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Can regenerating axons recapitulate developmental guidance during recovery from spinal cord injury?

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Abstract

The precise wiring of the adult mammalian CNS originates during a period of stunning growth, guidance and plasticity that occurs during and shortly after development. When injured in adults, this intricate system fails to regenerate. Even when the obstacles to regeneration are cleared, growing adult CNS fibres usually remain misdirected and fail to reform functional connections. Here, we attempt to fill an important niche related to the topics of nervous system development and regeneration. We specifically contrast the difficulties faced by growing fibres within the adult context to the precise circuit-forming capabilities of developing fibres. In addition to focusing on methods to stimulate growth in the adult, we also expand on approaches to recapitulate development itself.

CNS development and circuit generation have shared many similarities throughout evolution. Morphogens induce differentiation of discrete neural regions. Axon guidance molecules and target-derived factors direct extending fibres to connect with appropriate targets. Immature connections are refined by activity-dependent experience prior to becoming largely fixed in the adult. After this intricate process of development is completed, the CNS response to injury diverges widely among vertebrates. Several fish species retain the ability to completely regenerate transected spinal cords in adulthood, whereas adult human spinal cord injury (SCI) victims remain permanently paralysed.

Here, the obstacles to mammalian adult spinal cord regeneration are contrasted with the precision of neuro-development and the plasticity of youthful circuits. Regeneration itself encompasses several types of neuronal response to injury; direct regrowth of severed axons represents 'true' axonal regeneration, whereas sprouting from nearby uninjured fibres or proximal locations along severed axons has a compensatory role. Although recent advances have brought us closer to being able to clear some obstacles to regeneration, adult nerve fibres often display haphazard growth and are unable to efficiently reform functional circuits. To maximize the effectiveness of repair of the damaged spinal cord, a more faithful recapitulation of developmental pathfinding and circuit-refining mechanisms is likely to be beneficial.

We emphasize two approaches to recapitulating development in the injured CNS: re-establishing crucial developmental cues in the correct pattern to guide regenerating axons, and

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Competing interests statement

The authors declare no competing financial interests.

DATABASES

The following terms in this article are linked online to:

Entrez Gene: <http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=Gene> EphA4 | ephrin B3 | L1CAM | neogenin | NgR | Nogo | RGM-A | ROBO1 | ROBO2 | ROBO3 | Sema3A | SHH | WNT

OMIM: <http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=OMIMHGPPS>

maximizing the sprouting and plasticity of intact fibres through sensory feedback rehabilitation techniques.

CNS development: growth and guidance

Neuronal differentiation and migration

A set of diffusible signalling molecules directs the differentiation of ectodermal tissue into discrete regions along the early neural tube. Molecules that inhibit bone morphogenetic protein 4 signalling nudge ectodermal tissue down the neural pathway^{1,2}. Basic fibroblast growth factors (bFGFs) and WNT proteins stimulate differentiation into anterior neural structures, whereas retinoids stimulate posterior neural fates^{3–6}. In the developing spinal cord, the floor plate and nearby notochord secrete sonic hedgehog (SHH), which signals the ventral cord to differentiate into motor neurons and ventral interneurons^{5,7,8}.

More recently, many of these morphogens have been shown to also function as axon guidance molecules^{9–12} (see below). In addition, several morphogens persist after development, when they might continue to regulate stem cell division and differentiation^{13,14}. The role of adulthood morphogens in the context of CNS injury is not well characterized.

Traditionally, the ectodermal lineage was believed to branch into separate neuronal and glial lineages early during development. However, many studies during the past decade have identified radial glia as a multipotent progenitor cell type that gives rise to both neurons and glia in the subventricular zone and other regions of the CNS, including the spinal cord.

Remarkably, a population of radial glia persists in the adult, providing a source of new neurons for the hippocampal dentate gyrus, olfactory bulb and perhaps other regions^{15–18}. In addition, mature astrocytes can revert to a de-differentiated radial glia phenotype, serving as migratory scaffolds for newly generated or transplanted neurons after CNS injury^{19,20}. Harnessing the ability of radial glia reservoirs and de-differentiating astrocytes could provide powerful tools for emulating the favourable developmental environment after CNS injury.

Box 1 | CNS myelin-associated inhibitors

Injured CNS nerves fail to regenerate, whereas their peripheral counterparts recover relatively rapidly. Differences in the myelin sheaths between central and peripheral compartments explain much of this difference in regenerative ability. Peripheral myelin is produced by Schwann cells, whereas central myelin is produced by oligodendrocytes. Myelin-associated inhibitors (MAIs) are proteins expressed on oligodendrocyte surfaces that interact with axonal receptors to limit neurite outgrowth. Several of these MAIs and their receptors have been characterized in the past decade (for reviews, see REFS^{131, 190–192}).

Myelin-associated glycoprotein (MAG). MAG was identified as a MAI in 1994 (REFS^{193,194}). It is a member of the immunoglobulin superfamily that is present in both peripheral and central myelin^{126,193}. *Mag*^{-/-} mice do not show increased regeneration following CNS injury¹⁹⁵.

Nogo. Nogo cDNA was characterized in 2000 (REFS^{196–198}). It is a member of the Reticulon family and an antigen for IN-1 antibody¹⁹⁶. It has two separate inhibitory domains: a unique amino-terminal region and a conserved 66-residue loop^{196,197}.

Gene-disruption studies show variable amounts of regeneration following SCI^{199–201}.

Oligodendrocyte myelin glycoprotein (OMgp). OMgp was identified as a MAI in 2002 (REF. 127). It is a glycosylphosphatidylinositol (GPI)-linked protein. Genetic studies are in progress.

Nogo-66 Receptor (NgR). NgR was identified in 2001 (REF. 117). It is a GPI-linked protein that was also found to be the receptor for MAG and OMgp^{20,125–127,202}. Several putative co-receptors have been identified, including p75, LINGO1 and TAJ/TROY^{202–204}. *NgR*^{-/-} mice show varying amounts of regeneration following spinal cord injury (SCI)^{157,158}.

