CST regeneration[9–12].

The early 2000s saw a rapid expansion in understanding the biochemical signalling pathways mediating myelin inhibition. Three major myelin inhibitors, NOGO, myelin-associated glycoprotein (MAG) and oligodendrocyte myelin glycoprotein (OMGP), signal through a number of receptors and co-receptors, including NOGO receptor 1 (NGR1), NGR2, NGR3, paired immunoglobulin-like receptor B (PIRB), leucine-rich repeat and immunoglobulin-like domain-containing NOGO receptor-interacting protein 1 (LINGO1), p75 neurotrophin receptor (p75NTR) and the tumour necrosis factor receptor superfamily (TROY), to reorganize the cytoskeleton and inhibit axon growth via RHO and RHO-associated kinase (ROCK)[13,14]. Yet, gene knockout of many components in the pathway did not reproducibly enhance CST regeneration[2,15]. By contrast, targeting NOGO pharmacologically or genetically consistently enhanced CST sprouting[15–21].

Several clinical trials targeting myelin inhibitors are under way. The NOGO Inhibition in Spinal Cord Injury (NISCI) phase II, placebo-controlled, multicentre European trial is assessing the safety, tolerability, feasibility and preliminary efficacy of the anti-NOGO-A antibody NG-101 administered within 4–28 days after cervical spinal cord injury. The RESET trial is a multicentre, two-part phase I/II trial in the United States that is evaluating the safety, tolerability, pharmacokinetics and efficacy of AXER-204, an NGR1-based fusion protein as a trap for myelin inhibitors, in patients with chronic cervical injury. NISCI and RESET are scheduled to be completed by early 2023 and mid-2022, respectively. Because neutralizing myelin inhibitors as a monotherapy may be of limited benefit, a combinatorial strategy might be more effective, although such efforts have yielded complicated results in preclinical models[22].

Axon guidance molecules

During development, axon guidance molecules play key roles in guiding growth cones as they navigate through the embryonic and postnatal environment[23]. Axon guidance molecules can be attractive

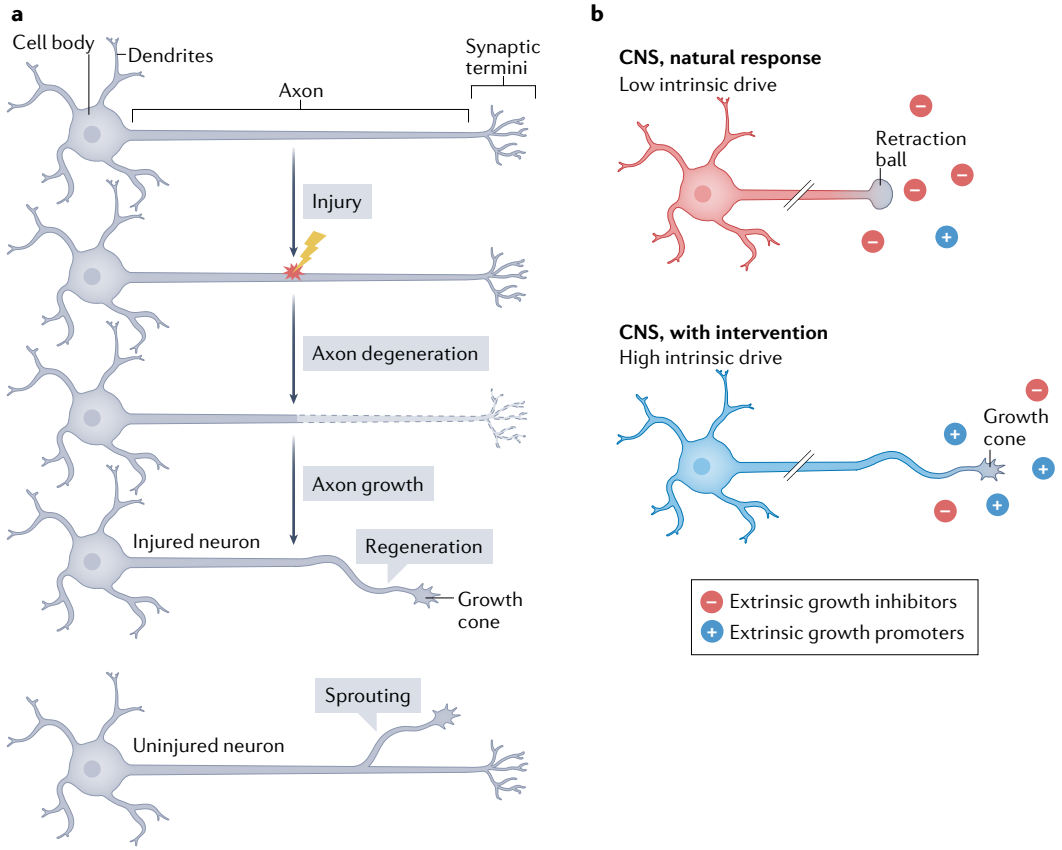


Fig. 1 | Forms of axonal repair and its regulation. a, Regeneration and sprouting are two forms of axonal growth after injury in the central nervous system (CNS). Regeneration is defined as axonal growth from injured neurons; sprouting is defined as axonal growth from uninjured neurons. Both forms of axonal repair may contribute to functional recovery. Spontaneous regeneration is very limited, whereas a baseline level of sprouting likely occurs after any CNS injury. **b**, Major aspects of the regulation of axonal repair. Axonal repair can be promoted by increasing the neuron-intrinsic drive for axonal growth, supplying growth-promoting factors and reducing growth-inhibiting factors in the CNS environment.

and repulsive at the same time. The response to these environmental cues depends on the receptor components and intracellular signalling mediators. Following spinal cord injury, some guidance molecules, such as ephrin B3 (EPHB3), semaphorin 4D (SEMA4D) and netrin, associate with myelin while others, such as WNTs and class 3 semaphorins, are present in a reactive extracellular matrix that forms at or near the injury site[24–28]. Genetic evidence indicated that WNT signalling mediated by the repulsive receptor RYK inhibits CST collateral sprouting, cortical remapping and functional recovery[29]. Exogenously supplied WNT4 causes the retraction of dorsal column sensory axons, whereas bone morphogenetic protein 4 (BMP4) promotes their regeneration[30,31]. Among the classic guidance molecules, there is pharmacological evidence for a role of repulsive guidance molecule A (RGMA) and SEMA3A in inhibiting axonal repair after spinal cord injury[32,33]. Genetic studies did not reveal a role for plexin A3 (PLXA3) or PLXA4 in inhibiting regeneration[34], which likely reflects signalling from class 6 rather than class 3 semaphorins in this context. Results on the neuronal role of EPH4, a receptor for multiple ephrins, in axonal repair were mixed[35,36]. Although EPHA4 was initially reported to regulate reactive astrogliosis and scar formation (Box 2), this was not corroborated in later studies[37–39]. Interestingly, genetic evidence indicates that plexin B2 expressed by macrophages and microglia is required to seal-off the lesion core by the astrocyte border[40]. Exogenously supplied guidance molecules have also been used to guide the growth of regenerating axons after injury[41]. Thus, axon guidance molecules can have complex roles in multiple cell types to influence spinal cord repair. In many cases, genetic studies are still required to verify their roles, preferably through inducible gene deletion experiments, since germline knockouts often exhibit developmental defects.

Inhibitory CSPGs

Chondroitin sulfate proteoglycans (CSPGs) in the extracellular matrix are another class of axon growth inhibitors that influence regeneration after spinal cord injury[42]. Traditionally, CSPGs were referred to as 'glial scar'-derived inhibitors (Box 2), although they are made by other cell types such as macrophages. CSPGs have a protein core and chondroitin sulfate sugar chains. Heterogeneity in both the core protein composition and sulfation patterns in the sugar chains makes it difficult to study CSPGs genetically[43]. Degrading the inhibitory chondroitin sulfate side chains of CSPGs with the bacterial enzyme chondroitinase ABC in vivo promoted axon regeneration in the brain and spinal cord[44,45], although the extent of regeneration was limited. Combining chondroitinase treatment with peripheral nerve grafts promoted more robust regeneration that was accompanied by functional improvement[46–48].

Genetically deleting protein tyrosine phosphatase-σ (PTPσ; a receptor of CSPGs) reduced dorsal column sensory axon retraction by 100 μm (ref. 49). Furthermore, a peptide that blocks the inhibitory action of CSPGs through PTPσ promoted serotonergic (5-HT) innervation beyond a contusion injury but not CST regeneration[50], whereas targeting CSPGs enzymatically reduced atrophy of injured corticospinal neurons and promoted compensatory sprouting of intact CST axons[51,52]. Among the therapeutic modalities studied to date, the therapeutic benefits of chondroitinase administration, while only limited, remain one of the most extensively replicated across different laboratories in rodent models.

Chondroitinase treatment in rhesus monkeys also enhanced CST sprouting (regeneration was not tested) and improved hand function after C7 lateral hemisection injury[53]. A phase I clinical trial in Australia is currently evaluating the safety and tolerability of NVG-291, a PTPσ-derived peptide that relieves CSPG-mediated inhibition, in healthy volunteers. Just as observed with the myelin inhibitors, the main effect of CSPGs on neural repair is through modulation of axonal sprouting[43]. However, while myelin inhibitors act mainly in the white matter, CSPGs are present throughout intact grey matter as perineuronal 'nets' in the extracellular matrix that surround neuronal cell bodies and dendrites[54].

