Guidance Molecules in Axon Regeneration

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The regenerative capacity of injured adult mammalian central nervous system (CNS) tissue is very limited. Disease or injury that causes destruction or damage to neuronal networks typically results in permanent neurological deficits. Injury to the spinal cord, for example, interrupts vital ascending and descending fiber tracts of spinally projecting neurons. Because neuronal structures located proximal or distal to the injury site remain largely intact, a major goal of spinal cord injury research is to develop strategies to reestablish innervation lost as a consequence of injury. The growth inhibitory nature of injured adult CNS tissue is a major barrier to regenerative axonal growth and sprouting. An increasing complexity of molecular players is being recognized. CNS inhibitors fall into three general classes: members of canonical axon guidance molecules (e.g., semaphorins, ephrins, netrins), prototypic myelin inhibitors (Nogo, MAG, and OMgp) and chondroitin sulfate proteoglycans (lecticans, NG2). On the other end of the spectrum are molecules that promote neuronal growth and sprouting. These include growth promoting extracellular matrix molecules, cell adhesion molecules, and neurotrophic factors. In addition to environmental (extrinsic) growth regulatory cues, cell intrinsic regulatory mechanisms exist that greatly influence injury-induced neuronal growth. Various degrees of growth and sprouting of injured CNS neurons have been achieved by lowering extrinsic inhibitory cues, increasing extrinsic growth promoting cues, or by activation of cell intrinsic growth programs. More recently, combination therapies that activate growth promoting programs and at the same time attenuate growth inhibitory pathways have met with some success. In experimental animal models of spinal cord injury (SCI), mono and combination therapies have been shown to promote neuronal growth and sprouting. Anatomical growth often correlates with improved behavioral outcomes. Challenges ahead include testing whether some of the most promising treatment strategies in animal models are also beneficial for human patients suffering from SCI.

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THE REGENERATIVE CAPACITY OF INJURED CENTRAL NERVOUS SYSTEM IS LIMITED

In higher vertebrates, including humans, the regenerative capacity of neurons in the injured adult central nervous system (CNS) is extremely limited. Depending on the location and severity of the injury, trauma to the CNS may cause substantial damage to nervous system tissue that results in permanent neurological deficits. In the spinal cord, for example, injury often results in an interruption of vital ascending and descending pathways causing a range of functional deficits. The long-term goal of spinal cord injury (SCI) research is to develop strategies to improve or restore these deficits. One key step toward this goal is to reestablish neuronal innervation interrupted by SCI.

Reinnervation may be established by one of three strategies: (Fig. 1A) long-distance axonal regeneration followed by synapse formation on appropriate (pre-injury) target cells; (Fig. 1B) short-distance axonal regeneration and synapse formation to create relays to distal targets; or (Fig. 1C) sprouting of spared axons that maintain connectivity beyond the injury site (Fig. 1). Interestingly, evidence suggests that the limited spontaneous recovery that is observed following CNS injury is most likely a result of sprouting and compensation from spared systems. As discussed below, long-distance axon regeneration often occurs following peripheral nervous system (PNS) injury but does not occur spontaneously in the injured adult CNS. Thus, in mammals, injured neurons of the PNS and CNS show quite distinct adaptive strategies to injury. The disparity between neuronal responses following PNS and CNS injury is due in part to both intrinsic (cell-autonomous) and extrinsic factors.

In this article we focus on the role of axon guidance molecules in the mature CNS with an emphasis on regeneration following injury. We also discuss two classes of inhibitory molecules,