NgR2. NgR2 was characterized as a NgR homologue in 2003 (REFS 205,²⁰⁶). It binds MAG, but not Nogo or OMgp²⁰⁷.

Ephrin B3. Ephrin B3 was identified as a MAI in 2005 (REF. 39). It is a member of the transmembrane ephrin B ligand family that binds to the EphA4 receptor on corticospinal tract (CST) axons⁵⁶. It has a developmental role in CST guidance⁵⁴. A role for myelin-derived ephrin B3 *in vivo* has not yet been found.

Repulsive guidance molecule A (RGM-A). RGM-A was previously characterized in a retinotectal pathway^{37,38}, and was proposed to act as a MAI in 2006 (REF. 40). Antibodies to RGM-A have improved CST regeneration and SCI recovery in rats⁴⁰. However, expression of the neogenin receptor by spinal cord axons has not been demonstrated. Genetic studies are in progress.

Tract directors: axon guidance molecules

Incredibly, a relatively limited set of guidance factors and their receptors mediate the guidance of trillions of axons to their diverse targets. Although various extracellular matrix (ECM) molecules affect axon guidance, we focus on several well-characterized classic guidance molecules: the netrins, semaphorins, the SLIT family, ephrins and repulsive guidance molecules (RGMs)²¹.

It is crucial to note that most of the molecules involved in guiding growing axons persist after development is completed²². However, differences in their distribution, especially after CNS injury, present regenerating axons with a drastically altered signalling environment. Of more than 50 guidance factors and receptors included in our search of the literature, the majority maintains some expression in the adult, but none maintains identical distribution (TABLE 1; see online Supplementary information S1 (table)). The rearrangement of these cues makes the task of accurately guiding regenerating axons in injured adults even more difficult. Therefore, re-establishing at least part of the developmental pattern of guidance molecule expression would contribute to any regenerative approach for treating adult CNS injury.

Netrins are homologous to the laminin ECM molecule²³. Functionally conserved from flies to humans, netrins act as diffusible midline cues in the developing CNS^{23–25}. Netrin signalling through DCC (deleted in colorectal cancer) receptors generally mediates attractive responses, whereas signalling through UNC5 receptors mediates repulsion along with DCC^{23,24}.

The SLIT family is another conserved group of guidance factors with prominent midline activity, and a predominantly repellent effect on axons^{26–29}. Developing commissural axons extending from the dorsal spinal cord suppress the surface expression of the SLIT receptors ROBO1 and ROBO2 until they cross the midline. The more recently identified ROBO3 isoform has the distinct role of suppressing SLIT sensitivity until after axons have crossed the midline³⁰. After crossing the midline, ROBO1 and ROBO2 surface expression is upregulated and ROBO3 expression is downregulated, leading to increased SLIT sensitivity, which prevents commissural axons from re-crossing the midline³¹. The embryonic floor plate has a

crucial role in producing SLITs, netrins and other midline guidance factors. One of the difficulties in recapitulating the developmental guidance factor milieu will be to mimic the floor plate's function in the adult CNS.

An evolutionarily economical mechanism for generating a broad range of position-dependent signalling cues from a limited set of molecules is gradient formation³². The membrane-associated signalling family of ephrin ligands and Eph receptors has diverse roles in both the developing and adult CNS^{33,34}. In the developing retinotectal system, complementary gradients of ephrin A ligands and EphA receptors are essential for forming a topographic map between the retina and the tectum^{21,35,36}. In the chick, and possibly in mammals, there is redundancy in the use of gradients to form topographic maps: RGM-A expression also exhibits a gradient from the anterior to the posterior tectum³⁷, and its receptor, the DCC-related protein neogenin, is found in a nasal to temporal retinal ganglion cell gradient³⁸. Recent publications propose roles for ephrin B3 and RGM-A in inhibiting axon regeneration following SCI^{39,40} (BOX 1). Intriguingly, the ordered gradients noted during development might be more accurately retained by adults of lower vertebrate species than mammals^{22,41}, correlating with their better ability to regenerate after CNS injury.

Semaphorins comprise another predominantly repulsive family of guidance molecules^{42,43}. Both secreted and membrane-bound isoforms interact with receptor complexes composed of plexins, neuropilins and/or integrins^{42,44}. As detailed below, ephrins and semaphorins also have crucial roles in corticospinal tract (CST) development.

A recurring theme shared by many of the ligand–receptor combinations directing tract guidance is that interactions can result in growth cone attraction or repulsion, depending on the receptor subtype and intrinsic state of the neuron^{45–48}. This intrinsic state differs among not only neuronal subtypes, but also developing neurons, adult neurons and regenerating neurons. Generally, developing neurons possess the intrinsic state most suited for rapid axonal elongation and target finding^{49–51}.

Pushing and pulling corticospinal fibres

As the main tract that mediates voluntary control of limb movements in primates, the CST has received heavy attention in the context of both development and SCI. In rodents and humans, CST motor neurons in cortical layer 5 send axons to the ipsilateral brainstem via the internal capsule⁵². After coursing through the ventral brainstem, CST axons decussate at the medullary–cervical junction¹⁰. In humans, crossed CST fibres proceed contralaterally down the spinal cord in the lateral funiculus, whereas in rodents the CST fibres deflect dorsally at the decussation, proceeding contralaterally down the dorsal funiculus⁵³ (FIG. 1). CST fibres finally synapse on interneurons and motor neurons within segmental grey matter contra lateral to the originating cortex⁵⁴.

