REVIEW

Determinants of Axon Growth, Plasticity, and Regeneration in the Context of Spinal Cord Injury

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The mechanisms that underlie recovery after injury of the central nervous system have rarely been definitively established. Axon regrowth remains the major prerequisite for plasticity, regeneration, circuit formation, and eventually functional recovery. The attributed functional relevance of axon regrowth, however, will depend on several subsequent conditional neurobiological modifications, including myelination and synapse formation, but also pruning of aberrant connectivity. Despite the ability to revamp axon outgrowth by altering an increasing number of extracellular and intracellular targets, disentangling which axons are responsible for the recovery of function from those that are functionally silent, or even contributing to aberrant functions, represents a pertinent void in our understanding, challenging the intuitive translational link between anatomical and functional regeneration. Anatomic hallmarks of regeneration are not static and are largely activity dependent. Herein, we survey mechanisms leading to the formation of dystrophic growth cone at the injured axonal tip, the subsequent axonal dieback, and the molecular determinants of axon growth, plasticity, and regeneration in the context of spinal cord injury. (*Am J Pathol* 2018, 188: 53–62; <https://doi.org/10.1016/j.ajpath.2017.09.005>)

During development, the axons of the central nervous system (CNS) form an elaborate circuitry. These axons fail to regenerate after injury in the adult CNS (Figure 1), with the exception of the olfactory sensory projections within the olfactory bulb,^{1,2} monoaminergic axons of the mediobasal hypothalamus,³ and serotonergic axons.⁴ The pioneering work of Santiago Ramón y Cajal⁵ demonstrates that, despite attempts to navigate the lesion environment, injured axons fail to regenerate and, ultimately, form dystrophic end bulbs that persist indefinitely in the lesion border.⁶ There has been an extensive amount of research to understand why most axons in the CNS fail to regenerate after injury, especially when compared with axons of the peripheral nervous system (PNS), which have a much greater capacity for regeneration. Two major mechanisms have been proposed to account for this difference in regenerative capability. One major field of study is understanding the intrinsic limitations on the growth potential of the adult CNS neuron, compared with the growth potential of immature neurons in the CNS or adult neurons of the PNS.⁷ The other major area of study is identifying the environmental factors that restrict axon growth potential.

Axonal outgrowth has been studied as a basis for axonal plasticity and regeneration in different modeling paradigms.⁸ We will survey the axon's response after injury, including axonal dieback and degeneration, then discuss the current understanding of intrinsic pathways affecting CNS axon growth potential, and finally describe the extrinsic factors that have been identified as inhibitors of axonal regeneration. Conversion of axonal growth-promoting interventions into functionality is context dependent (Figure 2), relying on both timing and activity.⁹

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Figure 1 The pattern of axon behavior after injury. During development, axons extend and form an elaborate circuitry that leads to a functional nervous system. As the central nervous system (CNS) matures, signaling that was active during development begins to down-regulate to prevent unwanted growth and plasticity. After injury, regeneration-associated genes (RAGs) are up-regulated to regrow injured fibers to their original targets. The initial response to injury results in calcium influx and an intrinsic retraction from the lesion site within the first 4 hours after injury. During the acute phase after injury, the microtubules within the proximal end of the axon destabilize, whereas the distal end undergoes wallerian degeneration. This destabilization allows the axon to dieback away from the lesion, an intrinsic property of the neuron itself. As the neuron enters the subacute phase of injury (2 to 14 days after injury), macrophages enter the lesion core, contact the axon, and cause the axon to dieback further. The dieback ceases when the injured axon tip reaches the surface of neural-glial (NG)²⁺ cells in the lesion penumbra, where they become entrapped. This entrapment contributes to regeneration failure. As the glial scar develops during this time, there is an up-regulation of various extracellular molecules that further impede the axon's regeneration potential. CSPG, chondroitin sulfate proteoglycan; Jak, Janus kinase; LAR, leukocyte common antigen related; MAG, myelin-associated glycoprotein; mTOR, mammalian target of rapamycin; NgR, Nogo receptor; OMgp, oligodendrocyte-myelin glycoprotein; PTEN, phosphatase and tensin homolog; PTP, protein tyrosine phosphatase; RGM-A, repulsive guidance molecule-A; SOC, suppressor of cytokine signaling 3.