Lack of extrinsic growth factors

The lack of production of growth factors or neurotrophic factors in appropriate spatial and temporal gradients has long been appreciated as a hurdle to axon regeneration after spinal cord injury[55]. During development, neurotrophic factors support neuronal survival, target finding and synaptic stabilization. Throughout adulthood, growth factors continue to influence a variety of functions in the nervous system, including neuronal survival, neurotransmitter production and release, and synaptic plasticity[56]. In the mid-1980s, exogenously administered neurotrophic factors were shown to promote neuronal survival in the

Box 1

Three major theories of CNS regeneration failure: a historical perspective

Ancient Egyptians first noted, in ~2,500 BC, that "a dislocation of a vertebra of his neck" is "a disease not to be treated"[170]. Scientific studies of axon regeneration started with Santiago Ramón y Cajal and his pupil Jorge Francisco Tello in the early twentieth century. In his seminal work *Degeneration and Regeneration of the Nervous System*, Ramón y Cajal described many astute observations on axonal behaviours in response to injury, such as retraction balls, that remain largely accurate today[1]. In a classical experiment, Tello transplanted a piece of peripheral nerve into the brain and observed that central nervous system (CNS) axons could regenerate in the peripheral nerve graft[1,171]. This result was substantiated by Aguayo and colleagues in the 1980s using modern tract tracing technologies in both the brain and the spinal cord[6,172–174].

These nerve bridge experiments led to the idea that the peripheral nervous system (PNS) presents a permissive environment for axon regeneration while the CNS presents an inhibitory one, hence the first major theory of CNS regeneration failure: the extrinsic inhibitor theory. This theory states that neuron-extrinsic factors, such as myelin-derived inhibitors (for example, NOGO) and glial scar-derived inhibitors (for example, chondroitin sulfate proteoglycans), impede axon regeneration and predicts that their removal or neutralization would unleash the regenerative potential of CNS neurons. This was the predominant theory for years, but many experts in the field of CNS regeneration now believe that broader mechanisms may need to be targeted to improve regeneration, either distinct from or in addition to glial-associated inhibitors.

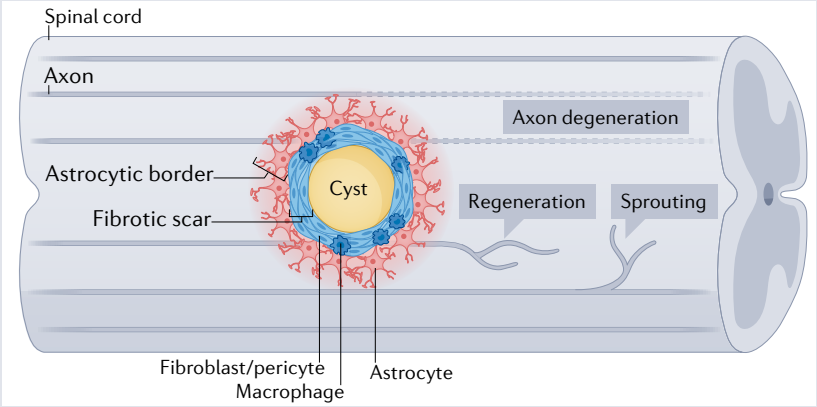
A second theory, the neuron-intrinsic theory, focuses on the limited intrinsic ability of adult CNS neurons to regenerate axons following injury. Scientists have long noted that developing neurons robustly grow axons, as do neurons in the adult PNS after injury. Dorsal root ganglion sensory neurons (with cell bodies residing in the PNS) extend one peripheral branch and one central branch. The peripheral branch regenerates following injury, while the central branch does not. If the peripheral branch is cut either at the same time or prior to a central branch injury, the regeneration of the central branch is enhanced, a phenomenon known as the 'conditioning lesion' effect[175–177]. Likewise, a prior peripheral axon injury enhances the regenerative response following a second peripheral axon injury (that occurs more proximal to the cell body). This effect was later partially attributed to levels of the intracellular second messenger cyclic AMP (cAMP)[178,179]. Around the same time, developmental decline in the ability of retinal ganglion cells to grow axons became well documented[180]. However, it was not until the identification of the phosphatase and tensin homologue (PTEN)–mammalian target of rapamycin (mTOR) pathway[72] that the neuron-intrinsic theory took centre stage in the field. Since then, an expanding list of neuron-intrinsic regulators of regeneration have been identified.

The third theory, the growth factor theory, was coined to emphasize the lack of growth-promoting factors in the adult CNS. Neurotrophic factors, such as nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF) and neurotrophin 3 (NT3), were initially discovered in developmental studies, where they support both neuronal survival and axon growth. In the PNS, they are produced primarily by Schwann cells after injury[181,182] and are essential in supporting peripheral nerve regeneration. They have also been extensively tested for a role in axon regeneration after CNS injury[66,183]. Many growth factors play a role in supporting neuronal survival and axon growth in the context of experimental spinal cord injury and are often applied in combination with other strategies such as cell transplantation and implantation of nerve bridges in animal studies.

The three theories are not mutually exclusive. For instance, the effects of extrinsic growth-promoting factors must be mediated through neuron-intrinsic signalling pathways.

Box 2

The injury scar



After spinal cord injury, a 'scar' forms at the injury site, often surrounding a fluid-filled cavity. The injury scar consists of two compartments: an inner fibrotic lesion core (also known as the fibrotic scar) composed of fibroblasts, macrophages and other blood-borne cells that clearly limit axonal regeneration, and an outer astrocyte border consisting of dense astrocytic cell bodies and reactive cellular extensions[184] (see the figure). Astrocyte borders, depending on their physical orientation, may either block or support axon regeneration[185]. The injury scar has complex roles in injury resolution and repair as it involves a variety of cell types and extracellular components whose functions may change at different stages of injury.

The astrocyte border has been a much-debated topic for spinal cord injury and repair. There is now general agreement that the astrocyte border functions to enclose the non-neural lesion core and minimize secondary injury by limiting the spread of inflammation[186]. Ablating proliferative astrocytes results in an enlarged injury site and worsens pathological and behavioural outcomes[187,188]. Attenuating astrocyte reactivity by manipulating signalling pathways, such as signal transducer and activator of transcription 3 (STAT3) and leucine zipper-binding kinase (LZK), also reduces reactive astrocytosis and enlarges the injury[107,111,112]. However, the precise role of the astrocyte border in axonal repair is still unclear. Traditionally, the astrocyte border had been considered a barrier to regeneration[189], yet it can also serve as a bridge to facilitate regeneration; regenerating serotonergic or corticospinal axons often closely associate with glial fibrillary acidic protein (GFAP)-positive processes when traversing the injury site[34,76,78,190]. Genetically suppressing astrocyte reactivity reduces rather than enhances axon regeneration[185]. Reactive astrocytes could be a major source of inhibitory chondroitin sulfate proteoglycans (CSPGs) after injury but some studies found that the primary source of CSPGs are macrophages and oligodendrocyte progenitors that migrate to the injury site[191]. Hence, at present, there is an evolving view on the role of astrocytes after spinal cord injury[192,193]. While it is clear that, soon after the primary injury, reactive astrocytes help limit the spread of secondary injury, how their traditional role as a source of inhibitory CSPGs reconciles with a role in supporting regeneration remains to be clarified. Furthermore, enzymatic digestion of CSPGs at and around the injury site was recently shown to enhance immune cell clearance and reduce pro-inflammatory gene and protein expression, indicating that prolonged expression of CSGPs at the injury site limits the resolution of inflammation[194].

The fibrotic scar has received limited attention until recently. There is still uncertainty whether the primary stromal cells of the fibrotic scar are fibroblasts, pericytes or both[195,196]. Attenuating fibrotic scar formation through genetic manipulation of pericytes has two opposing consequences on spinal cord repair: applied at a moderate level, it can be beneficial to axonal repair; however, more extensive attenuation leads to a failure to seal the injury epicentre, which in turn exacerbates the secondary injury response[197]. The effects of genetically reducing fibroblast-derived scarring have not been investigated. Pharmacological treatment with the microtubule-stabilizing drugs Taxol or epothilone B reduces fibrotic scar formation and promotes axonal growth after spinal cord injury[133,134]. However, whether these drugs enhance axonal repair primarily through fibroblasts or directly through modulating the neuronal cytoskeleton remains to be elucidated.

Two recent studies shed new light on the injury scar debate. In neonatal mice, microglia have been shown to orchestrate scar-free healing that allows for long distance axonal growth through the injury site[198]. Neonatal microglia accomplish this by expressing and secreting molecules to bridge the severed ends of the spinal cord and to resolve inflammation. Likewise, adult spiny mice (*Acomys cahirinus*) exhibit reduced scarring that is accompanied by spontaneous axon growth and functional recovery after spinal cord injury[199]. Transcriptomic analysis revealed a pro-regenerative proteoglycan signature at the injury site in *A. cahirinus* that features elevated levels of keratan sulfate proteoglycans. The injury scar remains an important area of investigation in promoting neural repair. Figure adapted with permission from ref. 200, Elsevier.

injured CNS[57]. This led to the hypothesis that reduced neurotrophin signalling in the adult CNS impedes recovery after injury.