A growing number of morphogens, axon guidance molecules and cell adhesion molecules (CAMs) have been implicated in CST development (FIG. 1). Initially, the axon of the pyramidal cell must be directed away from the cortical surface to start its descending journey. A semaphorin 3A (Sema3A) gradient decreasing from the pial to ventricular surface has been postulated to act through neuropilin 1 receptors to orient axons (repelled by Sema3A) and dendrites (attracted by Sema3A) in the developing cortical plate⁵⁵. Defects in two netrin receptors affect CST development at the decussation. *Unc5h3* mutations result in the termination of most CST fibres just rostral to the decussation⁵³. The few fibres that project to the spinal cord do so ectopically — either ipsilaterally, or in the contralateral dorsal grey matter⁵³. A mutant allele of *Dcc* also leads to a failure of CST decussation, with a resultant ipsilateral spinal cord projection⁵³. Mice with homozygous mutations in netrin 1, the ligand for DCC and UNC5H3, also display abnormalities at the decussation⁵³. Once past the

decussation, CST axons are propelled down the spinal cord by a gradient of two WNT isoforms acting through the RYK receptor¹⁰.

Proper CST development also requires Eph–ephrin signalling. Knockout of either ephrin B3 or its receptor EphA4 leads to the defective repulsion of EphA4-expressing CST axons by ephrin B3 in the spinal cord midline^{52,54}. In the case of EphA4 knockouts, most CST axons terminate within the medulla, with several ectopic projections to the ipsilateral spinal cord⁵². In the case of ephrin B3 knockouts, CST axons decussate normally, but terminate on both sides of the segmental grey matter rather than remaining restricted to one side⁵⁴ (FIG. 1). In both cases, knockout mice display a peculiar ‘kangaroo’ gait characterized by simultaneous rather than alternating movement of the limbs on opposite sides. Although this phenotype correlates with defective CST development, it results primarily from aberrant midline crossing of segmental EphA4-positive excitatory fibres in the lumbar cord⁵⁶.

L1CAM is another membrane-associated signalling molecule that is essential for proper CST development. As with the mutations described above, L1CAM mutations result in many CST fibres aborting prior to the decussation, with a few fibres projecting to ectopic spinal cord locations⁵⁷. Mutations of the human *L1CAM* gene result in CST misdevelopment and a clinically variable phenotype that includes a spastic, uncoordinated gait⁵⁸. Intriguingly, L1CAM appears to act as a receptor in conjunction with neuropilin 1, transducing a signal from *Sema3A* that drives CST fibres dorsally as they cross the ventral medullary–cervical junction⁵⁹ (FIG. 1). Finally, mutations in the human *ROBO3* gene lead to the disorder of horizontal gaze palsy with progressive scoliosis (HGPPS), in which the CST and other tracts completely fail to cross the midline⁶⁰.

Circuit connections and plasticity

Pruning based on target-derived factors

The earliest form of synaptic remodelling occurs during embryogenesis. In many developing tracts, a surplus of axons initially reach their targets^{61,62}. Many preliminary synapses form only to retract soon after. Winners of this game of musical synapses are determined by competition for limiting target-derived growth factors (TDGFs). Eventually, excess neuritic branches are pruned, successful neuritic branches stabilize, and many cell bodies that lack victorious nerve terminals undergo apoptosis^{61,62}. Seminal work by Hamburger, Levi-Montalcini and others used tissue ablation and grafts in developing chick embryos to support the ‘neurotrophin hypothesis’, showing the target-dependent nature of embryonic pruning and cell death^{63–66}.

Neurons of different types, locations and, most importantly, different developmental ages, respond differently to varying combinations of TDGFs (for reviews, see REFS ^{67,68}). For example, embryonic dorsal root ganglia (DRG) neurons depend on nerve growth factor (NGF) for survival⁶⁹, whereas their adult counterparts depend on NGF for other aspects of neuronal outgrowth and metabolism⁷⁰. Many details of the changing responsiveness to TDGFs over time, and their potential reversibility, remain to be determined.

The flexibility of immature circuits

Once axon pathfinding and the pruning process have selected for the formation of appropriate synaptic connections, further plasticity occurs in an activity-dependent manner. This results in the potentiation of some connections and inhibition of others. Through experience-dependent plasticity, neural networks become progressively more organized, as the organism ‘learns’ behavioural and motor responses⁷¹.

Disruptions of sensory experiences within certain age windows result in circuit reorganization, allowing the organism to adapt within the new sensory environment. For example, if barn owls are fitted with prismatic spectacles, their precise coordination of auditory and visual localization is initially disrupted⁷². However, over several weeks they learn to adapt, not only regaining auditory–visual coordination but also visual–motor coordination⁷². The ability to adapt correlates negatively with age — owls over 200 days old are unable to regain auditory–visual coordination under these circumstances⁷³. Critical periods define the age windows during which various circuits retain the plasticity to adapt to sensory deprivation (TABLE 2).

Ocular dominance represents another plasticity paradigm relating to vision. Synaptic terminals relayed from each eye segregate to form ocular dominance columns in each visual cortex⁷⁴. During monocular deprivation, the ocular dominance columns served by the non-deprived eye expand and partially replace those of the deprived eye^{75,76}.

This model provides a powerful system with which to study the mechanisms of plasticity. Analogous to the competition for TDGFs during development, the synaptic inputs from each eye compete through both spontaneous and stimulus-dependent activity⁷¹. Rather than TDGFs, GABA (γ -aminobutyric acid) serves as the arbitrator of this competition⁷¹. If synaptic GABA is inhibited or genetically reduced, ocular dominance shifts no longer occur in response to monocular deprivation⁷⁷. This defect can be rescued by pharmacological GABA agonists⁷⁸. Conversely, early GABA-mediated signalling results in the premature closure of the critical period window^{79,80}.

Synaptic structural dynamics have an important role in plasticity throughout life. To a limited degree, axonal protrusions and dendritic spines form and retract depending on variations in synaptic activity. Recent advances in two-photon *in vivo* imaging have revealed these dynamics in dramatic fashion^{81–84}.

Generating spinal cord plasticity

In addition to cortical sensory circuits, plasticity also applies to the intrinsic spinal cord circuits that mediate locomotion. Central pattern generators (CPGs) mediate coordinated activity between groups of agonist and antagonist limb muscles on opposite sides (for reviews, see REFS 85–87). CPGs allow ambulation to become a nearly automatic neural program⁸⁷. For example, decerebrate cats and anencephalic human infants display coordinated stepping movements^{85,86}. The role of axon guidance molecules in coordinating proper CPG circuit connections was highlighted above in the case of EphA4–ephrin B3 mutations^{52,54}.