Intracellular Mechanisms of Regeneration Impairment

Historically, it has been difficult to distinguish between regenerating fibers and spared fibers using axonal tracing. Postmortem analysis only allows for snapshots of fiber position at certain selected time points, making it difficult to know whether these fibers were spared, degenerating, or regenerating at that moment after injury. *In vivo* imaging after spinal cord injury (SCI) has vastly improved our understanding of axonal dieback and degeneration. Kerschensteiner and colleagues¹⁰ used *in vivo* imaging and a transgenic mouse strain expressing green fluorescent protein under a neuron-specific promoter (thymus cell antigen-1) to visualize a subset of neurons, including those of the dorsal root ganglion. After identifying a particular axon, using blood vessels as a landmark, the authors used a micro-manipulator to selectively transect the labeled axon of interest (along with some surrounding axons). The first phenomenon observed was a sudden fragmentation of both the proximal and distal ends of the transected axon, extending approximately 300 µm, which the authors termed acute axonal degeneration. Acute

axonal degeneration, which happens within the first hour after injury, is followed by axonal retraction. Within 24 to 30 hours after injury, both the proximal and distal ends of the severed fiber dieback symmetrically from the lesion site. By 48 hours after injury, dieback of the proximal end slows, although the authors noted that the end bulb continues to change shape. The proximal end eventually stabilizes approximately 300 µm from the lesion site, whereas the distal end begins to undergo wallerian degeneration. Within the first day after injury, many axons mount an initial growth response that ultimately results in a dystrophic growth state. Axonal injury may not only be a yes or no phenomenon. Recent findings report that axonal injury can be reversible^{11,12} and postulate an inherent self-preservation process in contused axons. Further studies using *in vivo* imaging will help elucidate the mechanisms underlying these abortive attempts at regrowth.

Dystrophic Growth State

On injury, axons exhibit dystrophic growth cones, first described as sterile clubs by Ramón y Cajal.⁵ The axon no longer extends

Figure 2 Functional gains of axonal outgrowth are not linearly dependent on time and activity after injury. Interventional studies combining rehabilitative and molecular progrowth strategies during the first 1 to 2 weeks after injury are ineffective in contributing to functional improvement and provide evidence for a critical time window for repair. The early postlesional circuitry during the first 1 to 2 weeks is considered unstable. Destabilizing this further by unlocking sprouting of new fibers does not result in improved voluntary motor function. Instead, sequential designs applying molecular progrowth strategies during the first week, followed by rehabilitative training to stabilize newly formed circuitry starting after 1 to 2 weeks after injury, result in improved outcomes. SCI, spinal cord injury.

lamellopodia, but rather persists in an active, stable state at the edge of the lesion. This dystrophic growth state is at least partly induced by the glial scar that forms around the lesion environment. In a set of microtransplantation studies, adult dorsal root ganglion neurons were able to grow 1 mm per day within white matter tracts of the CNS for long distances, but their growth halted abruptly as they contacted the glial scar.¹³ There is evidence that these fibers remain responsive, but are unable to progress beyond the lesion core. Evidence from the laboratory of Bradke and colleagues⁶ suggests that these fibers can persist near the lesion core for up to 42 years after injury, addressing the question of whether these fibers continuously dieback over time or stabilize. Further evidence of this active, persistent dystrophic state can be seen using time-lapse microscopy and an *in vitro* model of the glial scar. The glial scar is characterized by a considerable up-regulation of chondroitin sulfate proteoglycans (CSPGs; described later), which have been shown to inhibit regeneration. Work from the laboratory of Silver and colleagues¹⁴ demonstrated that as the neurons extend neurites into a gradient of increasing CSPG and decreasing laminin, they become dystrophic. However, they remain active, although they do not make forward movement into the gradient, continually endocytosing membrane and periodically extending and retracting short filopodia.^{10,15}

Studies to understand the mechanisms underlying the formation of the dystrophic growth cone demonstrated that, in this state, the growth cone contains disorganized microtubules.¹⁶ Work by Ertürk and colleagues¹⁷ furthered these observations when they demonstrated that destabilizing microtubules cause active growth cones to become dystrophic and stabilizing microtubules prevent dystrophic growth cone formation.