The CNS growth factors include the neurotrophin family consisting of nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin 3 (NT3) and NT4/5. Additional growth factors particularly relevant to regeneration include glial cell-derived neurotrophic factor (GDNF), insulin-like growth factors (IGFs), fibroblast growth factors (FGFs) and ciliary neurotrophic factor (CNTF). Administration of neurotrophins, such as NGF, BDNF and NT3, often in the context of cell transplantation (Box 3), promotes axonal growth from particular neuronal populations[58–60].

A common theme when applying neurotrophic factors to promote axonal repair is that different growth factors often influence different axonal populations, depending on the expression pattern

of cognate receptors; for example, NGF influences small diameter nociceptive axons[61], NT3 and IGF1 influence CST axons[58,62], GDNF and CNTF act on alpha motor axons, and BDNF influences CST, reticulospinal, raphespinal and medium diameter sensory axons. The functional significance of cell type-specific regeneration following neurotrophic factor delivery was demonstrated by the recovery of specific sensory functions associated with a particular neurotrophic factor in a dorsal root rhizotomy model, where sensory axon regeneration across the dorsal root entry zone into the spinal cord was assessed[61]. Sometimes, exogenously supplied receptors (for example, TrkB for BDNF) are required to boost a growth response due to the reduced receptor expression in adult neurons[63]. At other times, injured neurons may not be responsive to growth factors unless they are sensitized, for example, osteopontin sensitizes adult corticospinal neurons to IGF1 (refs. 64,65).

Locally applied neurotrophic factors could guide regenerating axons beyond a lesion to re-innervate appropriate targets[66]. In this regard, providing a trophic gradient with increasing concentration at the distal end is particularly effective in attracting axonal growth into and beyond a lesion site[67,68]. Neurotrophic factors are often combined with other, complementary strategies to promote axonal repair, for example, tissue or cell bridging at the injury site. This bridge can consist of peripheral nerve grafts, fibroblasts, bone marrow stromal cells, stem cells or bioengineered matrices[46,66,68–71].

Neuron-intrinsic factors

In the past decade, substantial progress has been made in understanding neuron-intrinsic mechanisms of axonal repair after injury. Multiple strategies have been pursued to identify neuron-intrinsic regulators of axon regeneration, including candidate gene approaches, genetic studies in model organisms and 'omics' approaches sometimes coupled with in vitro screens. Next, we trace the discovery of some of the key neuron-intrinsic factors to illustrate the different aspects of neuron-intrinsic control of axonal repair, such as transcriptional, translational and epigenetic control, injury signalling, energy metabolism, and cytoskeletal dynamics (Fig. 2a). A main theme is that many of the molecular pathways that were active during development to enable axonal growth are downregulated in the adult CNS.

Translational control

The discovery of the protein phosphatase and tensin homologue (PTEN)–mammalian target of rapamycin (mTOR) signalling pathway in retinal and corticospinal axon regeneration demonstrated the critical importance of protein translation in CNS axon repair. In a seminal candidate gene study, adeno-associated virus–Cre recombinase-mediated gene deletion in the mouse retinal system was used to examine the role of over a dozen cell growth control genes, including *Trp53* and *Pten*, in retinal axon regeneration[72]. Following optic nerve crush, which is known to cause the death

Box 3

Cell transplantation as a strategy for spinal cord repair

Cell transplantation has been investigated as a strategy for spinal cord repair since the 1970s[201]. Initial studies focused on tissues and cells from the developing nervous system, demonstrating potential for growth of host axons into grafts but inconsistent functional improvement. In the 1990s, progress in stem cell biology and developmental neurobiology contributed to define the various cell types along their developmental paths, including neural stem cells (NSCs; also known as neural progenitor cells), neuronal-restricted precursors and glial-restricted precursors[202]. Accordingly, the field moved away from embryonic or fetal cells to more developmentally defined cell types. Despite somewhat limited survival and fill of spinal cord lesion sites, transplants of neuronal or glial-restricted precursors could survive, connect with host cells and improve some functional outcomes, although the underlying mechanisms remained unclear[203–205].

In 2007, transplantation of green fluorescent protein (GFP)-expressing embryonic donor brain cells into adult mouse cortical lesion sites demonstrated axon growth from the grafted neurons as far distally as the spinal cord[206]. Subsequent studies grafted GFP-expressing spinal cord neural progenitor cells from E14 rats into sites of T3 complete transection[69]. These neural progenitor cells were implanted with a cocktail of growth factors (for example, brain-derived neurotrophic factor (BDNF), fibroblast growth factor 2 (FGF2) and vascular endothelial growth factor (VEGF)) and an anti-apoptosis small molecule to enable graft survival. Remarkably, tens of thousands of GFP-expressing axons extended from the injury site for distances up to 50 mm into the distal, denervated host spinal cord, synapsing with host neurons. Similar findings were obtained with human NSC grafts. In turn, host corticospinal axons regenerated into the grafts, forming neural relays that supported functional improvement (discussed in main text section: Newly emerging hypotheses)[146,151]. Transplantation of human spinal cord NSCs into a C7 hemisection lesion site in non-human primates led to extensive axon growth from the grafts (150,000 axons grew up to 50 mm) that was accompanied by significant improvement in hand function[207]. The extent of axonal growth from NSC transplants was unprecedented compared to other approaches. Whereas adult central nervous system (CNS) axons are inhibited by white matter, adult myelin stimulates the growth of NSC-derived axons[208]. This work is advancing to human clinical trials[209].

Remyelination using oligodendrocyte progenitor cells[210,211] is also being pursued as a treatment strategy for spinal cord injury. These cells have entered early-phase clinical trials but progress has been slow, possibly due to limited efficacy. Other cell types that have been tested in models of spinal cord injury include (1) fibroblasts to deliver trophic factors[66] (although these may impede regeneration on their own); (2) mesenchymal stromal cells, for example, from bone marrow or the umbilical cord, to promote tissue sparing[212]; (3) Schwann cells to promote axon growth and remyelination[213] (this has moved into early-phase clinical trials but may be limited as a monotherapy)[214,215]; and (4) controversial olfactory ensheathing cells[216,217]. To date, none of these approaches has exhibited efficacy in humans. A multi-site early-phase trial of potentially remyelinating stem cells indicated the safety and feasibility of transplanting human CNS stem cells into patients with complete and incomplete spinal cord injury[218].

