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REGENERATIVE BIOLOGY OF THE SPINE AND SPINAL CORD
Rahul Jandial and Mike Y. Chen

Regenerative Biology of the Spine and Spinal Cord

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DEDICATIONS

For Mr. Jett—the quintessential teacher.

—Rahul Jandial, MD, PhD

This book is dedicated to my father, Kang-shen Chen, PhD, who taught me to dream big and never give up, and to my mother, Shu-jen Chen, PhD, who taught me the importance of knowledge and sacrifice.

—Mike Y. Chen, MD, PhD

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Bad men can do good deeds. —Anonymous

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CHAPTER 1

FRONTIERS OF SPINAL CORD AND SPINE REPAIR:

Experimental Approaches for Repair of Spinal Cord Injury

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Abstract: Regeneration of injured CNS neurons was once thought to be an unachievable goal. Most patients with significant damage to the spinal cord suffer from permanently impaired neurological function. A century of research, however, has led to an understanding of multiple factors that limit CNS regeneration and from this knowledge experimental strategies have emerged for enhancing CNS repair. Some of these approaches have undergone human translation. Nevertheless, translating experimental findings to human trials has been more challenging than anticipated. In this chapter, we will review the current state of knowledge regarding central axonal growth failure after injury, and approaches taken to enhance recovery after SCI.

INTRODUCTION

In the field of spinal cord injury (SCI) research, the terms "regeneration" and "sprouting" both describe axonal growth responses. These forms of growth have to be distinguished because they serve separate functions and are controlled by distinct mechanisms (Fig. 1). "Regeneration" refers to new growth at the tip of a transected axon, and generally suggests propagation of growth over some distance, of the sort that occurs spontaneously after peripheral nerve injury. "Sprouting", on the other hand, refers to new growth that can arise from either a transected or an intact axon, can occur anywhere along the length of the axon (Fig. 1), and generally extends over a relatively short

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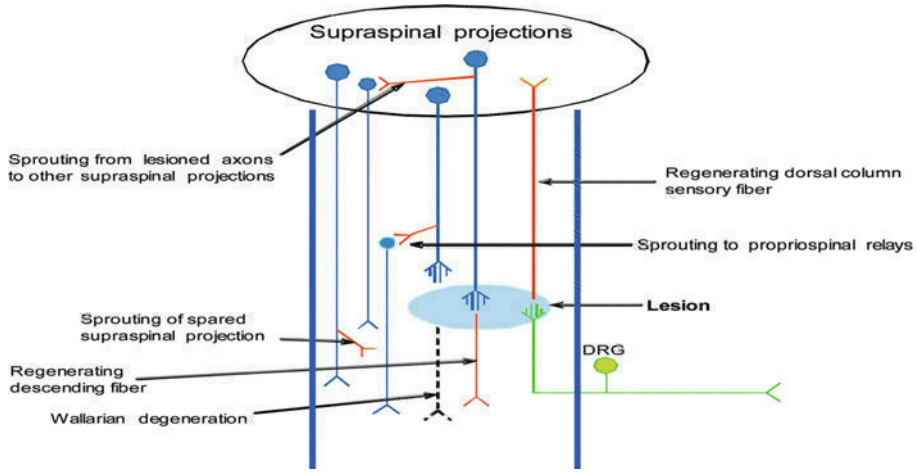

Figure 1. Anatomical plasticity of CNS neurons after injury. Following damages to descending and dorsal column sensory spinal tracts, the distal segment undergoes Wallarian degeneration and the proximal do not regenerate unless experimentally induced. Modified from Blesch A et al. Trends Neurosci 2009; 32:41-47;[1] ©2009 with permission from Elsevier.

distance. Axonal sprouting spontaneously occurs in both the PNS and the CNS and can be a robust phenomenon throughout life.[1,2] For example, axons sprout extensively in the human hippocampus during the course of Alzheimer's disease,[3] after spinal cord injury,[4-7] and in the cerebral cortex after stroke.[8,9] Sprouting can sub-serve substantial functional recovery, depending on the extent of the original injury and intervention with rehabilitative therapy.[10,11] However, axonal regeneration occurs rarely if at all after adult CNS injury, leading to the permanence of many functional deficits after SCI.