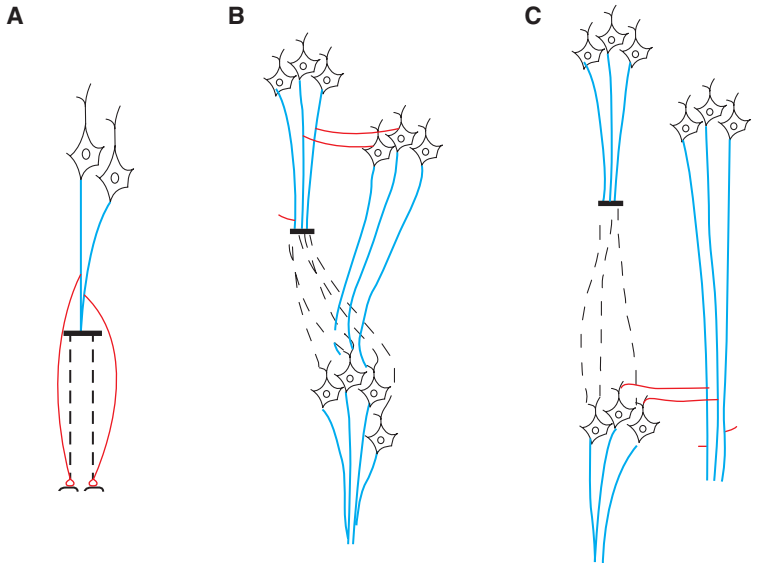

Figure 1. Strategies to reestablish neuronal innervation following injury. (*A*) Long-distance axonal regeneration. Following axon transection, the distal segment of the nerve undergoes Wallerian degeneration (dotted black lines). New axons (red) sprout from the proximal axon segment (blue) and reestablish synaptic contact with preinjury targets. (*B*) short-distance growth of injured axons. Collateral sprouts (red) form synaptic contact with neighboring neuronal elements to by-pass the injury site. (*C*) Sprouting of spared axons to maintain connectivity beyond the injury site. The strategy shown in *A* is typically observed following compression injury in the PNS, whereas neuronal responses shown in *B* and *C*, have been observed in the injured adult mammalian CNS.

the chondroitin sulfate proteoglycans (CSPGs), and the prototypic myelin inhibitors MAG, Nogo, and OMgp. Both classes of molecules are profoundly inhibitory for neurite extension in vitro, and evidence suggests they also restrict neuronal growth and plasticity in the injured adult mammalian CNS in vivo. We discuss the role of neurotrophic factors and growth-permissive molecules in the adult CNS, and provide evidence for their growth-promoting effects in models of CNS regeneration.

WHY DON'T SEVERED CNS AXONS UNDERGO SPONTANEOUS REPAIR?

The regenerative capacity of the PNS and CNS is remarkably different. Following PNS injury, sensory and motor axons can and often do regenerate over long-distances, supporting substantial axonal regeneration and functional recovery. Why is regenerative axonal growth in the CNS so limited? Thirty years ago, an elegant series of transplantation experiments by Aguayo and colleagues established that some populations of adult CNS neurons possess the capacity to extend long axons following injury when provided with a favorable growth environment. In the presence of a peripheral nerve graft, CNS neurons could extend axons over several centimeters into the grafted tissue (Aguayo et al. 1981). We now know, however, that this capacity does not extend to the most important motor system for primate motor function, the corticospinal projection (Grill et al. 1997; Hollis et al. 2009a). Conversely, optic nerves transplanted into the PNS poorly support growth of denervated sciatic nerves. Few sciatic nerve axons entered the optic nerve transplant, whereas most of them bypassed the transplant before reentering the distal peripheral nerve stump (Aguayo et al. 1978). Collectively, nerve transplantation experiments uncovered two important principles for axonal regeneration: (1) some populations of CNS neurons retain a capability for long-distance axon growth throughout adulthood, and (2) the PNS milieu, but not the CNS milieu, is conducive for long-distance axon regeneration in vivo.

Subsequent studies revealed that CNS myelin formed by mature oligodendrocytes is profoundly inhibitory for neurite outgrowth. CNS myelin contains several factors that inhibit neurite outgrowth (Caroni and Schwab 1988), including the inhibitors myelin-associated glycoprotein (MAG), the reticulon family member RTN4a/Nogo-A, and oligodendrocyte myelin glycoprotein (OMgp) (Filbin 2003; Schwab 2004; Yiu and He 2006). In addition, several growth inhibitory molecules belonging to families of canonical axon guidance molecules are found in CNS myelin, including members of the netrin, ephrin, and semaphorin families (Bolsover et al. 2008; Löw et al. 2008). Although the growth inhibitory nature of CNS myelin is well established, regenerating CNS axons are faced with a number of additional obstacles. Within days following injury, a glial scar forms around the injury composed of reactive astrocytes, microglia, and meningeal fibroblasts that migrate into the lesion site. This "scar" formed at the lesion is thought to pose a physical barrier to axonal regeneration (Fig. 2). In addition, scar-associated molecules, including CSPGs, function as chemical inhibitors that block axon regeneration (Bradbury et al. 2002). Thus, the extensive expression of multiple classes of inhibitory molecules in injured CNS tissue is believed to constitute a major hurdle for regenerating axons (Table 1).