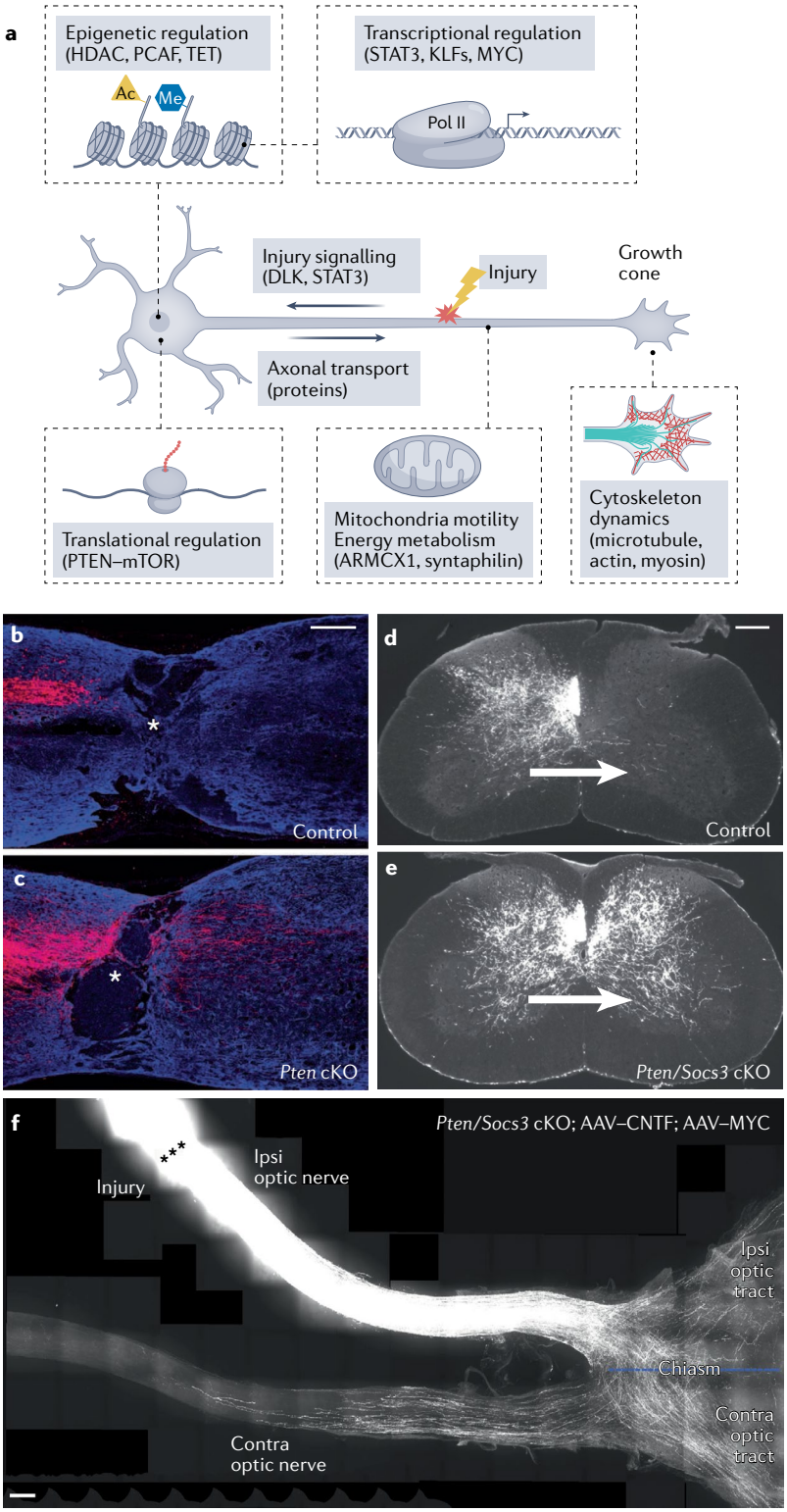


Fig. 2 | Neuron-intrinsic control of axonal repair. Many aspects of basic cell biology can regulate axon regeneration, including epigenetic, transcriptional and translational regulation, injury signalling, axonal transport, mitochondrial motility and energy metabolism, and cytoskeleton dynamics (part **a**). Examples of factors for each aspect are shown in parentheses. In mice, *Pten* deletion induces corticospinal axon regeneration after spinal cord injury (parts **b**,**c**). In *Pten* and *Socs3* conditional knockout (cKO) mice, robust corticospinal axon sprouting across the midline in the cervical spinal cord is observed after unilateral pyramidotomy injury (parts **d**,**e**). *Pten* and *Socs3* cKO and adeno-associated virus (AAV)-mediated ciliary neurotrophic factor (CNTF) and MYC overexpression synergize to promote robust retinal axon regeneration after optic nerve crush injury (part **f**). Scale bars = 200 µm (parts **b**–**e**), 100 µm (part **f**). The spinal cord crush site is indicated with an asterisk. The optic nerve crush site is indicated with three asterisks. contra, contralateral; DLK, dual-leucine zipper-bearing kinase; HDAC, histone deacetylase; ipsi, ipsilateral; KLF, Krüppel-like factor; mTOR, mammalian target of rapamycin; PCAF, P300/CBP-associated factor; PTEN, phosphatase and tensin homologue; STAT3, signal transducer and activator of transcription 3. Parts **b**,**c** are adapted from ref. 76, Springer Nature Limited. Parts **c**,**d** are adapted from ref. 85, CC BY 4.0 (https://creativecommons.org/licenses/by/4.0/). Part **f** is adapted with permission from ref. 86, Elsevier.

of ~80% of retinal ganglion cells (RGCs), *Pten* deletion in RGCs led to increased cell survival and substantial retinal axon regeneration[72]. By contrast, *Trp53* deletion increased RGC survival but not axon regeneration, indicating that increased survival does not automatically lead to increased regeneration. Deleting other genes, including *Rb* (encoding retinoblastoma), increased neither cell survival nor regeneration.

PTEN is a negative regulator of the phosphatidylinositol 3-kinase (PI3K)–AKT–mTOR signalling pathway, which normally promotes cell growth by increasing protein synthesis. *Pten* deletion counteracts reductions in mTOR signalling and protein synthesis in injured neurons, thereby promoting regeneration. As protein synthesis provides the building blocks of regeneration, the PTEN–mTOR pathway is considered to regulate the regenerative competence of neurons[73]. In addition to protein synthesis in the cell body, local translation in axons has been proposed as a mechanism for axon regeneration in the PNS[74,75]. A role for local translation in CNS axon regeneration remains to be demonstrated.

In addition to the retinal system, *Pten* deletion promotes corticospinal axon growth after spinal cord injury (Fig. 2b,c), indicating that some mechanisms discovered in the retinal system are effective in the injured spinal cord[76]. Not only does *Pten* deletion promote the regeneration of injured corticospinal axons after spinal cord injury but it also promotes the sprouting of spared corticospinal axons after unilateral pyramidotomy injury. In chronic injury, *Pten* deletion also promotes corticospinal regeneration but to a lower extent than after acute injury[77].Viral delivery of short hairpin RNA against *Pten* enhances corticospinal regeneration and even limited functional recovery after spinal cord injury[78,79].

The discovery of the PTEN–mTOR pathway in CNS axon regeneration represented a watershed moment in CNS regeneration research allowing the identification of additional neuron-intrinsic pathways. For the first time, a single genetic manipulation led to clear and reproducible axon growth, both in the optic nerve and the spinal cord, serving as a benchmark case for future regeneration studies.

However, there are several limitations in manipulating PTEN to promote axonal repair. First, the degree of regeneration is still limited: relatively few axons grow through or around an injury site, and the distance of regeneration is limited to several millimetres, at most, in both the optic nerve and spinal cord. Second, functional recovery is also limited and not universally observed[15,79]; however, this is a challenge with any molecular intervention. Third, while targeting PTEN in young mice promotes overt regeneration, there is an age-dependent decline in the regeneration-promoting effect[80]. Fourth, sustained deletion or suppression of PTEN is a risk factor for the induction of malignant transformation of cells, making it useful to explore less risky targets in this signalling pathway[81]. It is clear that other molecular pathways and biological processes important for regeneration remain to be identified and that PTEN–mTOR manipulation will likely need to be combined with complementary pro-regenerative approaches, such as the use of a cellular or non-cellular bridge placed in the lesion cavity, to assemble a more comprehensive strategy to promote regeneration.

Transcriptional control

Several pathways involved in axonal repair illustrate the importance of transcriptional control for axon regeneration. The second pathway to arise from the candidate in vivo screen using the retinal system described above was suppressor of cytokine signalling 3 (SOCS3). SOCS3 is a negative regulator of the Janus kinase (JAK)–signal transducer and activator of transcription (STAT) pathway. Similar to *Pten* deletion, *Socs3* deletion in RGCs leads to retinal axon regeneration after optic nerve injury[82]. Further studies depicted a model whereby injury-induced cytokines and growth factors, such as CNTF and IL-6, signalling through gp130, activate the JAK2–STAT3 pathway, which in turn leads to the expression of pro-regenerative genes. This pathway is normally suppressed by SOCS3 in the injured CNS. In the absence of SOCS3, the suppression is released, and the pathway is activated for a pro-regenerative transcriptional response. CNTF administration further enhances axon regeneration induced by *Socs3* deletion. SOCS3 thus serves as a brake for an injury signalling process that, when unrestrained, leads to transcription of pro-regenerative genes. Interestingly, overexpressing wild type *Stat3* or even a constitutively active variant leads to very limited regeneration of spinal cord dorsal column sensory axons; this effect has been attributed to enhanced growth initiation without sustained axon extension over time[83].

The PTEN–mTOR and SOCS3–STAT3 pathways have synergistic effects when manipulated in combination[84]. After optic nerve crush and with CNTF administration, *Pten–Socs3* double deletion in RGCs induced robust, sustained axon regeneration all the way to the optic chiasm, with a small percent of axons regenerating beyond the chiasm and sometimes in the wrong direction, back towards the uninjured retina. This synergy is also observed in the spinal cord where *Pten–Socs3* double deletion in corticospinal neurons led to substantial sprouting of uninjured corticospinal axons across the spinal cord midline after unilateral pyramidotomy and improved locomotor function[85] (Fig. 2d,e). Combining *Pten–Socs3* deletions and *Cntf–Myc* overexpression yielded perhaps the most dramatic retinal axon regeneration observed to date[86] (Fig. 2f).

The Krüppel-like factors (KLFs) are a family of transcription factors with C2-H2 zinc finger DNA-binding domains. The best-known member of the family is KLF4; KLF4 as well as octamer-binding protein 3/4 (OCT3/4), SOX2 and MYC constitute the original Yamanaka factors to reprogramme induced pluripotent stem cells[87]. Microarray-based gene expression profiling identified E17 and P21 as two developmental stages that represent vastly different axon growth abilities of RGCs[88]. A total of 111 differentially expressed genes were screened with in vitro neurite growth assays, identifying *Klf4* as the most effective suppressor of neurite growth. Additional analyses led to the discovery that multiple members of the KLF family regulate neurite growth, with some suppressing (*Klf4*, *Klf9*) and others activating (*Klf6*, *Klf7*) growth. Cre-mediated *Klf4* gene deletion in RGCs led to retinal axon regeneration[88].