MECHANISMS UNDERLYING AXONAL GROWTH FAILURE

There are several mechanisms that contribute to the failure of axonal regeneration in the adult CNS after injury. (1) Extensive inflammation and cell death occurs around sites of SCI. (2) Permissive tissue substrates of bridges fail to form in injury sites, leading to an inability of injured axons to adhere to and grow through the injury site. (3) A number of molecules in the injured adult spinal cord actively inhibit axonal regeneration, including molecules in the extracellular matrix and proteins associated with CNS myelin. (4) There is a failure of production of growth-stimulating molecules, such as growth factors, around sites of central injury. (5) Intrinsic neuronal programs to activate growth are deficient in adult neurons.[12]

1. After first mechanical insult, additional damage accumulates within the first few hours by a variety of reactive processes.[13] Interruption of blood flow and massive inflammatory responses result in cell death and cell loss at or adjacent to the epicenter. The majority of cellular loss is due to necrosis in the first few hours post-injury and to apoptosis in the following days and weeks. Damage and death

of oligodendrocyte occur afterwards contributing to demylelinating phenotype and axonal degeneration at and adjacent to the injury site.[14] Mechanisms that may trigger apoptosis in the injured spinal cord include vascular abnormalities, excitotoxic events, and inflammation.[15] These findings have provided a base for neuroprotective strategies in repair of spinal cord following injury.

2. One of the pathological outcomes of SCI is the formation of cavities in the spinal cord. After days to weeks, a CNS injury can expand in size leading to a scar-encapsulted cavity[16-18] in which injured axons are unable to adhere and growth through the lesion site. The underlying causes of cyst formation are not fully understood although various phenomena including ischemia, hemorrhage (Wallace, 1987), or macrophage infiltration and inflammation[18,19] were suggested to be contributors. Cellular implants and their genetic modification have been key strategies to provide substrates for axonal growth. Olfactory ensheathing cells, Schwann cells, neural stem cells, and transplants from fetal spinal cord[20-23] as well as fibrin or hydrogel loaded with growth factors have been studied as therapeutic approaches.
3. The damaged tissue around the lesion site reacts to injury with proliferation, and activation of microglia and macrophages recruited from the bloodstream and with reactive astrocytosis. Reactive astrocytes stabilize the injury site and limit the spread of injury. However, they also form a gliar scar with microglia and fibroblasts accompanied by accumulation of proteoglycans and tenescin C, fibronectin, laminin and collagen, which are the noncellular components of the scar. In parallel, disrupted myelin proteins, including the transmembrane proteins NogoA, the myelin associated glycoprotein (MAG) and the oligodendrocyte myelin glycoprotein (Omgp) bind to the Nogo receptor complex in neurons and activate a downstream signaling cascade that, through activation of a small GTPase, RhoA and its downstream ROCK ultimately leads to growth cone collapse and inhibits axonal growth. Successful outcomes that axonal growth after experimental CNS lesion was enhanced by neutralizing such inhibitory factors advanced to the development of therapeutic interventions.[2,24-26]
4. One of factors that influence the disparity between axonal regeneration following PNS and CNS injury is production of growth-stimulating molecules, in particular growth factors around injury site. Neurotrophic factors contribute to growth, guidance and survival of several neuronal populations during development. Clearly, rapid production of several growth factors by Schwann cells, including nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), Insulin-like growth factor (IGF), Ciliary neurotrophic factor (CNTF), Glial-cell line derived neurotrophic factor (GDNF) and others leads to successful axonal regeneration in the adult PNS.[27-32] In contrast, those factors are not expressed in temporal or spatial gradients supportive of regeneration in CNS.[33] These observations made neurotrophic molecules attractive candidates to enhance an intrinsic cell response of injured CNS neurons.[34-40]
5. In comparison with neurons at embryonic or early postnatal stage, intracellular genetic response for growth of adult neurons is very deficient; embryonic neurons show a better potential to regrow if lesioned and they are much less sensitive to inhibitory molecules. As mentioned above, even adult peripheral nerves regenerate with genetic program for growth activated robustly but adult CNS neurons lack intrinsic capacity for such pro-regenerative response.[41,42] Therefore,

a variety of strategies to unlock the genetic program of CNS neurons aimed at activating genes for growth are underway.

