Targeting Central Nervous System Regeneration with Cell Type Specificity

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KEYWORDS

• Spinal cord injury • Neuronal regeneration • Neural repair • Growth programs

KEY POINTS

- Axon regeneration is required for functional recovery following severe spinal cord injury.
- Functional recovery mediated by axon regeneration is not robust.
- Central nervous system (CNS) neurons are heterogeneous and possess multiple subtypes.
- A greater understanding of subtype-specific regenerative responses is needed.

INTRODUCTION

Spinal cord injury (SCI) lesions are broadly classified as either incomplete or complete. Incomplete SCI lesions spare surrounding neural tissue and residual axon projections. This spared tissue is capable of rescuing varying degrees of lost function, which is augmented through rehabilitation training in animals[1–3] and humans.[4–8] Conversely, complete injuries spare little to no neural tissue, resulting in permanent and irreversible loss of motor, autonomic, and sensory functions for which rehabilitation is not effective. It is universally agreed that biologic repair is necessary to reconstruct damaged circuits and restore lost function.

Understanding why central nervous system (CNS) axons fail to regrow following injury and developing repair strategies to overcome this failure has been a heavily studied topic for much of the past century. Pioneering work in the 1980s by Aguayo and colleagues[9–11] reported that CNS axons could be coaxed to regrow into peripheral nerve grafts. This suggested two things: the environment of the mature CNS is not supportive to axon regrowth, and the CNS environment created by trauma is devoid of growth supportive factors that are required for axon regrowth.

Research in the 1980s to 2000s focused heavily on identifying and trying to neutralize potential inhibitory molecules around CNS lesions and associated with degenerating myelin.[12–15] This was followed by work on digesting chondroitin sulfate proteoglycans, which were reported to be potent inhibitors of axon growth in vitro and are present around lesion sites and engulf perineuronal nets in the gray matter.[16,17] Although neutralizing inhibition was initially hoped to be a singular solution to axon regeneration, it has not held the test of time and replication, although its effect on axon sprouting has been well documented.[16,18,19] The search for other factors involved in suppressing regeneration led to the identification and manipulation of several growth-repressing signaling pathways[20–22] and growth factors[23–25] whose augmentation resulted in impressive degrees of axon regeneration. As discussed in more detail later, these technologies have transformed the field. It is now clear that promoting successful axon regeneration through regions of injury involves modulation of the traumatic environment and increasing neuronal regenerative capacity. Yet, despite impressive axon regeneration in models of severe SCI, robust functional recovery is lacking. Exactly what is missing remains to be

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discovered. This review outlines the neuropathologic response to SCI and argues that a more nuanced understanding of neuron subtype-specific regenerative responses is needed to mediate biologic repair capable of restoring lost functions.

PATHOLOGY: CELLULAR AND MOLECULAR RESPONSES TO SPINAL CORD INJURY

The pathologic response to SCI consists of the immediate mechanical injury of the spinal cord, followed by a secondary phase that takes place shortly thereafter, evolves over a period of months, and comprises a series of biochemical and cellular events.[26] The initial injury, generally in the form of compression or laceration of the spinal cord, is followed by an immediate immune response, subsequent cell proliferation, scar formation, and tissue remodeling.[27] During the immediate inflammatory phase, cells within the lesion die from internally programmed suicide (apoptosis), and neurons go through Wallerian degeneration, whereby axons "die-back" from the lesion (**Fig. 1**A). Axons at the epicenter of the injury are transected, whereas those in the periphery become demyelinated. During the initial inflammatory phase, various molecules and then cells penetrate the injury site to begin the process of debris clearance. Blood-borne molecules are the first to infiltrate and signal to local cells to produce components of the extracellular matrix, such as laminins, fibronectins, and collagens, which then function as a scaffold by which inflammatory macrophages then enter.