Although basic CPG circuits can develop without sensory input⁸⁸, effective CPG functioning depends on plasticity through either descending voluntary inputs or incoming sensory afferents^{86,87,89}. Temporary deprivation of sensory feedback to the rat CPG in the postnatal period results in permanent walking and swimming deficits, defining a critical period for CPG plasticity⁹⁰ (TABLE 2). Encouragingly, spinal cord-mediated behaviours display some plasticity throughout life. For instance, adult cats demonstrate both monosynaptic and CPG-mediated plasticity after thoracic spinal cord transection^{91–93}. This intrinsic plasticity allows functional recovery in the absence of significant regeneration⁸⁷. The beneficial role of sensory feedback on CPG plasticity following SCI is discussed below.

As in the visual cortex, inhibitory neurotransmitters have a crucial role in CPG plasticity. The transition from synchronous to alternating bilateral rhythmic limb movement late in gestation depends on glycinergic signalling⁹⁴. Serotonin, acting through 5-hydroxy-tryptamine (5-HT) receptors, delays the maturation of GABA-mediated inputs into lumbar versus brachial spinal circuits⁹⁵.

Closing the plasticity window

Understanding the mechanisms underlying critical period closure will form the basis for approaches to re-establishing plasticity in regenerating adult nervous systems. We have referred to the role of neurotransmitters in defining plasticity windows. What about the role of non-neuronal components of the CNS?

Extracellular chondroitin sulphate proteoglycans (CSPGs), produced mainly by astrocytes, form perineuronal nets around inhibitory interneurons in the visual cortex, coincident with the closure of the critical period window^{96–98}. CSPGs inhibit neurite outgrowth, probably blocking the synaptic structural dynamics that partly underlie plasticity⁹⁷. Enzymatic CSPG removal re-opens the critical period⁹⁷. Similarly, maturation of cortical myelination roughly coincides with the closure of the critical period⁹⁶. Genetic disruption of the action of myelin-associated inhibitors (MAIs) prevents critical period closure⁹⁶. Therefore, maturing astrocytes and oligodendrocytes have a role in consolidating neuronal plasticity.

Unfortunately, the mechanisms that evolved to consolidate plastic circuitry directly contribute to the inability to regenerate after CNS injury. Hence, plasticity and regeneration are intertwined. The most effective therapeutic approaches will untangle these pathways to allow the regeneration of injured circuits without disrupting the consolidation of existing intact circuits.

Starting over: neural response to injury

Injury and disease wreak havoc on the intricately choreographed neural circuitry. Unlike the many similarities across species in patterns of neural development, responses to injury differ greatly. Responses to injury also vary in individual organisms, depending on time, anatomical location and type of injury (FIG. 2). The most effective injury responses occur in lower vertebrate species, at younger developmental ages, and in the PNS rather than the CNS. A detailed understanding of the factors responsible for these differences provides insights into the challenge of improving treatments for adult mammalian CNS injury.

Lower vertebrates fully regenerate

The ability of lower vertebrate species to regenerate injured CNS tracts is extensively reviewed elsewhere^{99,100}. Adult salamanders can completely regenerate a transected spinal cord whereas tailless amphibians such as *Xenopus laevis* lose CNS regenerative capability after larval stages⁹⁹. Even mammals retain some capacity for spinal cord regeneration during development. The opossum can fully recover from spinal cord transection up to ~1 week postnatally¹⁰¹. Important lessons can be learned from these examples of successful CNS regeneration and applied to the adult mammalian context.

The cost of complexity

Evolutionarily speaking, it is tempting to wonder why more advanced vertebrate species would lose the ability to regenerate after CNS injury. Clearly, this capability would greatly benefit victims of stroke and SCI. However, this line of reasoning ignores the likelihood that, in feral animals, selection pressure has little or no role in favouring CNS regenerative capability. The types of injury that would damage the CNS would almost certainly lead to rapid demise in the wild before regeneration could take place. Therefore, the more likely evolutionary explanation for the 'loss' of CNS regenerative capacity is that this is an unselected by-product of gaining the increasingly complex nervous systems that selection pressures have favoured over time. Now that long-term care for neurologically disabled patients is possible, both the potential and the pressure to overcome this evolutionary side effect have grown.

Nervous system complexity increases not just across phylogeny but also across ontogeny. The loss of regenerative capability with age further demonstrates the cost of this complexity. However, even in the adult mammalian CNS, transected nerve fibres appear to at least attempt to regenerate, as shown in recent publications^{102,103}. Serial live two-photon imaging of spinal axons following needle transection shows proximal stumps undergoing a variable, haphazard regenerative phase, often abruptly terminating or even reversing direction without crossing the lesion site¹⁰³. Inevitably, these attempts to regenerate end in failure, marked by the retraction bulbs first described by Ramón y Cajal¹⁰⁴. Here, we summarize the knowledge regarding some of the barriers to successful axonal regeneration in the mammalian CNS.

Intrinsic limitations of mature neurons

Cell-autonomous mechanisms partially explain the failure of injured adult CNS fibres to regenerate. When neurons derived from animals of varying developmental ages are cultured under optimal growth conditions, postnatal neurons display considerably less neurite outgrowth than their embryonic counterparts^{49,105,106}. One explanation is that older neurons have decreased levels of cyclic AMP (cAMP)⁴⁶. cAMP affects neuronal responses both acutely (for example, by converting a repulsive signal into an attractive signal⁴⁷) and over the longer term, through activation of transcription factors such as CREB (cAMP responsive element-binding protein)^{107,108}.

Interestingly, one method to increase cAMP levels and regenerative ability in mature CNS neurons is to create a conditioning lesion on a peripheral branch several days before injuring the central branch^{107,109}. Furthermore, although injury induces the expression of regeneration-associated genes in both peripheral and central neurons, adult CNS neurons lack the appropriate downstream effectors to translate these signals into successful regeneration^{110–115}. The importance of the immediate early gene *c-jun* in stimulating expression of regeneration-associated genes was shown by Raivich and colleagues¹¹⁶.