The first step to regenerating a severed axon is to reestablish a growth cone at the tip.¹⁸ In mammals, injured axons of the peripheral nervous system are able to generate a new growth cone

within hours after being cut.^{19,20} Conversely, injured CNS axons in mammals retract 200 to 300 μm and form retraction bulbs. Only one third of these injured fibers are able to form a new growth cone at the terminal or at a node of Ranvier.^{10,17} The first step to generating a new growth cone comes from the disruption of the membrane, resulting in a calcium influx. This passive influx of calcium is further propagated by the activation of voltage-gated ion channels, resulting in an elevated intracellular calcium concentration. This calcium influx causes microtubule and actin depolymerization retrogradely down the axon,^{21,22} as well as activation of calpains. The proteolytic activity of calpains peaks rapidly after axotomy, within 10 to 30 minutes, and then returns to baseline by 60 minutes.²³ This brief calpain activation is responsible for restructuring the cytoskeleton of the axotomized tip.^{22–24} The calcium influx is also necessary for resealing the membrane, as demonstrated by the findings that in low calcium conditions ($<100 \mu\text{mol/L}$), membrane sealing does not occur and axon degeneration results.^{25,26} The neuron must act quickly to repair the ruptured membrane to restore ion homeostasis. This process happens within minutes to hours after axotomy. Calcium-mediated membrane collapse seals off the severed end of the injured axon, which allows for calcium removal mechanisms to set in to restore ion homeostasis. Once the membrane has been sealed, a new growth cone can form, beginning with microtubule and actin repolymerization.

Axonal Dieback

Axons exhibit different responses to inhibition and injury. When the axon tip is unable to make forward progress, but also does not retract, the axon is said to be in a state of growth cone dystrophy, in which the axon tip persists where it is, turning over membrane and forming vesicles.¹⁴ Growth cone collapse involves a short and temporary retraction from the growth cone's initial position, losing lamellapodia and filopodia. However, the growth cone will usually begin to extend again. In the case of axonal retraction or dieback, the axon tip regresses away from the lesion over a much greater distance than growth cone collapse, eventually stabilizing in proximity to the lesion border.^{27,28}

Axonal dieback was first described by Ramón y Cajal⁵ when he observed severed axon tips retracting away from the site of injury over time, which he believed was intrinsic to the neurons themselves. Evidence now shows that two phases of axonal dieback exist. The initial phase of axonal dieback appears to be an intrinsic property of neurons themselves, as demonstrated using *in vivo* imaging. Ascending sensory axons retract several hundred micrometers within hours after injury before they stabilize for several days.¹⁰ The secondary phase of axonal dieback correlates with the infiltration of activated macrophages and appears to be contact dependent^{15,28,29} (Figure 1).

Inflammation in the CNS: Macrophage-Mediated Axonal Dieback

Neuroinflammation plays a major role after SCI, but its exact function in regeneration failure remains controversial.

Immediately after injury, the blood-brain barrier breaks down.³⁰ Resident CNS cells up-regulate proinflammatory cytokines and chemokines within the first hour after injury.³¹ Neutrophils infiltrate the lesion site within 3 hours, phagocytose debris, and remain elevated for 3 days.³² Resting microglia, which normally survey the CNS for insult, become activated within 24 hours after injury, resulting in changes in their morphology and the release of chemokines and cytokines.^{30,33} CD68⁺ cells increase dramatically after injury within the first 7 days, then slowly increase until 2 to 4 weeks after injury.^{28,30} CD68 labels both activated microglia and infiltrating macrophages, so it is difficult to distinguish between these two cell types based on this marker alone. Along a similar time frame, T lymphocytes increase at the site of injury, although not to the extent of macrophages.³⁴