Cell proliferation begins approximately 2 days postinjury and leads to the formation of the fibrotic scar and surrounding astroglial scar-border. Fibrotic scar is formed by the proliferation of non-neural cells including endogenous fibroblasts, pericytes, and endothelial cells that occupy the central compartment of CNS lesions,[28] whereas the astrocyte scar-border serves to demarcate areas of damaged tissue and to sequester viable (neural tissue) from nonviable (fibrotic) tissue.[29–31] The end result of this scarring segregates the spinal cord lesion into three major lesion compartments: (1) the central lesion cavity, referred to as the nonneural lesion core or fibrotic scar; (2) the surrounding astroglial scar-border; and (3) a region of spared but reactive and reorganizing neural tissue[27,32,33] (see **Fig. 1**A). The following sections discuss in more detail astrocyte scar-border and fibrotic scar formation, and highlight some of the challenges these are thought to present in regards to axon regeneration.

CELL REACTIVITY: ASTROCYTE SCAR-BORDER FORMATION

Astrocytes are a type of glial cell that tile the entire CNS, where they occupy individual, nonoverlapping territories and assist in maintaining normal circuit function. Long thought to be mere support cells for neurons, astrocytes have now been implicated in almost every facet of neurologic function.

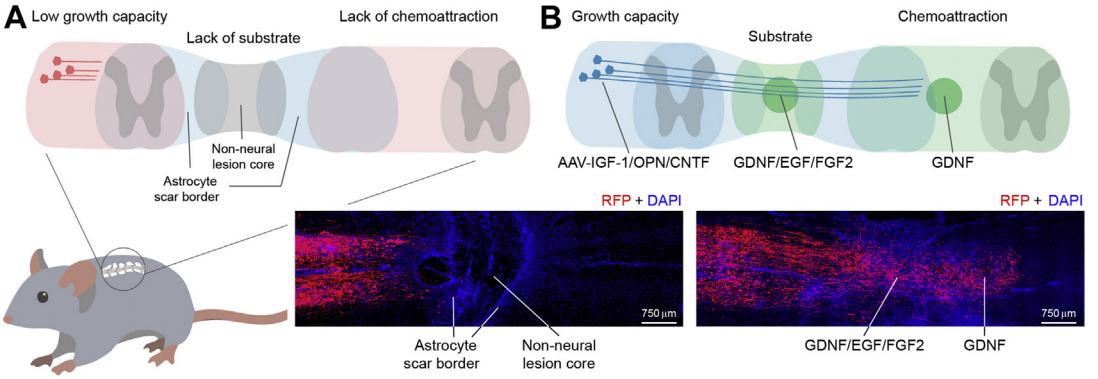

Fig. 1. Axon regeneration after SCI. (*A*) Scheme depicts axon regenerative failure following SCI. The nonneural lesion core is surrounded by an astrocyte scar border that demarcates damaged tissue from reactive and remodeling neural tissue that has been spared from the injury. Axons fail to regrow because of a combined lack of (1) neuronal growth capacity, (2) supportive substrate, and (3) chemoattraction. Photomicrograph depicts RFP-labeled propriospinal axons stopping at the lesion scar border. (*B*) Scheme depicts biologic repair strategy that targets (1) upregulation of intrinsic neuronal growth capacity by viral overexpression of IGF-1/OPN/CNTF, (2) upregulation of supportive substrate via biomaterial delivery of EGF/FGF2, and (3) chemoattraction via biomaterial delivery of GDNF. Photomicrograph depicts RFP-labeled propriospinal axons growing into and through nonneural lesion cores and toward chemoattractant below the lesion.

Astrocytes are widely known for their response to injury and disease, which they respond to with a process referred to as astrogliosis. Previously believed to be a homogeneous and detrimental event resulting in scar formation, evidence gathered over the past 30 years has demonstrated the contrary.[34] It is now clear that astrogliosis is not a single uniform event, but is a highly heterogeneous process that depends largely on the severity and type of CNS insult. At the lower end of the spectrum, mild to moderate astrogliosis results in cell hypertrophy and changes in gene expression that are reversible and subside over time. Severe astrogliosis, such as what occurs after SCI, results in cell proliferation, scar-border formation, and permanent tissue reorganization. This is a major and well-studied pathology of many neurodegenerative diseases and is a prominent feature of SCI.[16,31,32,35]