Conversely, mature neurons express higher levels of the signal transduction machinery for inhibitory extra-cellular factors. For example, Nogo-66 receptor (NgR) is drastically upregulated in adult relative to embryonic neurons^{117,118}, whereas the receptors that mediate attractive responses to netrin are downregulated in adults and following injury^{119,120}.

Extrinsic adult CNS barriers

Whereas cell-autonomous mechanisms contribute to limiting adult axon growth, extrinsic factors appear to have an even more crucial role in blocking adult CNS regeneration. Classic experiments by Tello and Ramón y Cajal as well as David and Aguayo demonstrated the more inhibitory nature of the CNS for axon outgrowth^{104,121}. Subsequent experiments by Schwab and others suggested that this inhospitable milieu results primarily from the presence of CNS myelin-specific inhibitory factors rather than a lack of positive factors^{122,123}. Furthermore, the age at which most species lose the ability to regenerate after SCI coincides with spinal cord myelination¹²⁴. Several major MAIs have been discovered and extensively studied during the past decade. When expressed on oligodendrocyte cell surfaces, these molecules interact with axonal receptors, triggering growth cone collapse *in vitro* and blocking outgrowth *in vivo* (BOX 1). Surprisingly, several structurally unrelated MAIs bind to the same axonal receptor, NgR^{117,125–127}.

Unfortunately, myelin is not the only extrinsic barrier to adult CNS regeneration. CNS injury induces reactive astrocytes to release many molecules that inhibit regeneration, including CSPGs and other glial scar components. Furthermore, breakdown of the blood–brain barrier results in the recruitment of inflammatory cells and cytokines that have a more complicated effect on CNS regeneration. Interestingly, as with many axon guidance molecules, several

MAIs and CSPGs are expressed during development as well as in the adult. For example, Nogo isoforms are expressed by both central and peripheral neurons at developmental stages before the onset of oligodendrocyte Nogo expression^{118,128,129}. The role of neuronally expressed Nogo and other inhibitory factors remains to be determined¹³⁰. For a more in-depth discussion of astroglial inhibitors and MAI, see the review by Yiu and He in this issue¹³¹.

Overcoming barriers to CNS regeneration

Depending on the type of CNS injury, attempts at regeneration might need to recapitulate all or only some of the stages of development described above. For example, full regeneration after stroke or neurodegenerative disease would require the replacement of lost neurons, followed by the regeneration and guidance of projections over the entire distance covered by the absent tracts. By contrast, significant recovery from SCI could occur through encouraging sprouting and guidance from spared tracts, as well as maximizing plasticity of spared and regenerated circuits (FIG. 3). Multiple promising approaches against each of the barriers discussed in the last section are being pursued. It is widely accepted that no single approach will prove sufficient for successful regeneration — a methodology combining the most effective individual therapies is required¹³². The review by Thuret, Moon and Gage in this issue covers in more depth the therapeutic approaches to promoting recovery from SCI¹³³. The review by Yiu and He gives a more detailed discussion on approaches to overcoming extrinsic inhibitors¹³¹.

Providing new neurons

The advent of embryonic stem cell (ESC) lines that can be perpetuated in culture has sparked intensive investigation into methods for differentiating these cells into neurons suitable for therapeutic transplantation. Such ESC-derived neurons should more faithfully replicate the growth-favouring intrinsic state of immature neurons. Approaches to ensuring that pre-differentiated neurons remain as immature as possible continue to be refined. Much has already been learned from numerous studies involving transplants of embryonic neural tissue. Despite the many formidable extrinsic obstacles to successful regeneration, embryonic neural transplants can survive and even thrive within the adult host CNS^{134–137}.

Several factors appear to maximize the ability of transplanted neurons to incorporate into the host CNS: the absence of inflammation in the area to be transplanted¹³⁸; the administration of neurotrophins, especially fibroblast growth factor 2 (REFS 136,^{139,140}); the incomplete loss of host neurons in the area to be transplanted^{138,141}; and at least a partial retention of the host pre- and postsynaptic target circuit^{19,138,141,142}. The third point suggests that surviving neurons in incomplete injuries release signals that promote the engraftment of replacement neurons. The fourth point suggests that the presence of surviving projections to postsynaptic targets enhances the ability of newly extending fibres to reach those targets.

Tapping endogenous stores of new neurons

An ideal (especially for socio-political reasons) stem cell-based approach to CNS regeneration entails the stimulation of endogenous stem cell reserves to replace lost tissue. There is still controversy surrounding whether endogenous stem cells fully replace adult neurons outside the hippocampal dentate gyrus and olfactory bulb^{16,18}. However, several groups have succeeded in inducing the birth of new neurons following minimally invasive targeted apoptosis of several different cortical regions^{15,142–145}. Apparently recapitulating all of the developmental steps described above, these ‘adult-born’ neurons migrate to the precise areas vacated by lost neurons, presumably along processes formed by astrocytes that have de-differentiated into radial glia^{15,19}. They then extend projections towards the denervated targets of the original neurons^{15,142}. Whether a similar mechanism could occur in the adult spinal

cord remains unclear. Similarly, the extent to which projections from host neurons innervate the replacement neurons remains poorly understood.

As with exogenous cell transplants, endogenous neural replacements seem to integrate more efficiently when inflammation is minimized, adjuvant neurotrophins are administered and the targeted area is only partially destroyed^{15,142,145}. This suggests that ongoing pre- and postsynaptic activity in the targeted area has an important role in guiding the axons that extend from adult-born neurons^{17,18,146}.

Rejuvenating neurons

Intraneuronal cAMP levels decrease with age, correlating with decreased regenerative potential^{46,51}. Consequently, agents that increase neuronal cAMP levels have been used to increase regenerative capacity, both *in vitro* and *in vivo*^{47,51,107,147,148}. Aside from antagonizing intrinsic inhibitory pathways, another approach to enhancing neuronal regenerative potential is to increase the activity of stimulatory pathways. GAP43 and CAP23 are growth cone-associated proteins, the expression of which increases in injured nerves that are attempting to regenerate^{114,149}. Transgenic overexpression of these proteins in the DRG leads to increased regeneration of ascending fibres after SCI¹⁴⁹.