Although macrophages phagocytose debris³⁵ and can secrete neurotrophic factors,^{36,37} previous work from the laboratory of Silver and colleagues^{28,29} suggests these cells induce axonal dieback through direct contact with the dystrophic endings of axons. After SCI, long-distance retraction of injured axons coincides with the infiltration of activated macrophages. Depletion of infiltrating macrophages using clodronate liposomes significantly reduced axonal dieback, but did not enhance regeneration.²⁸ The authors found that the growth state of the axon was important to facilitate this axonal retraction, because only dystrophic axons were susceptible to macrophage-mediated dieback *in vitro*. Inhibiting matrix metalloproteinase-9, degrading proteoglycans at the site of injury, and enhancing the growth potential of injured axons through the use of a conditioning lesion were all sufficient to block this macrophage-mediated dieback.²⁹ Using a radiation chimera model and high-resolution *in vivo* imaging, Evans and colleagues¹⁵ were able to distinguish between the actions of resident CNS microglia and infiltrating blood-derived macrophages and demonstrated that blood-derived macrophages alone are responsible for this secondary axonal dieback after SCI. A milestone contribution by Kigerl and colleagues³⁸ was that the effect of microglia/macrophages being neurotoxic or growth promoting was largely dependent on their polarization toward different subsets.

Intrinsic Growth Capacity for Injured CNS Neurons

After dieback, injured neurons of the adult CNS are only capable of modest sprouting and minimal regeneration, even with neutralizing various inhibitory components of the extracellular environment, suggesting their intrinsic growth capacity is reduced over time. To initiate regeneration, the injured axon must transmit an injury signal back to the soma, where the neuron begins transcribing various regeneration-associated genes.³⁹ Axotomy leads to a rapid influx of ions, increasing the intracellular calcium concentration, which can act as an injury signal.⁴⁰ Injury also induces cytokine expression that can act as a signal for downstream signaling. In particular, the family of cytokines that signal through the glycoprotein 130 receptor

has been implicated as an injury signal in the periphery, resulting in signal transduction through the Janus kinase/STAT pathway. These cytokines, including ciliary neurotrophic factor and IL-6, are expressed after axotomy and promote regeneration in the periphery.^{41–43} This work has been expanded to spinal cord injury. IL-6 has been implicated in proinflammatory signaling, with both positive⁴⁴ and negative⁴⁵ effects on regeneration reported. Leukemia inhibitory factor has been implicated in oligodendrocyte preservation,⁴⁶ and when leukemia inhibitory factor was delivered directly into the dorsally hemisectioned spinal cord, it was able to enhance the growth of injured corticospinal axons.⁴⁷ Ciliary neurotrophic factor has also been shown to mediate stroke-induced neurogenesis,⁴⁸ further suggesting that this cytokine family can have an important role in regeneration signaling.

To promote regeneration, efforts have been made to enhance the intrinsic growth capacity of adult CNS neurons. One method that has augmented the regenerative ability of neurons in animal models is the use of a conditioning lesion. Injuring the peripheral branch of a sensory neuron in the sciatic nerve enhances the ability of the central process to regenerate beyond a central lesion.⁴⁹ This effect can be achieved even if the conditioning lesion is performed immediately after the central injury.^{50,51} Although the exact mechanism of how distal conditioning stimulates intrinsic growth of central processes remains under investigation, this method to enhance growth has elucidated several regeneration-associated transcription factors, including c-Jun, CCAAT/enhancer binding proteins, cAMP responsive element binding, STAT3, activating transcription factor 3, SRY-box 1, and Smad1.³⁹ A study with dorsal root ganglion neurons found that STAT3 activation is necessary to produce the conditioning lesion effect.⁵² There is some work to suggest that the conditioning lesion effect is partly achieved through the glycoprotein 130 signaling described above.^{53,54} Several other methods have been used to try to replicate this conditioning effect without the negative consequences of harming peripheral nerves. For instance, inducing inflammation within the dorsal root ganglion (DRG)⁵⁵ or the vitreous of the eye⁵⁶ has also been shown to enhance intrinsic growth of these neurons. Furthermore, adding cAMP⁵⁷ or electrically stimulating the intact sciatic nerve⁵⁸ has also been shown to replicate the conditioning lesion effect.