EXPERIMENTAL EFFORTS TO ENHANCE REGENERATION OF THE INJURED SPINAL CORD

Most experimental spinal cord studies are conducted as combined anatomical and functional analyses. Experimental efforts have been designed to target the multiple mechanisms of SCI pathology identified above. These experimental approaches include,[43]

a. placement of molecular and cellular bridges in the lesion cavity;[44]
b. stimulation of the injured spinal cord with growth factors;[45]
c. neutralization of myelin or ECM-related inhibitors;[46,47] and
d. "conditioning" of neurons to activate intrinsic genetic programs and proteins related to an active growth state.[48-51]

Table 1. Growth factor sensitivities of spinal cord axons

Growth Factors	Injured Axons	References
NGF	Nociceptive spinal axons	Tuszynski et al (1994, 1996) Ramer et al (2000)
BDNF	Cerulospinal axons	Tuszynski et al (1994, 1996)
	Rubrospinal axons	Kobayashi et al (1997) Ye and Houle (1997) Liu et al (1999)
	Raphespinal axons	Bregman et al (1997) Menei et al (1998)
	Coerulospinal axons	Menei et al (1998)
	Reticulospinal axons	Ye and Houle (1997) Jin et al (2002)
NT-3	Vestibulospinal axons	Jin et al (2002)
	Local motor axons	Lu et al (2001)
	Local sensory axons (CGRP)	Lu et al (2001)
	Corticospinal axons	Schnell et al (1994) Grill et al (1997)
	Dorsal column sensory axons	Bradbury et al (1999)
NT-4/5	Local motor axons	Blesch et al (2004)
	Coerulospinal axons	Blesch et al (2004)
	Reticulospinal axons	Blesch et al (2004)
	Propriospinal axons	Blesch et al (2004)
GDNF	Local motor axons	Blesch and Tuszynski (2003)
	Propriospinal axons	Blesch and Tuszynski (2003)
	Dorsal column sensory axons	Blesch and Tuszynski (2003)
	Nociceptive spinal axons	Ramer et al (2003)

Adapted from Lu P et al. Exp Neurol 2008; 209:313-320;[43] ©2008 with permission of Elsevier.

Effects of Growth Factors and Bridges

In recent years, several groups in a series of studies have examined the ability of injured adult spinal axons to respond to growth factors (Table 1). Growth factors were provided using techniques of direct infusion of protein, or gene delivery wherein genes encoding growth factors are expressed using viral gene therapy vectors. In many cases, the lesion cavity is filled with cellular grafts to support axon penetration. Critical points established from these studies are: (I) injured adult axons exhibit enhanced growth after exposure to growth factors after injury. For example, several brainstem neural projections that modulate motor function in the spinal cord exhibit sensitivity to the growth factors brain-derived neurotrophic factor (BDNF)and neurotrophin-3 (NT-3).[20,52-54] (II) Patterns of growth factor sensitivity of adult axons after injury are comparable to that of developmental growth factor sensitivity. (III) The combination of cell grafting to reconsistute a cellular bridge in a lesion site, and growth factor administration by gene therapy, results in substantial axonal regeneration within a spinal cord lesion site.[43,55-57] Aguayo and colleagues[58] showed several years ago that central axons can extend in peripheral nerve grafts placed into sites of SCI; these grafts are now known to contain substantial quantities of the growth factors nerve growth factor (NGF), BDNF, NT-3, ciliary neurotrophic factor (CNTF), and glial cell line-derived neurotrophic fator (GDNF) secreted by Schwann cells.