Transgenic and genomic techniques have allowed progress to be made in understanding the mechanisms of astrocyte function and scar-border formation. Following severe CNS insults, astrocytes divide and form a dense meshwork of interwoven cells surrounding the lesion perimeter. Scar-border-forming astrocytes do not migrate from other areas of the CNS, but rather derive from newly proliferated astrocytes located at and near the injury site. There has been some controversy suggesting that scar-border-forming astrocytes originate from ependymal cells located in the central canal.[36,37] However, recent work has reported that when the ependyma is not directly severed, ependymal cells contribute extremely minimally to astrocyte scar-border formation.[38]

Loss of function studies have demonstrated that the role of astrocyte scar-border formation is to seal off the lesion area to prevent inflammation from further damaging areas of healthy tissue. Preventing or attenuating astrocyte scar-border formation results in increased inflammation, cell death, demyelination, and worsened behavioral recovery.[29,30,35] However, this protective function has long been balanced by the widespread belief that scar-border formation is a key contributor to regenerative failure. The notion of astrocyte-mediated inhibition was initially caused by its barrier-like appearance and was further propagated by reports that astrocytes upregulate chondroitin sulfate proteoglycans, which inhibit axon growth in vitro and in vivo.[16,39]

Recent evidence supports the notion that scar-border-forming astrocytes may not be the primary inhibitor to axon regrowth that they were once thought. Loss-of-function studies have directly tested this hypothesis and have reported that attenuation or ablation of scar-border-forming astrocytes does not result in spontaneous axon regeneration. Rather, when intrinsic neuronal growth capacity is stimulated and chemoattractive growth factors are provided, the formation of the astroglial scar-border in fact supports the regrowth of regenerating sensory axons into non-neuronal SCI lesion cores.[31] Preventing or attenuating astrocyte scar-border formation also attenuates this stimulated regeneration. This similar concept has also been found to be true for propriospinal axons (**Fig. 1**B),[25] suggesting that the growth-supportive nature of scar-border-forming astrocytes is likely beneficial for most if not all types of CNS axons.

CELL REACTIVITY: FIBROTIC SCAR FORMATION

Considerably less attention has been given to the fibrotic component of the scar. However, recent studies have highlighted the inhibitory nature of the fibrotic scar,[20,40,41] which document regenerating axons specifically avoiding areas of fibrotic tissue. Mechanistic information regarding the origin and function of the fibrotic scar is gradually accumulating.

Following CNS injury, fibroblasts from locally damaged meninges are recruited by macrophages and then migrate into the lesion site where they proliferate and form the fibrotic scar.[42] The fibrotic scar is characterized by an array of nonneural cells (predominantly fibroblast-lineage cells, endothelia, fibrocytes, pericytes, and inflammatory cells) and these produce extracellular matrix molecules. Similar to the astrocytic scar-border, this fibrotic component is thought to serve protective and detrimental roles following CNS injury. It protects nearby tissue and assists in resealing the blood brain barrier (BBB).[43] However, it is also believed to be inhibitory for axon growth.[27,44] Fibroblasts have been reported to express multiple inhibitors of axon growth, including NG2, phosphacan, tenascin-C, semaphorin 3A, and EphB2. Strategies targeting the attenuation of fibrotic scar have reported beneficial results.

The microtubule stabilizing pharmacologic compounds taxol[45] and epothilone B[46] have been reported to reduce fibrotic scar formation, which was correlated with enhanced sensory and serotonergic axon innervation and functional recovery following SCI.[45] Another interesting study[47] reported that moderate reduction of type A pericyte scar formation results in less extracellular matrix deposition and enhanced regrowth of corticospinal tract (CST) and RST: reticulospinal tract (RST) axons into and around SCI lesions, resulting in increased electrophysiologic connectivity and

functional recovery. This suggests that it may be possible to achieve functional axon regeneration in the absence of promoting neuronal growth capacity. However, therapeutic strategies targeting fibrotic scar need to strike a delicate balance between mild to moderate attenuation, because too much attenuation has been reported to cause the failure of wound healing and expand the injury site, negatively impacting neural repair and functional recovery.[37,47,48]