In a series of pioneering studies from Zhigang He's laboratory in the late 2000s, phosphatase and tensin homolog, a negative regulator of the mammalian target of rapamycin pathway, emerged as a major determinant of CNS axon regeneration. One of the most notable studies on enhancing intrinsic growth capacity came from Park et al,⁵⁹ which demonstrated that a targeted deletion of phosphatase and tensin homolog in adult mice allowed robust regeneration after optic nerve injury. Using intravitreal injections of adeno-associated viruses expressing Cre, the authors managed to express Cre in >90% of retinal ganglion cells to conditionally knock down expression of *Pten*, resulting in remarkable regeneration of these optic nerve fibers through cholera toxin B tracings. This pathway was shown later to be important for regeneration of adult corticospinal neurons as

well, in which a conditional phosphatase and tensin homolog deletion enabled unprecedented regeneration beyond the lesion site.⁶⁰ The phosphatase and tensin homolog/mammalian target of rapamycin pathway is required for tumor suppression and is thought to be turned on after development to stop unwanted growth and sprouting within the CNS. Therefore, despite the promising results in achieving regeneration, caution should be exercised before moving these findings into therapeutics. A recent study examined the long-term effects of deleting *Pten* in the sensorimotor cortex of neonatal mice.⁶¹ After a spinal cord injury in these mice as adults, the authors reported robust regeneration of corticospinal tract (CST) axons, similar to what was observed in earlier studies.⁶⁰ However, the purpose of this study was to assess the long-term consequences of this targeted deletion. The authors observed larger layer V neurons in mice lacking *Pten* than in those that maintained *Pten* expression, but they did not observe significant differences in open field activity, RotaRod (TSE Systems, Chesterfield, MO) motor performance, tumor formation, or other neuropathology, suggesting this early deletion of *Pten* caused little neuropathology in adult mice. Better understanding of these molecules and pathways will provide new avenues for regenerative research.

Extracellular Mechanisms of Regeneration Failure

Enhancing the intrinsic growth capacity of CNS neurons is a major obstacle in achieving regeneration. Another impediment to growing CNS axons is the inhibitory extrinsic factors that exist in and around the lesion environment. By using peripheral nerve grafts to connect the medulla to the spinal cord, David and Aguayo⁶² demonstrated that central axons from both levels were able to regrow 30 mm through this peripheral environment, illustrating the inhibitory nature of the CNS environment. Extensive research has been performed to understand the differences between the CNS and PNS environment that allow axons to regrow in the periphery but not in the CNS. CSPGs and factors associated with CNS myelin are among the leading groups of molecules studied for their inhibitory properties, but other factors are up-regulated after injury as well and contribute to regeneration failure. Together, these molecules are found in and around the glial scar, generating a hostile environment for axon growth.

Formation of the Glial Scar

Immediately after SCI, extensive inflammation occurs at the site of injury, leading to cavitation that extends the primary insult. Over time, reactive astrocytes surround this lesioned area to form what is known as the glial scar. This scar is made up of two distinct components: the lesion core that is composed of a variety of cell types, including infiltrating macrophages, neural-glial (NG)2⁺ cells, fibroblasts, and pericytes; and the penumbra, which is made up of hypertrophic astrocytes that surround the