BDNF particularly must be noted for its growth promoting effects on a broad range of neuronal populations including cerulospinal, rubrospinal, raphespinal, coerulospinal and reticulospinal axons (Table 1). In addition to supporting extension of axons, BDNF has also been demonstrated to prevent degeneration of the red nucleus[59] and even the death of corticospinal neuronal cell bodies by administration into the cortex.[60] Yet corticospinal axons, which are essential for human voluntary movement,[61] remain among the most refractory axonal systems from which to elicit regenerative responses. One possible explanation for the limited ability of corticospinal axons to regenerate is the absence of high level expression of growth factor receptors on the tips of injured axons.[62] This speculation was recently supported by the observation that over-expression of the BDNF receptor, trkB, in layer V motor cortex resulted in corticospinal axon regeneration into a cortical lesion site.[63]

NT-3 has also been implicated in promoting growth of injured corticospinal axons[35,37,38,55,64-67] as well as stimulating the regeneration of ascending dorsal column sensory axons.[35,55,64-67]

Other growth factors including insulin-like growth factor-1 (IGF-1), the fibroblast growth factor (FGF) family and GDNF have axonal growth-promoting effects. However, the fact that GDNF along with NGF enhances growth of nociceptive spinal axons indicates a potential to cause dysfunctional sprouting and pain after SCI. Most likely, these growth factors should be avoided in experimental efforts to treat SCI.

Neutralizing Inhibitors to Axonal Regeneration

Two classes of environmental molecules contribute to failure of axonal growth: Proteins associated with degenerating myelin (myelin-associated inhibitors) and proteoglycans associated with glial scarring, including the chondroitin sulfate proteoglycans (CSPGs).

More than 20 years ago, early studies by Schwab and colleagues identified an axon growth-inhibiting activity in CNS myelin.[68] Two membrane protein fractions of 35

and 250 kDa in CNS myelin were found to inhibit neurite outgrowth in vitro.[69] Such inhibitory proteins, cloned later as NogoA, MAG (myelin associated protein) and OMgp (oligodendrocyte myelin glycoprotein), mediate their signaling through the so-called Nogo-66 receptor (NgR1) with a complex of coreceptors that include p75/Lingo-1/Taj.[70-73] Most recently, ligands have been shown to bind to another inhibitory receptor, paired immunoglobulin-like receptor B (PirB).[74] Binding of MAI to the receptor activates RhoA-mediated stimulation of Rho-associated kinase (ROCK) that regulates actin cytoskeletal dynamics[75] resulting in inhibition of neurite outgrowth and growth cone collapse.[76] Effects of blocking inhibitory signaling from MAI-NogoR on axonal regrowth in vivo have been extensively examined in rodent models of SCI. For example, some studies reported enhanced corticospinal, rubrospinal and raphespinal axon regeneration in NgR1 knockout mice after SCI,[77] whereas other studies reported no effect of nogo receptor deletion.[78,79] Antagonizing the inhibitory activity of NogoA pharmacologically (using the monoclonal antibody (IN-1), peptide NEP1- 40, or soluble version of NgR1) was reported to result in significant growth of damaged CST axons with recovery of locomotion in some studies[80,81] but not in others.[82] The ROCK inhibitor Y-27632 was also reported to promote sprouting of CST fibers and improve locomotor recovery after injury.[83] Despite some of this controversy regarding the precise role of nogo neutralization in axonal regeneration after SCI, clinical trials of nogo antibody infusions are currently underway in the United States and Europe, and results are expected sometime in 2012.

In addition to the myelin-inhibitory molecules, extracellular matrix molecules such as chondroitin sulfate proteoglycan (CSPG), hyaluronan and tenascin are deposited after spinal cord injury and these molecules restrict axonal growth.[84,85] CSPGs consist of a core protein to which a variable number of unbranched sugar (glucosaminoglycan; GAG) side chains are covalently attached. Degradation of CSPGs with the bacterial enzyme chondroitinase (CHASE) turns an inhibitory surface for axon growth to a more permissive environment.[86-88] Rho-ROCK signaling also appears to mediate inhibitory signals from CSPG binding, as observed with myelin-associated inhibitors.[89,90] Therapeutic effects of CHASE on axon regeneration have been demonstrated in many studies; intrathecal delivery of CHASE enhanced axonal growth of both ascending sensory projections and descending motor projections, resulting in functional improvement after spinal cord injury.[88] Interestingly, transgenic mice expressing CHASE in astrocytes reported regenerated corticospinal axons into a lesion site, but not caudal to the lesion site; functional improvement did not occur.[91]

Finally, the receptor "protein tyrosine phsophatase sigma" (PTPsigma) was recently identified as a receptor for CSPGs.[92] PTPsigma gene disruption enhanced penetration of axons into a CSPG-rich scar tissue after SCI. This discovery provides new directions for targeting inhibitory CSPG molecules to enhance axon regeneration.