In the spinal cord, overexpression of either *Klf6* or an engineered activated form of *Klf7* (*VP16-Klf7*) also promotes growth of corticospinal axons[89,90]. Just as PTEN–mTOR and SOCS3–STAT3 signalling synergize when manipulated in combination, synergistic effects are also observed between KLFs and SOCS3–STAT3. KLF4 physically interacts with phosphorylated STAT3 (p-STAT3), which leads to the suppression of STAT3-dependent gene expression and increased axon growth. *Klf4* deletion promotes RGC axon regeneration through STAT3 and synergizes with CNTF administration or *Socs3* deletion in promoting retinal axon regeneration[91]. On the other hand, KLF6 co-occupies regulatory DNA elements with STAT3, and *Klf6* overexpression synergizes with STAT3 activation (via overexpressing an engineered *VP16-Stat3* construct) in promoting neurite outgrowth from postnatal cortical neurons in culture[90]. The in vivo significance of this interaction remains unknown.

SOX11 is a transcription factor that has a peculiar effect on regeneration: in the retinal system, SOX11 promotes regeneration of some RGCs but kills others[92]; in the spinal cord, overexpressing *Sox11* promotes regeneration but impairs functional recovery[93]. These results illustrate neuronal subtype-specific effects of signalling pathways, which remain to be elucidated in the spinal cord, and re-emphasize the principle that axon growth or regeneration does not necessarily translate into functional improvement.

Injury signalling

The discovery of the dual leucine zipper-bearing kinase (DLK) pathway in axon regeneration exemplifies the utility of invertebrate genetic model organisms in the study of axon regeneration mechanisms. In the early 2000s, technological advances in laser axotomy spurred the use of invertebrate model organisms, and especially *Caenorhabditis elegans*, to study axon regeneration[94]. Two labs independently identified DLK as a critical regulator of axon regeneration in *C. elegans*[95,96]. DLK (known as DLK1 in *C. elegans*; distinct from another gene encoding delta-like 1 homologue) belongs to the mitogen-activated protein kinase kinase kinase (MAP3K) family of proteins. MAP3Ks, MAP2Ks and MAPKs constitute a phosphorelay module that receives signals from external and internal stimuli and activates downstream cellular responses to challenges or stresses[97]. The *Drosophila melanogaster* DLK homologue (also known as Wallenda) is also required for axon regeneration[98].

There are two mammalian homologues of *C. elegans* DLK: DLK (also known as MAP3K12) and leucine zipper-bearing kinase (LZK; also known as MAP3K13). DLK is important for efficient regeneration and especially for the conditioning lesion effect in the PNS, where prior axonal injury enhances the regenerative response after a second injury[99] (Box 1). DLK is required for retrograde transport of phosphorylated STAT3, presumably carrying an injury signal to the cell body. In the optic nerve, DLK was shown to be required for *Pten* deletion-induced retinal axon regeneration[100]. Paradoxically, DLK also promotes RGC death in the retinal system after optic nerve injury and in a mouse model of glaucoma[100,101]. While LZK itself is not required for RGC death under pathological conditions, it provides partial functional compensation for DLK in RGCs[102]. During development, DLK also promotes apoptosis of motor and sensory neurons[103,104]. Furthermore, DLK promotes distal axon degeneration after axonal injury in both *D. melanogaster* and the mammalian PNS[105]. Thus, DLK may mediate apparently divergent responses in the context of neural injury, including axon regeneration, axon degeneration and cell death.

Similar to DLK, LZK also promotes axon growth in culture[106]. However, the first in vivo role ascribed to LZK was in mediating astrocyte responses after spinal cord injury. Deleting *Lzk* in astrocytes reduces astrogliosis, leading to an expanded (worsened) injury site; overexpressing *Lzk* in astrocytes enhances astrogliosis, leading to a more compact injury site[107]. Other studies implicate DLK in the microglial responses to injury or diseases[108,109], although it is not yet known if DLK acts cell-autonomously in microglia under these conditions. Most recently, neuronal DLK and LZK have been shown to mediate CST axon regeneration and sprouting in the mammalian spinal cord[110]. This study further indicated that DLK-mediated and LZK-mediated injury signalling function in parallel to PTEN–mTOR-mediated regenerative competence (rather than in a linear pathway), and that these two processes are independently required for successful regeneration. Taken together, these studies indicate that DLK and LZK signalling pathways regulate the injury responses of multiple cell types, including neurons, astrocytes and, possibly, other glia in the mammalian spinal cord. This feature is not unique to DLK and LZK: the SOCS3–STAT3 pathway and, to some extent, the PTEN–mTOR pathway have also been shown to regulate responses to injury in astrocytes[111–113]. Thus, there is an emerging theme that neuron-intrinsic pathways that regulate axonal repair may also regulate glial responses to CNS injury.

Epigenetic regulators

Epigenetic regulation has been actively investigated in many areas of biology. Through chromatin modifications (for example, DNA methylation and histone acetylation) without changing DNA sequences, epigenetic regulation is thought to underlie lasting changes in gene expression. Specific epigenetic marks are often associated with open or repressed states of the chromatin. Pharmacological inhibition of the histone deacetylase HDAC1 provided evidence suggesting a role for histone modification in dorsal column sensory axon regeneration after spinal cord injury[114]. P300/CBP-associated factor (PCAF), which has histone acetyl transferase activity, was shown to mediate the peripheral nerve conditioning lesion effect, and overexpressing *Pcaf* promotes dorsal column sensory axon regeneration after spinal cord injury[115]. Exposure to an enriched environment promotes sensory axon regeneration after spinal cord injury, which is dependent on CREB binding protein (CBP)-mediated histone acetylation[116]. Genetic or pharmacological inhibition of HDAC3 also promotes dorsal column sensory axon regeneration after spinal cord injury[117]. Epigenetic and expression profiling of dorsal root ganglion (DRG) neurons reveals an increase in chromatin accessibility and histone H3 acetylation that correlate with a robust transcriptional response after peripheral injury; CTCCC-binding factor (CTCF), a chromatin organizer known for its insulator activity, was shown to contribute to peripheral axon regeneration[118]. The role of CTCF in CNS neurons remains to be demonstrated.

Regarding DNA methylation, the levels of TET3 (an enzyme mediating DNA demethylation) and 5-hydroxymethylcytosine (5hmC) modifications are elevated after peripheral but not central lesions of DRG axons. TET3 is important for peripheral axon regeneration, while TET1 is important for PTEN deletion-induced retinal axon regeneration[119,120]. Forced expression of three Yamanaka factors (*Oct4*, *Sox2* and *Klf4*) reprogrammes RGCs to an immature DNA methylation pattern and promotes axon regeneration after injury in a TET1-dependent and TET2-dependent manner[121]. However, it is unclear how the growth-promoting function of KLF4 here would reconcile with the previously reported negative role of KLF4 in axon regeneration[88,122]. Ubiquitin-like-containing PHD and RING finger domains protein 1 (UHRF1), which normally recruits DNA methyltransferases to DNA, has been shown to be important for peripheral axon regeneration by mediating the repression of PTEN and REST[123]; this mechanism has not been tested in the CNS.

Distinct from epigenetic modifications are RNA modifications such as N^6-methyladenosine (m^6A), which mediates epitranscriptomic regulation through RNA stability and protein translation[124]. m^6A and enzymatic components required for this RNA modification have been shown to mediate peripheral and PTEN deletion-induced retinal axon regeneration[125]. In addition to histone modifications and DNA methylation, pioneer factors, which bind condensed chromatin and facilitate the binding of other transcription factors, may be key targets in a combinatorial approach to initiate a pro-regenerative transcription programme[126]. *Lin28*, which regulates *let-7* microRNAs, promotes axon regeneration after peripheral, optic nerve and spinal cord injury when overexpressed[127,128], representing another epigenetic mechanism regulating regeneration.

Cytoskeleton dynamics and axonal transport

All axon repair strategies rely on changes in cytoskeletal dynamics in growth cones to achieve growth. Inhibiting the actin-binding protein non-muscle myosin II, leads to reorganization of both actin and microtubules in the growth cone, resulting in axonal extension in cultured neurons[129]. Consistent with this, deleting non-muscle myosin IIA and IIB promotes retinal axon regeneration after optic nerve injury without affecting the expression of pro-regenerative genes[130]. There is contrasting evidence that either stabilization (reduction) or activation

of microtubule dynamics increases regeneration. Knocking down expression of Fidgetin, a microtubule-severing enzyme that trims the labile domain of microtubules, promotes sensory axon regeneration into the spinal cord following a dorsal root crush[131]. Genetic gain-of-function and loss-of-function analyses indicated that profilin 1, an actin-binding protein, promotes axon regeneration after peripheral nerve or spinal cord injury, which may occur through increasing actin retrograde flow, microtubule polymerization and invasion into filopodia[132]. On the other hand, both the microtubule-stabilizing drug Taxol and a blood–brain barrier-permeable alternative, epothilone B, enhance axon regeneration and functional recovery[133,134]. Because these microtubule-stabilizing agents also reduce the fibrotic scar, it is not clear whether enhanced regeneration following their application can be attributed more to effects on neurons or on the fibrotic scar. In RGCs, doublecortin-like kinases (DCLKs) promote neuronal survival and axon regeneration through distinct mechanisms, including microtubule dynamics and retrograde injury signalling[135].