lesion core and wall off the injured area from surrounding healthy tissue.⁶³

The glial scar is a complex environment of extracellular matrix and cell types that intermingle to form a dense structure. This structure serves to protect neighboring tissue, but also acts as a major impediment to regenerating axons. Using a nitro-cellulose support to remove an intact glial scar from an adult rat after CNS trauma, Rudge and Silver⁶⁴ demonstrated, *in vitro*, that this glial scar explant was inhibitory to purified rat hippocampal neurons. Further work *in vivo* showed that micro-transplanted adult DRG neurons were able to grow long distances through white and gray matter of the CNS, only halting growth abruptly on entering the lesion site containing up-regulated extracellular matrix molecules, such as CSPGs.¹³ This work demonstrated, for the first time, that the glial scar inhibits regeneration of adult CNS neurons. Since then, extensive research has been performed to try to overcome the inhibitory nature of the glial scar, specifically CSPGs (described *CSPGs and Their Receptors*). However, recent work from Michael Sofroniew's laboratory is challenging the dogma that the glial scar impedes functional recovery.⁶⁵ Beginning with a study in 2004, Faulkner et al⁶⁵ used mice expressing herpes simplex virus thymidine kinase under a glial fibrillary acidic protein promoter to ablate reactive astrocytes in the vicinity of the lesion on treatment with the antiviral agent ganciclovir. The authors report widespread tissue disruption, enhanced cellular degeneration, demyelination, greater cell death of both neurons and oligodendrocytes, and motor deficits when astrocytes were ablated after small stab injuries or moderate crush injuries, suggesting astrocytes are crucial to protect surrounding tissue from the spread of injury. Together, these studies demonstrate the importance of the glial scar in containing the inflammatory processes to the site of injury and protecting surrounding healthy tissue. The early immature scar may be even implicated in sprouting responses because immature astrocytes are, for example, known for exerting growth-promoting capacities.⁶⁶

CSPGs and Their Receptors

One major component of the glial scar is a family of extracellular matrix proteins known as CSPGs, which consist of a protein backbone and glycosaminoglycan (GAG) chains. The varying number, length, and sulfation of GAG chains attached to the core protein provide each CSPG with its unique properties.⁶³ CSPGs are present throughout the developing and adult CNS; however, their expression is dramatically increased after injury, in the vicinity of the disrupted blood-brain barrier.⁶⁷ Reactive astrocytes are responsible for producing and secreting many of the CSPGs after injury, and their up-regulated expression persists for months.^{68,69} These molecules have been shown to inhibit neurons in development,⁷⁰ in culture,⁷⁰ and after injury.⁶³ Because neurons extend processes into an increasing gradient of CSPG, their growth halts and their growth cones become dystrophic.¹⁴ CSPGs have also been implicated in the formation of perineuronal nets, which prevent aberrant synaptic plasticity in uninjured adult animals.⁷¹ Much of

their inhibition is thought to be mediated through their GAG chains, because removing these structures leads to regeneration and functional recovery after SCI.⁷² Recent studies have implicated CSPGs in remyelination deficits as well, further contributing to regeneration inhibition. CSPGs have been shown to inhibit the process outgrowth and differentiation of oligodendrocyte progenitor cells *in vitro*.⁷³ By inhibiting CSPG synthesis, a recent study was able to accelerate remyelination in a focal demyelination mouse model.⁷⁴ Therefore, by direct interaction with injured axons or through indirect effects on myelination, CSPGs have an established role in regeneration failure.

It was thought that CSPGs inhibited neurons through nonspecific mechanisms. Many believed the negatively charged boundary formed by CSPGs repelled neurons⁷⁵ or that neurons were inhibited because of substrate occlusion⁷⁶ caused by CSPGs. However, the recent discovery of several CSPG receptors has shifted the thought about how CSPGs inhibit neurite outgrowth. The leukocyte common antigen-related family of receptor tyrosine phosphatases has been identified to specifically bind CSPGs and mediate inhibition. Receptor protein tyrosine phosphatase σ ⁷⁷ was identified first, followed by fellow leukocyte common antigen-related family member leukocyte common antigen related.⁷⁸ Protein tyrosine phosphatase δ is also assumed to be a receptor, because of its high sequence homology with leukocyte common antigen related and protein tyrosine phosphatase σ , but this has yet to be described. These members all contain a canonical GAG binding motif composed of a cluster of lysine residues.⁷⁹ Nogo receptor (NgR)1 and NgR3 have also been identified as CSPG receptors.⁸⁰ Although knocking out either NgR1 or NgR3 individually was not sufficient to overcome CSPG-mediated inhibition, their combined loss allowed for significant regrowth of severed retinal ganglion cells. This study provides a link between two different classes of inhibitory molecules, those associated with myelin and with CSPGs.