Nourishing neurons

Exogenous neurotrophin expression through multiple routes of administration has produced many positive results both in culture and in animal models of CNS injury^{147,150–154} (for reviews, see REFS^{136,155}). Proposed mechanisms of action in this context include axonal regeneration^{147,150,151}, increased neuronal survival^{152,153}, improved remyelination¹⁵⁴ and the stimulation of endogenous stem cells¹⁵⁶.

As sole therapeutic agents, neurotrophin effects might be limited to cell sparing and local axon sprouting. However, strategic neurotrophin expression could be an adjunctive component of any therapy for successful CNS regeneration. TABLE 1 illustrates the difficulty of pharmacologically emulating the geographically precise endogenous signalling cues present during development. Strategies to trigger the injured host to re-express endogenous neurotrophins at appropriate levels and locations need further exploration.

Clearing the path for neuritis

The discovery that three major MAIs bind to the same receptor, NgR, has provided a clearly defined target for overcoming myelin inhibition of CNS regeneration^{117,126,127} (FIG. 2a). Several approaches to NgR inhibition have yielded varying results. Genetic NgR deletion improves the ability of serotonin-containing but not CST fibres to regenerate in mouse SCI^{157,158}. Pharmacological Nogo or NgR inhibition has resulted in more robust and reproducible effects in various CNS injury models^{159–163}. Antagonists of the Nogo–NgR pathway are likely to enter clinical testing in the near future. Although the removal or antagonism of myelin's inhibitory effects is essential for fostering CNS regeneration, remyelination of regenerated fibres remains necessary to achieve effective conduction^{132,164}.

RhoA GTPase acts downstream of NgR to help mediate its inhibitory signal¹⁶⁵. RhoA probably also serves as the intracellular inhibitory gateway for CSPGs¹⁶⁶. Therefore, RhoA represents another attractive target for blocking the influence of both myelin and CSPGs on CNS regeneration. Several small-molecule inhibitors of Rho or Rho-associated kinase mediate increased neurite outgrowth both *in vitro* and *in vivo*^{167–171}.

Optimizing plasticity of spared fibres

Although great strides are being made in fostering the regeneration of injured spinal cord tissue, the ideal of total regeneration remains far off, if not impossible. Fortunately, most SCIs that occur outside the laboratory spare a variable proportion of nerve fibres and cell bodies. A more feasible goal, which will improve outcomes regardless of regeneration, is to optimize the plastic responses of these uninjured fibres. This will involve not only antagonizing the signals that prevent regeneration, but also re-establishing an environment that is conducive to synaptic remodelling. Examples of success with this approach in ocular dominance plasticity need to be further adapted to the context of SCI^{96,97}. In this respect, ongoing work involving MAI antagonism in mice shows great promise in allowing plastic sprouting responses to compensate for different types of CNS injury¹⁷².

After the walls come down

As the potential of stem cells has been partnered with progress in eliminating extrinsic barriers to nerve regeneration, optimism in the SCI field has reached levels that were unimaginable only 15 years ago. To objectively evaluate this optimism, it is useful to consider the progress made in rodent CST injury. Blockade of MAIs, digestion of CSPGs, inhibition of RhoA signalling and glial cell transplants have all aided CST fibre growth and functional improvement after SCI^{160,162,173–176}. However, with the exception of one promising study, neither transplanted nor endogenous stem cells have been shown to functionally replace lost CST fibres¹⁵.

Crucially, the axonal growth that has been achieved through these approaches does not faithfully recapitulate the accurate guidance of developing fibres. Rather than re-establishing a highly fasciculated and directed pathway, regenerating CST fibres are extensively branched and dispersed throughout the spinal cord grey and white matter^{160,162} (FIG. 2).

Therefore, even if (and when) all the negative extrinsic influences to CNS regeneration are surmounted, there are other issues to be considered (FIG. 3): regeneration often proceeds haphazardly along ectopic pathways^{160,162}; it is often unclear whether ‘regenerating’ axons derive from transected fibres or sprout from neighbouring uninjured fibres; circuit reformation might occur through synaptic relays that do not exist in the intact nervous system¹⁷⁷; the formation of inappropriate synaptic connections might occur; and the plasticity and consolidation of appropriately regenerated synapses remains unexplored.

Despite these histologically apparent limitations on CNS regeneration, many animal SCI experiments have shown functional improvements in both locomotion and fine motor coordination. If translated to humans with SCI, these improvements would signify a large advance over current clinical therapy. However, to fully optimize the benefits of CNS regeneration, two other approaches will be necessary. First, as repeatedly emphasized throughout this review, the most crucial developmental guidance cues need to be characterized and perhaps re-expressed at the proper locations to guide regenerating axons. Second, plasticity of both intact and recreated circuits needs to be engaged. We conclude with a discussion of one approach that addresses the second issue — sensorimotor rehabilitation.

Use it or lose it: feedback therapy

The CPG is located in low thoracic or high lumbar cord segments in most species, below the level of the majority of traumatic SCI⁸⁶. Therefore, the CPG remains at least partially intact in most of these cases. This has been demonstrated repeatedly in paraplegic human patients, in whom involuntary locomotive leg activity can be induced by different types of sensory stimulation^{85,86}. In cats and other species, a short period of treadmill training following thoracic spinal cord transection results in an astounding level of recovery^{93,178–180}. This

recovery presumably occurs through CPG plasticity rather than the frank regeneration of severed spinal cord tracts⁹³.

Experiments in cats and humans demonstrate the dependence on sensory feedback for regaining ambulation after SCI^{181,182}. A cat that had regained ambulation through treadmill training following thoracic spinal cord transection was subjected to a series of staged lesions of hindlimb cutaneous nerves¹⁸³. Locomotion recovered after each subsequent lesion until the last cutaneous afferent was severed — without any sensory input, ambulation was no longer possible¹⁸³. In fact, sensory feedback is crucial for the proper functioning of various rhythmic motor systems⁸⁹.