NEURONAL HETEROGENEITY: LESSONS FROM THE OPTIC NERVE

CNS neurons are distinct in their morphology, physiology, gene expression, and function. Furthermore, different neuronal subtypes display heterogeneous regenerative responses and possess specific activation requirements.[25,49,50] A comprehensive understanding of the molecular architecture of CNS neurons is pivotal to devising targeted regenerative strategies to manipulate their growth. Retinal ganglion cells (RGCs) have been extensively characterized, and their mechanistic dissection over the past several decades provides an ideal conceptual framework that would be beneficial to apply to other CNS neurons.

Survival or Death

Before a neuron can regenerate, it must first survive axotomy. Work initially performed by David Aguayo's group[51] demonstrated that most (>80%) RGCs die following axotomy, and that this is at least partially caused by a lack of neurotropic support. RGC survival is enhanced via exogeneous delivery of brain-derived neurotrophic factor,[52] and axon sprouting is enhanced with certain growth factors.[53] This principle has been subsequently confirmed for corticospinal neurons[54,55] and spinal motor neurons,[56,57] where specific growth factors, but not others, enhance their survival following axotomy. Survival correlates with the distance of the neuronal soma from the site of axotomy. This may partially explain why most RGCs die following optic nerve crush,[52] and some short propriospinal neurons following SCI,[58] whereas long descending propriospinal,[58] corticospinal, and rubrospinal neurons[59,60] have been reported to survive following axotomy at the spinal level. Although some neurons respond to particular growth factors, this is dependent on the level of receptor expression in the soma, and neurons vary in the degree to which they express specific receptors.[54] A more thorough understanding of growth factor receptor expression levels across cell types is needed to develop targeted repair strategies (**Fig. 2**).

The recent revolution in single cell RNA sequencing has revealed multiple and previously unknown types of neurons within the optic nerve[61,62] and spinal cord.[63–65] More than 40 transcriptionally distinct types of RGC have been identified, and their ability to survive or die following axotomy varies greatly among subtypes. Although most RGCs that survive are αRGCs, certain subtypes of these also die, suggesting that factors beyond transcriptional proximity contribute to survival ability.[61] Although there are some correlative similarities in cellular morphology and physiology among surviving RGCs, no single factor predicted survival, suggesting that even within subtypes that survived, they did so via differing mechanisms. Recent advances in the analysis of bioinformatic datasets may prove valuable in prioritizing cell types based on their ability to respond to experimental perturbations,[66] and their development

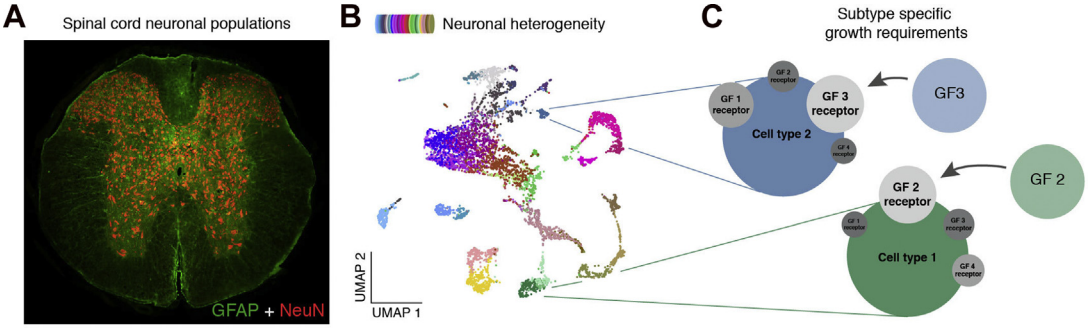

Fig. 2. Neuronal heterogeneity and subtype-specific activation requirements. (*A*) Photomicrograph depicting spinal cord neuronal populations on a cross-section of mouse spinal cord tissue immunohistochemically stained for GFAP (*green*) and NeuN (*red*). (*B*) Uniform manifold approximation and projection (UMAP) visualization of single-nucleus sequencing of mouse spinal cord neurons depicting more than 40 transcriptionally distinct neuronal subtypes. (*C*) Scheme depicting how different neurons within the spinal cord may have different receptors for specific growth factors (GF) to induce their regeneration.

and widespread use will shed light on how heterogeneous cellular populations respond to experimental manipulations and will be instrumental in guiding cell specific repair strategies.