Many different signaling pathways have been implicated in CSPG-mediated inhibition. When neurons contact CSPGs, the ρ / ρ -associated protein kinase signaling pathway is activated, and blocking this signaling attenuates inhibition.⁸¹ Protein kinase C has been implicated downstream of the ρ pathway, because inhibiting protein kinase C blocks signaling from ρ activation and promotes dorsal column regeneration.⁸² In addition, CSPG-mediated calcium influx activates the epidermal growth factor receptor and blocking this kinase function blocks inhibition, suggesting epidermal growth factor receptor also mediates CSPG inhibition.⁸³

Chondroitinase ABC (ch'ase) is an enzyme that selectively cleaves GAG chains from the protein core of CSPGs. Many studies have used this enzyme to reduce or eliminate inhibition both *in vitro* and *in vivo*, suggesting much of the inhibitory properties of CSPGs are attributable to their GAG chains.⁶⁷ Administering ch'ase intrathecally promoted regeneration of sensory and CST axons after a dorsal column lesion,⁷² restoring postsynaptic activity below the lesion and promoting functional

recovery of locomotor and proprioceptive behaviors. Ch'ase has also been shown to promote regeneration of axons in the Clarke nucleus projections and the rubrospinal tract.^{84,85} Ch'ase has also been used in many combinatorial strategies to improve regeneration,^{86–89} demonstrating the inhibitory properties of these molecules and the complexity of restoring regeneration.

Entrapment

Another reason for regeneration failure has been discovered recently. First described in 1985, Carlstedt⁹⁰ observed that fibers regrowing through the dorsal root were able to elongate until they reached the dorsal root entry zone, the interface between the PNS and CNS, where some fibers began to form synaptoid nerve terminals with a glial cell population. Further studies demonstrated that an active signaling pathway was responsible for this abrupt stopping behavior just as the root enters the spinal cord,⁹¹ which was termed the physiological stop pathway. In both studies, the authors believed the fibers were forming stable connections with reactive astrocytes. However, NG2⁺ cells were not yet discovered.⁹² *In vivo* imaging has been used to visualize this synaptic-like stabilization at the dorsal root entry zone.⁹³ Later studies found similar findings in the spinal cord, where injured fibers in the spinal cord cease retracting in an area of NG2⁺ cells.^{94,95} Herein, neurons become entrapped on the NG2⁺ cell surface, where they begin to form synaptic-like connections with this glial cell population.²⁷ This cell-cell interaction is facilitated by the laminin and NG2 molecules on the NG2⁺ cell surface and the receptors on the neurons. Although ch'ase treatment can release neurons from the NG2⁺ cell surface acutely in *in vitro* cultures, this interaction becomes difficult to break by 5 days *in vitro*. This emerging mechanism of regeneration failure begins to address the conflicting findings that, although CSPGs are known to be inhibitory, dystrophic axons tend to associate in an area of high CSPG expression chronically, causing us to rethink our understanding of how CSPGs inhibit growth.⁹⁶

Myelin Components and NOGO

The components of CNS myelin differ from those of PNS myelin and have been implicated in neurite outgrowth inhibition *in vitro*.⁹⁷ Although myelin-associated glycoprotein,^{98,99} oligodendrocyte-myelin glycoprotein,^{100,101} and Nogo¹⁰² are all present in CNS myelin and inhibit neurite outgrowth *in vitro*, only Nogo has been shown to inhibit regeneration *in vivo*.¹⁰³ However, the inhibitory properties of Nogo have been debated.¹⁰⁴ Knocking out all three receptors for Nogo, either singly or in combination, did not allow for meaningful enhanced regeneration.^{105,106} These diverging results are largely attributable to differences in methodology, as myelin-associated inhibitors affect axonal sprouting rather than axonal regeneration.¹⁰⁷ It appears that these inhibitors operate with modulators of intrinsic growth state in a sequential manner. Once the intrinsic state is elevated, extrinsic growth modulators including myelin-associated inhibitors are likely to serve as a subsequent barrier to regeneration.¹⁰⁷

Other Inhibitors of Axonal Regeneration

The extracellular environment contains other inhibitors along with CSPGs and components of CNS myelin. The repulsive guidance molecule-A, Ephrin and its receptors, semaphorins (Semas), the Wnt receptor Ryk, tenascin-R, and slits have all been implicated in inhibiting regeneration.