Intrinsic Determinants of CNS Regeneration

Transection of the spinal cord results in no spontaneous regeneration of the corticospinal tract.[93] In contrast, the raphespinal and rubrospinal projections, which also contribute to locomotion, exhibit a modest degree of regeneration into spinal cord lesion sites when provided with cellular bridges, trophic stimulation, and other experimental therapies.[52,94-97] The regenerative differences between corticospinal and other systems may be due to several potential mechanisms. For example, regeneration-associated genes (RAG) including GAP-43, c-JUN, Galectin-1, β-II-tubulin and α-1-tubulin are expressed

at only low levels in corticospinal neurons whereas expression of these genes is induced after injuries of raphespinal and rubrospinal systems.[98-102] In general, the ability of the neuron to reactivate expression of growth molecules in response to injury corresponds to its regenerative potential.[51] Intrinsic neuronal properties related to axon growth involve transcription of specific sets of genes and some autonomous processes within the axon including local mRNA translation, protein synthesis and conveyance of an injury signal to the cell soma via retrograde axoplasmic transport.[103]

Enhancing Intrinsic Growth Potential

In an attempt to boost intrinsic neuronal growth properties, two main methodological approaches have been used: (1) "conditioning" lesions, and (2) modification of genetic programs.

1. "Conditioning" lesions prime the growth state of injured neurons for regeneration. The dorsal root ganglion (DRG) neuron has served as a unique system to study the intrinsic potential of neurons to regenerate after injury. The peripheral branch of a DRG neuron can regenerate robustly following sciatic nerve injury, but the central branch cannot. Interestingly, an injury to the peripheral branch of a DRG neuron (a "conditioning" lesion) significantly enhances the regeneration of the central DRG branch if the spinal cord is subsequently lesioned.[48,104] This striking phenomenon is attributed to activation of genes associated with regeneration by the conditioning lesion. Those genes include GAP-43, CAP-23, SPRR1A (involved in cytoskeleton dynamics within the growth cone), Galectin-1 and BDNF. One key mediator known to mimic regenerative responses induced by the conditioning lesion is cyclic AMP (cAMP). Direct administration of cAMP into the DRG neuron recapitulates some, but not all, of the conditioning effect.[49,50] Polyamine metabolism through CREB has been shown to be a mechanism for enhancement of axon regeneration by cAMP elevation.[105]

   Lens injury, another form of a "conditioning" lesion, has also been reported to stimulate the activation of growth programs for retinal ganglion cells after axotomy. Such regenerative responses can be achieved by combined application of cAMP and oncomodulin, a calcium-binding protein secreted by macrophages into retinal ganglion cell bodies. Like conditioning lesions in the peripheral branch of a DRG neuron, upregulation of growth-associated genes is accompanied by regeneration of injured optic nerves.[51,106,107]
2. Modifications of genetic programs: Experimental manipulations at molecular level to enhance the intrinsic capacity of injured neurons to regrow.

   SCI differentially modulates the expression of thousands of genes encoding diverse proteins such as cell adhesion molecules, cytoskeletal proteins, survival factors, ion channels, neuropeptides, transcription factors, and others. To identify genetic mechanisms that could potentially promote axon regeneration, a number of screenings based on gene microarray methods have been conducted. This strategy was designed to monitor changes in gene profiling occurring before and after injury, in order to identify master control genes for regeneration.[32,108-112] Studies in DRG neurons following a conditioning lesion identified several candidate genes to promote axonal growth, including the transcription factors c-Jun,[113]