In human patients with SCI, body-weight-supported treadmill training (BWSTT) exploits this sensory–CPG loop to re-establish ambulation^{184,185} (for reviews, see REFS^{86,87}). Although the most carefully controlled clinical trial so far of BWSTT did not show a significant difference in outcome relative to conventional physical therapy, optimism continues to drive work in this area^{186,187}. Importantly, patients with incomplete SCI derive proportionally more benefit from sensorimotor rehabilitation than patients with complete SCI, emphasizing the significance of plastic sprouting from spared fibres^{184,188}.

Whether sensorimotor rehabilitation also assists the regeneration of severed fibres remains to be determined. Presumably, mechanisms could include local release of TDGFs that would attract regenerating fibres, or facilitated growth of regenerating fibres along active spared fibres. Therefore, when used as an adjunct to the other approaches discussed here, sensorimotor rehabilitation therapy could enhance clinical recovery to a much greater degree than when it is used in isolation^{188,189}.

Conclusion and goals

The biomedical community has made considerable progress in overcoming the barriers to recovery after mammalian CNS injury. This progress has enabled some damaged neurons and axons to regenerate. However, the mission has not yet been completed — nerves continue to face many obstacles on the road to recreating damaged circuits. These obstacles partly arise from the dramatically altered distribution of guidance cues in the adult as opposed to embryonic nervous systems.

To truly overcome CNS injury, we need to learn more about and re-apply basic developmental guidance mechanisms in the adult context. At the same time, spared circuits need to be fully recruited through pharmacological and rehabilitation techniques that encourage plasticity. In the case of SCI, only a small percentage of fibres needs to successfully reconnect with their targets to mediate substantial clinical recovery¹²⁴. Therefore, through the concerted effort of all these approaches, we will be able to help patients recover from CNS injury by recapitulating the precise growth, guidance and flexibility of the developing CNS.

Supplementary Material

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Glossary

Morphogens

Diffusible proteins that are involved in signalling the differentiation of cells into specific tissues and organs during embryogenesis. More recently, they have also been shown to have roles in axon guidance.

Radial glia

Progenitor cell type that gives rise to immature neurons and other radial glia. Immature neurons then migrate along radial glial processes.

Extracellular matrix

(ECM). Connective tissue produced largely by fibroblasts and astrocytes that provides diverse inhibitory and growth-promoting signals to neurons and their extensions.

Experience-dependent plasticity

The reorganization of neural circuits in response to excitatory and inhibitory synaptic influences. Involved in learning and adaptation to varying external stimuli.

Critical periods

Discrete phases early in life during which neural circuits exhibit maximal experience-dependent plasticity.

Ocular dominance

Neurons in the visual cortex respond electrophysiologically to light stimuli from one eye to a greater extent than to stimuli from the other eye. A model system for studying plasticity.

Monocular deprivation

Experimental model in which one eye is sutured shut during the critical period for ocular dominance plasticity, preventing experience-dependent changes.

Central pattern generators

(CPGs). Local circuits involved in coordinating largely automatic motor behaviours such as ambulation and swimming. Modulated by sensory feedback and descending voluntary inputs.

Chondroitin sulphate proteoglycans

(CSPGs). Carbohydrate-rich extracellular molecules with inhibitory effects on neurite outgrowth. Produced predominantly by astrocytes.

Myelin-associated inhibitors

(MAIs). Surface proteins expressed by oligodendrocytes that prevent neurite outgrowth or regeneration.

Regeneration-associated genes

Genes that are upregulated following axonal injury (for example, *Gap43*, *Sprr1a*, *Fn14* and *arginase 1*). Increased expression correlates with regeneration in peripheral but not central neurons.

Body-weight-supported treadmill training

(BWSTT). Physical therapy technique for SCI patients using a harness to partially support the patient's weight while therapists assist the patient's legs to ambulate on a moving treadmill.

Figure 1. Corticospinal tract development

Sagittal (a), coronal (b) and axial (c) views of a developing corticospinal tract (CST) fibre (blue), with neuronal receptors in grey and guidance molecules in red. The initial projection of CST axons away from the pial surface is facilitated by semaphorin 3A (Sema3A) activation of repellent NRP1 receptors. At the medullary–cervical junction, secreted midline signals Sema3A and netrin 1 interact with neuronal L1 and UNC5H3, respectively, to repel the CST fibre dorsally and contralaterally. Decussated fibres are then propelled caudally down the spinal cord by a gradient of WNT1/WNT5A interacting with neuronal RYK. Along the spinal cord midline, ephrin B3 prevents re-crossing by interacting with neuronal EphA4.

Figure 2. Corticospinal tract response to injury

a | Aberrant regeneration following spinal cord injury (red shading, region affected by dorsal hemisection). In untreated wild-type animals, spinal cord transection leads to the retraction of corticospinal tract (CST) axons followed by minimal abortive regenerative sprouts (green) rostral to the lesion, with no fibres detected caudal to the lesion. When myelin-associated inhibitors are antagonized either genetically or pharmacologically, significant sprouting and regeneration occur rostral to the lesion, with a few ectopically directed regenerating fibres detectable caudal to the lesion. **b** | Compensatory sprouting in stroke. Unilateral lesions affecting corticospinal motor neurons (red shading) result in degeneration of the entire CST projection from that side. The unlesioned CST sends sprouting fibres (green) contralaterally both in the brainstem at the red nucleus and at segmental spinal cord levels in an attempt to compensate for the lost CST. Treatments with Nogo-66 receptor blockers, anti-Nogo-A antibodies, or inosine that enhance regeneration and plasticity increase this sprouting response. **c** | Compensatory sprouting in pyramidotomy. Unilateral lesion of one CST just after decussation results in degeneration of the CST fibres caudal to the lesion. As in stroke, the unlesioned CST sends sprouting fibres contralaterally in an attempt to compensate for the lost CST fibres. Treatments with anti-Nogo-A antibodies or with inosine that enhance regeneration and plasticity increase this sprouting response.