Influencing Regeneration

Although several neurotropic factors (brain-derived neurotrophic factor, glial derived neurotrophic factor (GDNF), Ciliary neurotrophic factor (CNTF), and fibroblast growth factor-1 [FGF-1]) aid in promoting neuronal survival, most have classically failed at promoting any meaningful regeneration.[67] Nonetheless, some axons regenerate into peripheral nerve grafts,[11,68] suggesting a limited degree of growth capacity is maintained. RGCs undergo a developmental decline in their regenerative ability and adult RGCs do not extend axons by default but require the presence of neurotropic cues.[67,69–71] Various factors have been identified that promote a degree of regeneration, including zymosan,[67] angiotensin II,[72] and others, and combining these with peripheral nerve grafts further potentiates this growth.

Although initial successes in RGC regeneration seemed promising, the overall growth was modest.[72] Similar findings proved true in the context of SCI, where a combination of permissive cell grafts and neurotropic factors elicited a limited degree of regenerative growth.[9,49,73,74] Nonetheless, in both injury models and in all types of neurons studied, it was abundantly clear that an increase in growth by several orders of magnitude would be required to achieve a clinically meaningful result capable of restoring lost function.

Elegant work from Zhigang He's laboratory spearheaded the discovery and application of factors regulating intrinsic neuronal growth programs. Numerous molecular regulators of axon growth have been and continue to be identified. An in-depth discussion of these is beyond the scope of this review and has been discussed elsewhere.[75–77] Briefly, deletion of PTEN,[78] SOCS3,[79] c-myc,[80] DCLK,[81] and members of the KLF family[82] have been reported to robustly increase the amount of regeneration of RGCs following optic nerve crush, and combinations of these manipulations have been reported to further augment this growth.[21,80] Recent work has led to the identification of different combinations of growth factors, such as Insulin-like growth factor-1 (IGF-1), osteopontin (OPN), and CNTF, which act on similar molecular pathways and yield similar degrees of regeneration,[23–25,50] a key step toward eventual clinical translation.

Nevertheless, although these manipulations result in extensive regeneration, they do not act on all types of neurons equally, and subtype-specific responses have emerged.[25,50,83] PTEN deletion results in the survival and regeneration of RGCs following axotomy, but is restricted primarily to αRGCs, which constitute just 6% of the total RGC population.[50] PTEN acts by elevating mTOR activity, which declines following development. αRGCs have naturally high levels of mTOR activity, and this may partially explain their robust growth response in comparison with other types of RGCs. Forced overexpression of the growth factors OPN and IGF-1 results in a similar degree of regeneration as PTEN deletion, and αRGCs selectively express receptors for both these growth factors. Together, these findings suggest that signaling pathways regulating growth are not uniform among CNS neurons, or even among subtypes of the same class of neuron. Finding the appropriate permutations to elicit a more comprehensive regenerative response across RGC subtypes requires a highly nuanced understanding of the specific signaling pathways regulating subtype-specific growth, and needs to be balanced by positive and negative effects different perturbations may have. For example, overexpression of the transcription factor Sox11 results in the death of αRGCs, but in the regeneration of non-αRGCs.[83] Although more work needs to be done in this area, it is intriguing to imagine that given the limited amount of neurons currently capable of regenerating, the field has only seen a fraction of what is theoretically possible.