Repulsive guidance molecule-A is repulsive for axon growth during development¹⁰⁸ on binding to its receptor neogenin.¹⁰⁹ Repulsive guidance molecule-A is expressed by oligodendrocytes and reactive astrocytes after SCI and localized to CNS myelin and the developing glial scar.¹¹⁰ Repulsive guidance molecule-A inhibits mammalian CNS neurite outgrowth by a mechanism dependent on the activation of the ρ A- ρ kinase pathway.¹⁰⁸

Tenascin-R is believed to play a role in the formation of the perineuronal net, which functions in development and limits plasticity after injury. Tenascin-R increases after SCI. Tenascin-R-deficient mice have enhanced regeneration, which appears to be mediated through rearrangement of the perineuronal net rather than acting on the neuron for regrowth directly.¹¹¹ There is also some evidence suggesting slits are up-regulated after cortical injury, potentially affecting regeneration.¹¹²

Ephrin and Eph receptors have well-established roles in development, functioning in axon guidance and patterning. These molecules are also up-regulated after CNS injury, leading to speculation that these molecules play a role in regeneration failure. In particular, Ephrin-B expression on astrocytes and EphB expression on fibroblasts have been shown to segregate these cell types at the lesion site, aiding in formation of the glial scar and reestablishment of the blood-brain barrier.¹¹³ Beyond a possible role in scar formation, the interaction of ephrinB3 on postnatal mouse oligodendrocytes with EphA4 on cortical neurons inhibits these neurons, possibly playing a role in regeneration failure.¹¹⁴ Furthermore, EphA4 accumulates in CST axons after SCI, which may allow these axons to respond to ephrinB3 in myelin or ephrinB2 in scar tissue. Blocking this signaling with peptide enhanced sprouting, but not regeneration, of these axons.¹¹⁵

Semas have also been studied as inhibitory and repulsive guidance cues. When applied *in vitro*, Sema3A caused growth cone collapse of adult DRG neurons.¹¹⁶ Sema3A is also present within the lesion environment after SCI, where preconditioned adult DRG axons cease growing.¹¹⁷ Sema3A appears to inhibit in a CSPG-dependent manner, because studies blocking the interaction of Sema3s and CSPGs *in vitro* reduce Sema3A inhibition.¹¹⁷ Some studies with a small molecule (SM-216289), which blocks the binding of Sema3A with the neuropilin-1/plexinA receptor complex, reduced DRG inhibition *in vitro* and enhanced regeneration after olfactory nerve axotomy in rat.¹¹⁸ It also enhanced regeneration of serotonergic axons, but not CST or ascending sensory axons, after SCI.¹¹⁹ In addition to functioning as a guidance molecule, Sema3s function in

vascular remodeling,¹²⁰ the immune system,¹²¹ and cell death,^{122–124} which may all affect regeneration after SCI.

Finally, the Wnt family of developmental guidance molecules has been implicated in SCI, in particular with CST axons. Ascending and descending axons respond differently to Wnt stimulation, which is likely mediated through their different expression of receptors. Recently, the Wnt receptor Ryk has been identified as a negative determinant blocking neurite outgrowth, resulting in plasticity in lesion-remote areas.¹²⁵

The observation by Aguayo and colleagues¹²⁶ in the late 1970s/early 1980s was followed by an intense and successful search identifying growing numbers of molecules implicated in modulating axonal growth. Pertinent questions remain regarding the redundancy and functional hierarchy elicited by the increasingly large number of molecules.

Conclusion

Major progress has been made in understanding what impedes regeneration after SCI. A variety of different strategies have been used to achieve regrowth beyond the lesion center, and in some cases, this regrowth has been significant for a large, albeit still limited, number of fibers. Combinatorial strategies have been more successful in achieving this goal. However, as the field of regeneration makes great strides in enhancing the growth capacity of these injured axons, we must now think even more about how to reintegrate these growing fibers back into the circuitry that was established during development to restore proper function.

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