Related to the axonal cytoskeleton is microtubule-dependent axonal transport. Axonal transport has long been recognized as an important factor in peripheral axon regeneration and has gained increased attention in CNS regeneration[136]. Injury signalling from the injured axonal tip to the cell soma relies on retrograde transport[137,138], while anterograde transport provides distal axons with the building blocks for axonal growth. Cargos include proteins, vesicles and organelles such as mitochondria (see below). In the PNS, certain mRNA species are selectively transported into the axons, allowing for local translation that supports axon regeneration[139]. The degree to which this process can be manipulated to enhance CNS axon regeneration remains to be explored. The exclusion of growth molecules, such as integrins and their RAB11 carriers, from axons of mature CNS neurons has been proposed as a mechanism that hinders regeneration[136]. Overexpression of the scaffold protein protrudin increases the accumulation of integrins, RAB11 and endoplasmic reticulum in axons and promotes CNS axon regeneration in vitro and in vivo, highlighting the importance of selective axonal transport in CNS regeneration[140].

Energy metabolism

Mitochondrial motility and local energy deficits are established factors in limiting regeneration in the PNS. Genetically deleting the mitochondria-anchoring protein syntaphilin promotes mitochondrial transport and axon regeneration after sciatic nerve crush[141]. Recently, this finding was extended to the CNS where deleting syntaphilin promotes corticospinal, serotonergic and dopaminergic axon growth that is accompanied by functional improvement[142]. Further studies indicated that p21-activated kinase 5 (PAK5) positively regulates axonal mitochondria remobilization and replenishment after injury by phosphorylating syntaphilin and thereby suppressing mitochondrial anchoring. Accordingly, viral mediated expression of a constitutively active PAK5 enhances corticospinal axon sprouting after unilateral pyramidotomy[143]. Conversely, ARMCX1, a protein that mobilizes (rather than immobilizes) mitochondria, was found to promote axon regeneration of RGCs after optic nerve injury in genetic gain-of-function and loss-of-function analyses[144]. These studies illustrate the important roles of mitochondria motility and local energy metabolism in supporting axon regeneration in the CNS. In *D. melanogaster*, co-activating PI3K and epidermal growth factor receptor (EGFR) in glia increases aerobic glycolysis and enhances CNS axon regeneration via glia-derived metabolites such as L-lactate, whereas local application of L-lactate in the injured mouse spinal cord enhances CST axon regeneration[145]. Given the importance of energy metabolism for regeneration, it will continue to deserve attention in future studies.

Newly emerging hypotheses

Several new hypotheses have emerged from recent studies that highlight key principles of CNS regeneration.

Persistent immature state

Contrary to conventional wisdom first articulated by Ramon y Cajal[1], adult CNS neurons can revert to an immature state and regenerate axons if presented with an enabling growth environment, such as one provided by NSC transplantation (Box 3). Corticospinal axons, which are particularly resistant to regeneration-enhancing strategies, readily regenerate into and sometimes beyond NSCs transplanted to a spinal cord injury site[146]. This regeneration is dependent on the placement of a neural milieu in the injury site that is homologous to that in the spinal cord. For instance, a spinal cord NSC graft supports extensive corticospinal regeneration but a forebrain NSC graft does not[146]. Regenerating host corticospinal and sensory axons innervate appropriate self-organized motor and sensory interneuronal microdomains that develop within NSC grafts[147,148]. Remarkably, transcriptomic profiling indicates that injury alone without any intervention is sufficient to revert adult corticospinal neurons into an embryonic transcriptional state but only for a limited time (2 weeks) and that NSC transplantation prolongs this immature state by at least an additional 2 weeks[149]. Bioinformatic analyses on the injured versus regenerating corticospinal transcriptome reveals both known and potentially new regulators of axonal growth, including cAMP, cAMP-responsive element-binding protein 1 (CREB1), PTEN–mTOR, MAPK, P53, tumour necrosis factor (TNF), transcription factor 7-like 2 (TCF7L2) and huntingtin. Among these, huntingtin represents a hub in a regulatory network involving *Fos*, *Nfkb*, *Bdnf*, *Creb* and other growth-related genes. In vivo validation with genetic loss-of-function experiments confirmed the role of huntingtin in supporting NSC transplant-induced corticospinal regeneration[149]. Thus, CNS injury alone may induce an immature state in adult neurons but only temporarily; NSC transplantation prolongs this immature state, which could be key to regeneration (Fig. 3a).

Neuronal relays

Regenerating axons do not have to rebuild the exact original neural circuits to be functional as has been demonstrated in NSC transplantation studies. Transplant-derived neurons may serve as substrates to form neuronal relays across anatomically complete injuries as evidenced by improved electrophysiological and functional outcomes following severe spinal cord injury[69] (Fig. 3b). While the organizing principles for such relay formation remain to be elucidated, anatomical tracing studies indicate that most NSC-derived neuronal relays through the graft are polysynaptic in nature: host corticospinal axons terminate in the rostral part of the graft, while transplant-derived neurons in the caudal part of the graft extend axons and synapse on host neurons distal to the injury[150]. Thus, neuronal information can presumably be transmitted through new circuits that are mediated by multiple neuronal subtypes formed in the graft. Indeed, ex vivo and in vivo calcium imaging studies of the spinal cord demonstrated that host corticospinal neurons innervate graft neurons, which in turn innervate host neurons below the lesion; likewise, sensory stimulation of the host elicits neuronal responses in the graft[151]. Future studies are required to understand the organizing principles of neuronal relay formation and how they can be optimized for functional recovery.

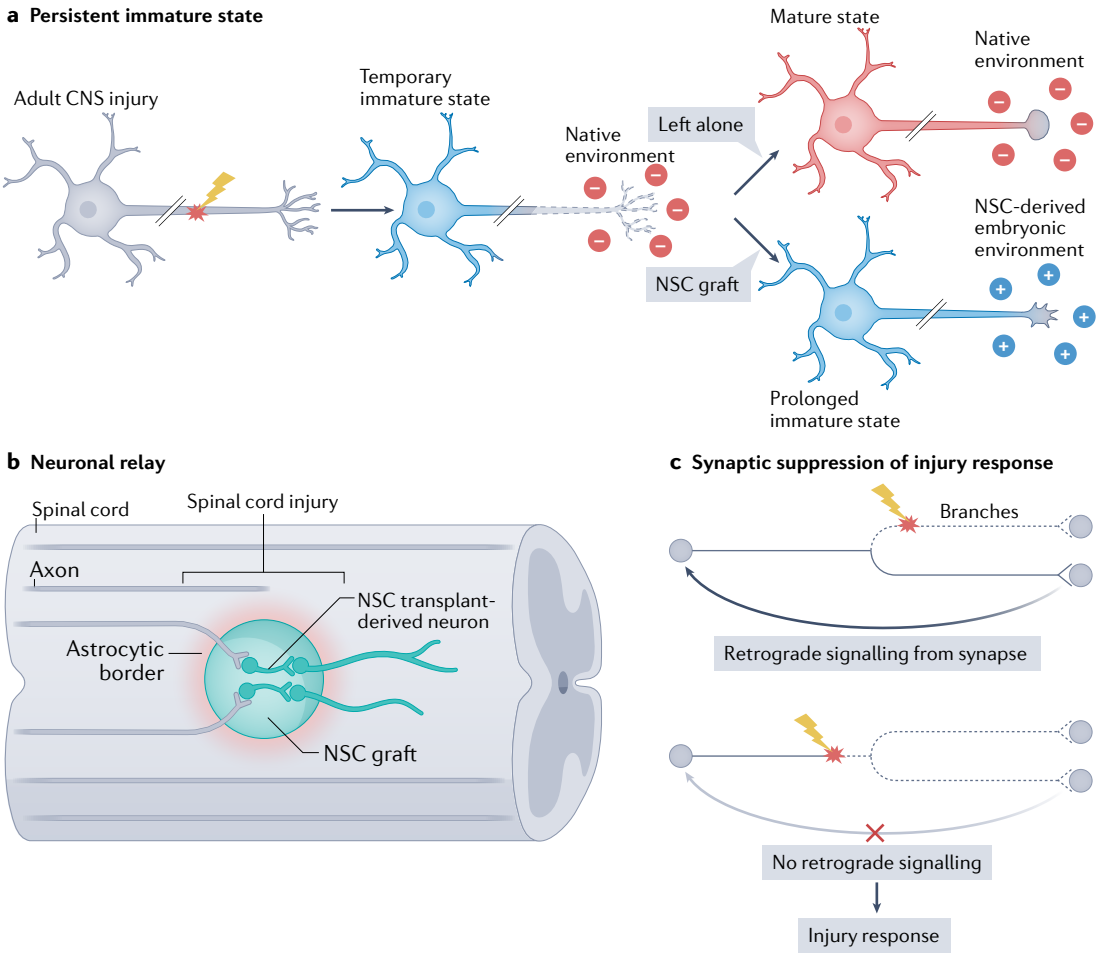


Fig. 3 | Newly emerging hypotheses for regeneration of the CNS. a, Injury induces a temporary immature neuronal state; achieving a more persistent immature neuronal state may be key to regeneration. Neural stem cell (NSC) grafts prolong this immature state and induce axon regeneration. Dots with + and − signs indicate growth-promoting and growth-inhibiting factors, respectively, in the growth environment. **b**, After NSC transplantation at the injury site, transplant-derived young neurons profusely extend axons into the distal host tissue. Host neurons also regenerate into the transplant-modified injury site, synapsing on transplant-derived neurons. Thus, a neuronal relay forms with the transplant, providing an intermediate target for injured host axons. Experimental evidence suggests that NSC-derived neuronal relays are mostly polysynaptic in nature, involving multiple transplant-derived neurons. **c**, After injury, some spared synaptic contacts may retrogradely signal onto neuronal cell bodies to suppress axon regeneration and other injury responses. When most synaptic outputs are eliminated, the absence of such a retrograde mechanism from the synapses allows for a strong injury response such as axon regeneration (or cell death, depending on other signalling events in the cell). CNS, central nervous system.