NEURONAL HETEROGENEITY: APPLICATION TO SPINAL CORD INJURY

Most effective manipulations currently applied to SCI were initially discovered from studies in the optic nerve. Compared with the eye, these manipulations are not as effective in the context of SCI, likely caused by differing lesion pathologies. Although lesions to the optic nerve result in a spared contiguous bridge of reactive astroglia along which regenerating axons grow across, severe and complete SCI lesions often do not have spared tissue bridges, and the nonsupportive nature of the fibrotic scar poses a significant barrier to regeneration.

SCI lesions are grafted with various cells, and combining these with delivery of neurotropic factors results in some growth of ascending and descending axons into grafts.[49] Similar to findings from the optic nerve, all neurons do not regenerate equally, and observations of heterogeneous growth responses are emerging. A traditional belief is that corticospinal neurons are the most refractory to regeneration, whereas dorsal root

ganglion neurons and raphespinal (serotonergic) neurons the most responsive to regeneration. This idea, however, is largely based on spontaneous and correlative growth responses, where corticospinal axons die back considerably, whereas serotonergic and sensory axons remain in close proximity to lesion borders.[31]

The notion of refractory versus responsive regeneration may be somewhat misguided. Emerging evidence suggests the issue lies more in dissecting appropriate requirements to trigger the regenerative programs of different subtypes of neuron. Although corticospinal neurons are considered the most refractory type of CNS neuron, PTEN deletion results in robust regrowth of corticospinal[20,22] axons following SCI, but is ineffective at promoting propriospinal axon growth,[25] which are considered to have a naturally high regenerative capacity.[84] Currently, a manipulation that induces robust regrowth from multiple tracts, including the corticospinal tract, is grafts of caudalized neural stem cells.[85,86] This is achieved partially by transforming the regenerating corticospinal neuron to an embryonic transcriptional state,[87] but the exact mechanisms of how the neuron regenerates, or what neurotropic cues are secreted by the graft, are not known.

Chemoattraction and growth factor specificity have also become recognized as important elements in overcoming regenerative failure. Achieving regeneration of propriospinal[25] and sensory[31] axons into nonneural lesion cores requires the presence of chemoattractive cues in combination with upregulation of intrinsic neuronal growth programs. Propriospinal neurons, activated by overexpression of IGF-1, OPN, and CNTF and chemoattracted by GDNF, grow robustly into and through SCI lesions (see **Fig. 1**B), whereas 5HT neurons do not.[25] This suggests that a major issue to solving regeneration lies in finding the right combination of factors that are specific to the cell population being studied (see **Fig. 2**C).

Although the field of SCI has made tremendous advances in achieving regeneration, the normalized percentage of regeneration relative to an intact spinal cord is still modest. The reason for this is not clear. One likely reason is that, similar to RGC neurons in the optic nerve, supraspinal and intraspinal neurons comprise a heterogeneous mix of cells that likely respond differently to growth factors and transcriptional manipulations. It has been reported that there are more than 40 different types of neurons in the lumbar spinal cord of the adult mouse,[64] and each of these subtypes vary in growth factor receptor levels (see **Fig. 2**A–C). Exploiting single cell technology to identify genetic programs that drive regeneration is important in finding candidate molecules or growth factors that can be used to target different neuron subtypes.

SUMMARY

There is now compelling evidence suggesting that different neuronal subtypes display heterogeneous regenerative responses and possess specific activation requirements. Although the level of regeneration currently achievable is impressive, it fails to yield robust functional recovery. Given what is known about cellular heterogeneity, current strategies are likely biased to cell types with a particular transcriptomic profile. More information on growth programs within specific neuronal populations is required to overcome this barrier to growth. Going forward, it will be useful to develop a deeper and more nuanced understanding of the genetic and functional diversity that exists within the numerous populations of CNS neurons. This will enable researchers to create tailored and cell type–specific regenerative interventions that have the potential to restore functions lost through SCI.

CLINICS CARE POINTS

- Biological repair strategies which stimulate axon regrowth across lesion sites will be key to allowing recovery of function following severe SCI.
- Currently, no such strategies exist for human patients with SCI.
- The requirements to achieve regeneration among different types of neurons are not the same, and a in depth dissection of these regeneration requirements will be necessary to foster functional recovery following severe SCI.

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DISCLOSURE

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