Figure 3. Modes of circuit regeneration and plasticity after axotomy

Two schematic motor circuits are shown. Note that one of the motor neurons is not directly influenced by the pictured descending neurons. After a partial lesion that severs one of the descending fibres (middle panel), the motor neuron controlled by that fibre loses voluntary function. Over time, and with the aid of regenerative strategies, several modes of recovery might occur (bottom panel): precise regeneration from the severed fibre to the original target (1); regeneration of the severed fibre to the original target through a haphazard/ectopic pathway (2); sprouting from unlesioned heterologous neighbouring fibres onto the denervated target neuron (3); formation of atypical synaptic relay circuits (4); sprouting of the injured fibre proximal to the lesion towards neurons neighbouring the denervated target neuron (5); and enhanced intrinsic plasticity through sensory feedback training (6).

Table 1

Developmental shifts in axon guidance factor distribution

Guidance factors	Development Spinal cord	Forebrain	Adult Spinal cord	Forebrain	Injury Spinal cord	Forebrain	Refs
Netrin 1	FP, ventral CC	Striatum, HC, OB, optic cup	D = V; neurons and OGD	Striatum, SN, CBL, retina	Dorsal hemisection: ↑ in invading fibros/macros	CBL lesion: ↑ in invading fibros/macros	120, 208–211
DCC	VH, commissural axons, dorsal CC	Cx, striatum, CBL, retina	V>D; grey = white (weak expression)	HC, CBL, retina (weak expression)	nd	ON injury: 44% ↓; CBL injury: no change	119, 120, 208, 211–214
UNC5H2	RP, DRG	Optic cup	D = V grey (stronger expression than in embryo)	CBL	nd	ON injury: 26% ↓	45, 119, 120, 215
Ephrin A2	+ (ns)	HC, OB, BS	+ (ns)	HC, OB, BS, retina	nd	ON injury: ↑ in caudal superior colliculus	216–218
EphA4	V>D	SVZ, BG, HC, CBL, retina	DH>VH	Cx, striatum, SN, HC, CBL, BS	Transsection: ↑ in proximal CST stumps and astrocytes	HC injury: no change	219–225
Ephrin B3	Midline	HC	OGD	Cx, HC, CBL	Transsection: mild ↓	ON injury: ↑ in RGC	39, 54, 219, 225, 226
EphB2	VH	Cx, HC, CBL, BS	Fibros	HC	Transsection: ↑ in fibros	HC injury: no change	219, 223, 227, 228
SLIT1	FP>MN	Diffuse	D>V	Diffuse	Dorsal hemisection: ↑ in macros/fibros	CBL injury: ↑ in macros	26, 211, 229, 230
SLIT3	FP, MN (weak expression)	HC, OB, BS	V>D	Diffuse	Dorsal hemisection: large ↑ in macros/fibros	CBL injury: no change	26, 211, 230
ROBO1	Commissural axons, MN	Diffuse	nd	Diffuse	nd	CBL injury: no change	26, 211, 230, 231
ROBO3	Commissural axons, ventral IN (not MN)	BS	nd	CBL	nd	CBL injury: no change	30, 211, 232
Sema3A	VH, ventral CC	Cx, HC	VH	HC, OB, CBL	Stab injury: ↑ in fibros	Cortical or olfactory stab injury: ↑ in fibros	233–238

Guidance factors	Development Spinal cord	Forebrain	Adult Spinal cord	Forebrain	Injury Spinal cord	Forebrain	Refs
Sema3F	nd	Cx, BG	Intermediate IN (weak expression)	nd	Stab injury: weak ↑ in fibros	nd	235, 236, 238, 239
NP1	DRG>dorsal funiculus>MN	Cx, HC, OB	DRG	CSMN	Dorsal hemisection: persist/no change in DRG	nd	233–235, 238, 240
PlexA1	Diffuse, sympathetic ganglia	nd	DRG	Diffuse Cx	Dorsal hemisection: persist/no change in DRG	nd	235, 238, 241
PlexB1 RGM-A	CC FP>VH	SVZ SVZ; optic mesenchyme	nd Neurons and OGD	SVZ, CBL nd	nd Dorsal hemisection: ↑ in macros and OGD	nd nd	242, 40, 243, 244
Neogenin	Ventral midline	Diffuse	VH>DH (weak expression)	+ (ns)	nd	nd	119, 212–214, 245

↑, increase; ↓, decrease; BG, basal ganglia; BS, brain stem; CBL, cerebellum; CC, central canal; CSMN, corticospinal motor neurons; Cx, cortex; CST, corticospinal tract; D, dorsal; DCC, deleted in colorectal cancer; DH, dorsal horn; DRG, dorsal root ganglia; fibros, fibroblasts; FP, floor plate; HC, hippocampus; IN, interneurons; macros, macrophages; MN, motor neurons; nd, not determined; + (ns), present but localization not further specified; OB, olfactory bulb; OGD, oligodendrocytes; ON, optic nerve; RGC, retinal ganglion cells; RP, roof plate; SN, substantia nigra; SVZ, subventricular zone; V, ventral; VH, ventral horn.

Table 2
Critical periods for experience-dependent neuronal plasticity

Organism	System	Open [*]	Close [‡]	Refs
Fly	Visual pathway atrophy	Pupal–adult transition	24 hours	246
Barn owl	Auditory–visual coordination	50 days	200 days	73
Zebra finch	Song learning	25 days	60 days	247
Mouse	Ocular dominance	19 days	32 days	248
Ferret	Ocular dominance	40 days	65 days	249
Cat	Ocular dominance	28 days	>120 days	250
Human	Ocular dominance	12 months	36 months	251,252
Rat	Ambulation	8 days	13–31 days	90

^{*} Open refers to the time of onset of the critical period after birth.

[‡] Close refers to the time point at which the critical period ends after birth.