Synaptic suppression of regeneration

The idea of glial inhibition has been so influential in the field that other extrinsic influences on regeneration may have been overlooked. The synaptic suppression hypothesis postulates that synaptic connectivity and/or transmission may suppress structural changes, such as axonal regeneration, within an injured neuron[152,153] (Fig. 3c). Because synaptic connections cannot be entirely neuron intrinsic in principle, they can be considered a form of extrinsic influence on regeneration. When regenerating axons encounter certain non-neuronal cells, such as NG2 cells, in the CNS, presynaptic or synaptic-like structures form and regeneration stops[154,155]. In vivo imaging in the mouse spinal cord indicates that the presence of a surviving axonal branch suppresses the regeneration of the injured branch but that regeneration occurs when both branches are injured with a complete loss of all synaptic contacts[156]. It makes economic sense for CNS neurons to preserve the remaining neuronal structure and function rather than devote resources to a regenerative process that is unlikely to be productive. Pharmacological inhibition of the α2δ2 subunit of voltage-gated calcium channels, which weakens synaptic transmission, enhances axon regeneration[157]. A recent study indicated that the presence of an active synaptic vesicle-priming machinery (including key components such as the presynaptic active zone protein Munc13) suppresses axon regeneration, lending further support to the synaptic suppression hypothesis[158]. Future work is required to extend these findings beyond the dorsal column sensory system and to elucidate how presynaptic structures or processes signal to the cell bodies, presumably retrogradely, to suppress regeneration. The synaptic suppression hypothesis could explain why the same molecular manipulations

(for example, PTEN–SOCS3 or IGF1–osteopontin) elicit more robust axon regeneration in the optic nerve than in the spinal cord: the grey matter in the spinal cord represents a source of potential synaptic contacts for regenerating axons.

Strategies to promote functional recovery

Currently, five strategies are being pursued to promote functional recovery following spinal cord injury; two of these do not involve axonal growth, whereas three do. These strategies likely need to be combined to maximize the potential for functional recovery.

Neuroprotection

Neuroprotection has been extensively investigated in the context of spinal cord injury, traumatic brain injury and stroke. The scientific premise is that the body's response (for example, the immune response) to the primary injury leads to secondary injury that causes additional neuronal loss and exacerbates the outcome. Mitigating this secondary injury response may preserve cells and tissues that would otherwise be lost due to secondary injury (Fig. 4a). Therapeutic goals include the rescue of neuronal loss, a reduction of inflammation and the preservation of tissue integrity at the injury site. Despite its seeming attainability, neuroprotection has not realized its potential in the clinic. The only drug that was once approved and regularly used clinically to treat spinal cord injury, methylprednisolone (a corticosteroid used to curb inflammation), is no longer an accepted standard of care due to a lack of aggregate evidence for its efficacy[159]. Systemic or local cooling (hypothermia) at the injury site has also been explored; however, consistent beneficial effects have not been established[160]. More robust, reproducible benefits in preclinical models will likely improve the chance of positive outcomes in clinical trials. The availability of small-molecule inhibitors of therapeutic targets may accelerate clinical translation[161].

Functional plasticity

Neuronal connections can be strengthened or weakened through changes to synapses, leading to changes in neural circuits and behaviour (Fig. 4b). This plasticity allows organisms to adapt to the changing environment to survive. Learning and memory are a prime example of functional plasticity. Similar functional plasticity occurs after spinal cord injury, underlying some spontaneous functional recovery. A well-known example of functional plasticity is the crossed phrenic phenomenon wherein a latent crossed phrenic pathway can be activated to mediate the recovery of respiratory function following a lateral injury to the high cervical cord. Most spinal cord injuries are not anatomically complete, even when assessed as 'clinically' complete. Intensive rehabilitation, often prescribed for patients with spinal cord injury, may strengthen neuronal connections to improve functional outcome for specific tasks, while the same training may weaken connections for other, untrained tasks as demonstrated in animal studies[162].

Electrical stimulation has long been pursued as a method to induce functional changes in the injured CNS. This area has gained renewed interest in recent years due to advances in engineering solutions that enable high-precision bio-adapted devices and stimulation protocols[163]. Many research groups are combining epidural electrical stimulation with rehabilitative training to improve functional recovery in people with spinal cord injury[164–166]. Approaches involving brain machine interfaces can be considered an extension of the native functional plasticity of the CNS. One principle for electrical stimulation (sometimes combined with chemical stimulation and together referred to as neuromodulation) is to enhance the functional state of the circuit so that the residual pathway can respond to supraspinal and/or sensory input that would otherwise be subthreshold. Applying the same logic but with a rather different approach, a recent study showed that potassium–chloride cotransporter 2 (KCC2) agonist administration can also convert a dysfunctional circuit into a functional state following incomplete spinal cord injury by suppressing spinal inhibitory neurons[167]. These converging pieces of evidence indicate that elevating the functional state of spinal circuits via intrinsic adaptive plasticity is a viable approach to promote recovery after incomplete spinal cord injury.

Structural plasticity

Here, we refer to structural plasticity as sprouting from uninjured neurons and readily detectable with light microscopy but does not represent true regeneration (Fig. 4c). Many of the molecular manipulations that promote regeneration also promote sprouting. Promoting sprouting is less challenging than promoting regeneration. In the course of studying myelin inhibitors and inhibitory extracellular matrix, sprouting was extensively documented. Indeed, in some earlier studies of spinal cord injury, what was described as regeneration turned out to be sprouting from spared axons. Perhaps the leading candidate sprouting therapy for clinical translation is chondroitinase given the replication of its effects by numerous independent groups and an efficacy study in non-human primates[43,53].

Regeneration of exogenous neurons

Various cell types have been explored as potential therapies in models of spinal cord injury, including fibroblasts, mesenchymal stem cells, oligodendrocyte progenitor cells and NSCs (Fig. 4d). Some of these cell types may provide tissue bridges to support axonal growth, trophic support or modulate the immune response. NSCs have the potential to contribute new neurons following transplantation and thus differ from mechanisms focused on endogenous repair. For NSC-derived neurons to be functionally relevant, they must form synaptic connections with host neurons and integrate into host neural circuits. As discussed above, recent studies provide evidence that NSCs form neuronal relays across sites of spinal cord injury. To achieve 'adaptive plasticity', the ensuing remodelled neural circuits would have to be capable of supporting functional recovery by 're-wiring' host neural circuits both above and below the injury. In other words, an active learning process by the CNS might be required to utilize new circuits effectively.

Regeneration of endogenous neurons

Endogenous regeneration refers to regeneration of host axons into and beyond an injury site, without involving transplant-derived neurons (Fig. 4e). Compared with exogenous regeneration, where long-distance axonal growth is readily accomplished by NSC-derived young neurons, endogenous regeneration relies on long-distance host axonal growth through or around the injury site by injured adult neurons; this remains a major challenge in the mammalian spinal cord. The past 14 years have witnessed tremendous advances in understanding the molecular regulation of axon regeneration, especially regarding neuron-intrinsic mechanisms. It is now generally accepted that optimal conditions for regeneration include a supportive intrinsic growth state in the neuron, a conducive growth environment within and surrounding the injury site, and the presence of factors distal to the injury site that may provide trophic support and/or guidance[68].

Conclusions and perspectives

In summary, substantial progress has been made in the past decade in understanding the molecular and cellular mechanisms of axonal repair after spinal cord injury. Conceptually, a glial inhibition-centric view has been superseded by the idea that a multifaceted approach, including neuron-intrinsic regulation, is required to support regeneration.

The NSC field has advanced rapidly and yielded degrees of growth that were unimaginable only a few years ago. Transcriptomic studies

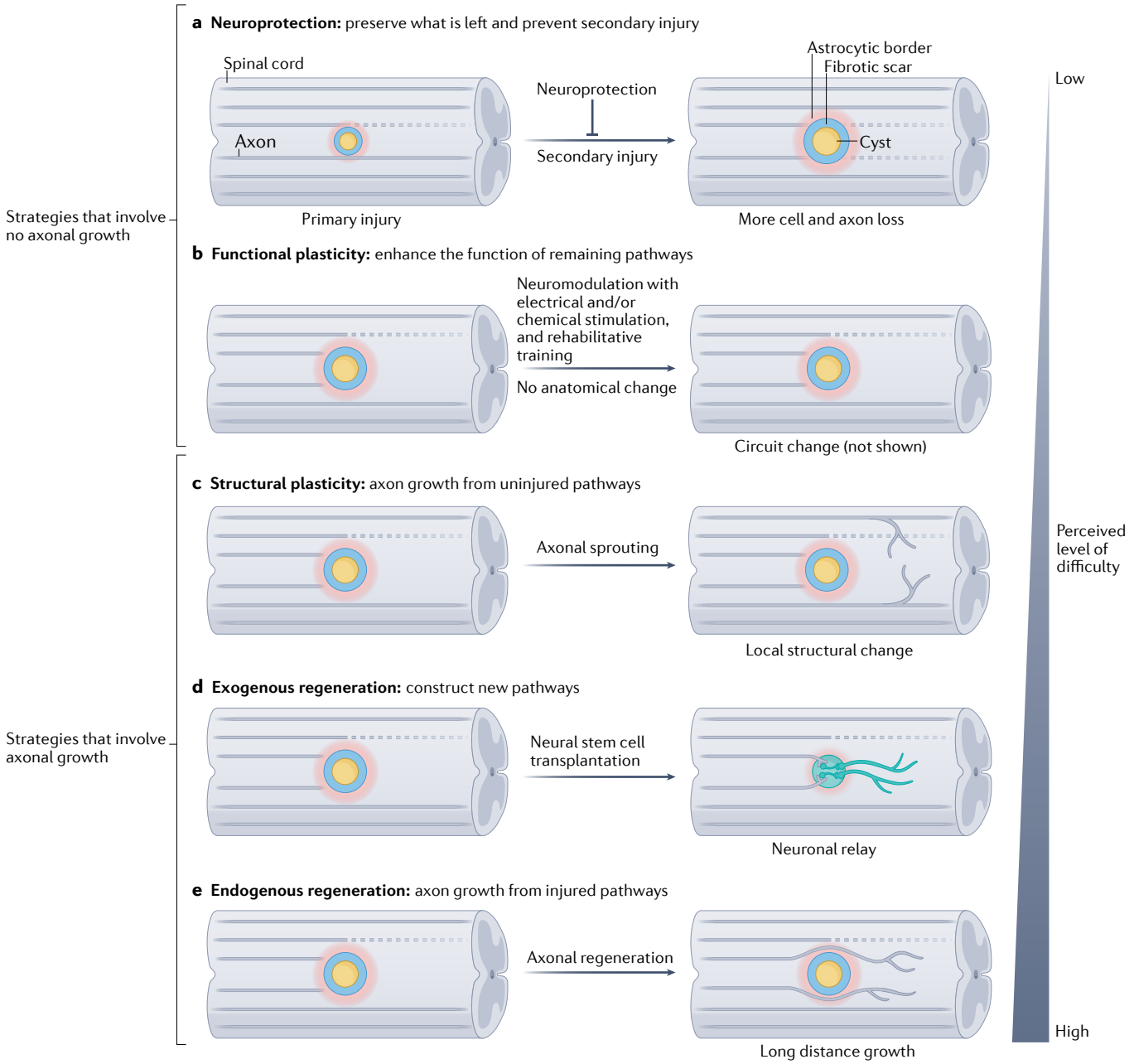


Fig. 4 | Five strategies to promote functional recovery after spinal cord injury. a, Neuroprotection aims to preserve remaining tissues and cells from secondary injury responses that would otherwise exacerbate outcomes. **b**, Functional plasticity refers to inducing changes in the neural circuits that do not rely on axonal growth; this is primarily achieved by neuromodulation with electrical and/or chemical stimulation, and rehabilitative training. **c**, Structural plasticity refers to changes involving axonal growth of uninjured neurons (that is, sprouting) but not the long-distance regeneration of damaged pathways. **d**, Exogenous regeneration involves axonal regeneration with the aid of transplant-derived neurons (for example, neural stem cells), which promote functional recovery through a neuronal relay. **e**, Endogenous regeneration relies solely on the regeneration of endogenous neurons without the aid of any transplant-derived neurons. Strategies in parts **a** and **b** do not involve axonal growth, whereas strategies in parts **c** and **d** involve axonal growth. There is some debate on whether exogenous or endogenous regeneration is more difficult to achieve, but both have their unique set of challenges.

Glossary

Astrogliosis
Also known as reactive astrogliosis, astrocytosis or astrocyte reactivity, refers to the astrocyte response to injury, disease or other insults and challenges in the central nervous system (CNS). Astrocytes proliferate, undergo hypertrophy and express increased levels of markers of reactivity, including glial fibrillary acidic protein (GFAP) and vimentin. At the injury border, highly reactive astrocytes form the astrocyte border that contains the fibrotic scar and lesion core.

Central nervous system
(CNS). Part of the nervous system that consists primarily of the brain and spinal cord. The optic nerve is unusual among the cranial nerves in that it is part of the CNS and is often used as a model to study CNS axon regeneration.

Corticospinal tract
(CST). Controls voluntary movement in humans. In rodents, the CST is often used as a model to study axon regeneration after spinal cord injury. The neurons that give rise to the CST are called corticospinal neurons, sometimes referred to as corticospinal motor neurons.

Chondroitin sulfate proteoglycans
(CSPGs). A group of molecules that have a protein core and a chondroitin sulfate side chain. Examples include neurocan, aggrecan, brevican, phosphacan and versican. CSPGs are considered inhibitory to axonal repair after central nervous system (CNS) injury.

Dorsal root ganglion
(DRG). Dorsal root ganglia are located outside the spinal cord and contain the cell bodies of sensory neurons that are pseudo-unipolar in morphology, meaning that they extend one axon from the cell body, but this axon soon bifurcates into two major axonal branches with one branch travelling in the peripheral nervous system (PNS) and the other extending into the central nervous system (CNS). This unique anatomical feature makes DRG neurons an appealing model to study the differential regenerative capabilities between the CNS and the PNS.

Growth cones
Hand-like structures at the tip of developing or regenerating axons. The outer region is mainly supported by the actin cytoskeleton and the inner region is mainly supported by the microtubule cytoskeleton. Growth cones are responsible for sensing, interpreting and responding to environmental cues. They are critical for axon growth and regeneration.

Janus kinase (JAK)–signal transducer and activator of transcription (STAT) pathway
This pathway responds to extracellular signalling molecules, such as cytokines and growth factors, to trigger cellular responses through the regulation of transcription. Ligand-receptor interaction activates JAKs, which then activate STATs, which in turn regulate transcription.

Neural stem cell
(NSC). Can give rise to a variety of cells of neural lineages, including neurons and glia. NSC transplantation has the potential to improve functional recovery by promoting regeneration and neuronal relay. In transplantation studies, NSCs may be referred to as neural progenitor cells due to the uncertain or mixed developmental stage of the transplanted cells.

Oligodendrocyte progenitor cells
These glial cells are marked by their expression of neural/glial antigen 2 (NG2) and can proliferate and differentiate into mature myelinating oligodendrocytes in injury or disease. Oligodendrocyte progenitor cells are also known to contribute to scar formation after spinal cord injury.

Peripheral nerve bridges
A piece of peripheral nerve is taken from the peripheral nervous system (PNS) and transplanted into the central nervous system (CNS), where it serves as a conduit or bridge for axons to regenerate through. This is based on the observation that the PNS provides an environment conducive to axonal regeneration.

Peripheral nervous system
(PNS). Part of the nervous system outside the brain and the spinal cord that comprises the nerves and the ganglia. The PNS has a much higher capacity for axon regeneration than the central nervous system (CNS).

Retinal ganglion cells
(RGCs). Neurons in the mammalian retina that convey information from the retina to the rest of the brain. Their accessibility and long axonal projections make RGCs an excellent model system to study axon regeneration after optic nerve injury.

have revealed that adult neurons revert to an immature state in which they are capable of regenerating using mechanisms previously employed during neural development. The field has advanced through the utilization of several model systems, including optic nerve injury, invertebrate model organisms and state-of-the-art 'omics' technologies. As the same molecular pathway may regulate the injury responses of multiple cell types, deciphering cell type-specific roles of various molecular pathways is important not only for understanding basic biology but also for clinical translation: it may be necessary to target the same pathway differently in distinct cell types at differing time points after injury. Meanwhile, manipulating multiple molecular pathways both intrinsic and extrinsic to neurons will likely be required to sustain long-distance regeneration, with or without cell transplantation. Biomaterials and 3D bioprinting are providing additional tools to aid spinal cord repair.

How regenerating axons make useful connections distal to the injury remains an important challenge. Future studies will address how axonal repair can be optimized so that useful synaptic contacts and circuits are established to support functional recovery. The timing for encouraging appropriate synaptic contacts may be key: for example, making synapses too early could stop axon regeneration[153], whereas making synapses too late may lead to the elimination of regenerated axons[86]. Methods to increase axon conduction and myelination of regenerating axons are also important considerations[168,169]. The recent realization that patients with severe spinal injury can regain some function following electrical stimulation and rehabilitative training has opened new possibilities for enhancing regeneration. The successful clinical translation of regenerative therapies by manipulating molecular and cellular events will almost certainly require combination with activity-dependent strategies such as intensive rehabilitative training and electrical stimulation[163].

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