AXON GUIDANCE MOLECULES REGULATE NEURONAL STRUCTURE BEYOND THE INITIAL PHASE OF NEURONAL NETWORK ASSEMBLY

Following nervous system development, expression patterns of numerous axon guidance molecules are decreased, or altered, whereas others retain embryonic expression levels and are present in abundance in the mature brain and spinal cord. The expression of these molecules in the adult implies additional roles for guidance cues beyond the initial phase of neuronal process outgrowth, growth cone navigation, and target innervation. Recent evidence indicates that axon guidance molecules

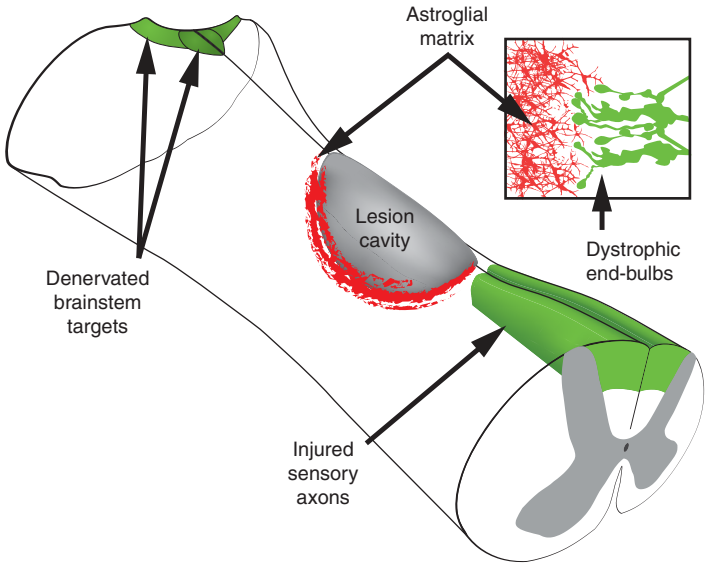

Figure 2. Schematic of adult rat spinal cord with dorsal lesion cavity as a result of injury. Transected axons of ascending sensory fibers (green) display dystrophic end-bulbs (inset) at the edge of the lesion cavity. The lesion cavity is surrounded by a glial scar (red) composed of reactive astrocytes, microglia and meningeal fibroblasts that migrate into the lesion site. Growth inhibitory molecules, including semaphorins and chondroitin sulfate proteoglycans (CSPGs) are enriched in the glial scar contributing to the growth inhibitory nature of injured adult mammalian CNS tissue.

participate in a number of network refinement processes that occur after the initial scaffold of connectivity has been established (Bagri et al. 2003; Morita et al. 2006; Fu et al. 2007; Paradis et al. 2007; Low et al. 2008; Xu and Henkemeyer, 2009). In addition, guidance cues can regulate aspects of neuronal excitability and synaptic function in the mature CNS (Klein, 2009; Pasterkamp and Giger 2009). Once fully developed, neuronal circuits in the mature nervous system become more stable; however, it is also important to point out that adult neuronal connectivity is not hardwired. Indeed, more restricted forms of structural plasticity persist throughout adulthood in response to experience, injury, and aging. Because many mature neurons continue to express receptors for guidance cues, it has been speculated that inhibitory and chemorepulsive axon guidance molecules play a role in synaptic stabilization and limitation of neuronal plasticity in adulthood.

Of significance for studies on nervous system regeneration is the up-regulation of the expression of many guidance cues with known inhibitory activity, including members of the semaphorin, ephrin, netrin, Wnt, and slit families. Conversely, neurotrophic factors and permissive guidance cues are thought to promote neuronal growth and structural changes in adulthood (Sofroniew et al. 1990). A tightly regulated balance between growth-promoting and growth-inhibiting molecules is likely to determine the extent and type of neuronal structural changes that may occur in the mature CNS.

CANONICAL AXON GUIDANCE MOLECULES CONTRIBUTE TO THE GROWTH INHIBITORY NATURE OF INJURED ADULT MAMMALIAN CNS TISSUE

Although a great deal is known about extracellular molecules and signaling pathways that regulate axonal growth and pathfinding during development, comparatively little is known

Table 1. comparison of factors that influence axonal regeneration following injury to the adult mammalian peripheral (PNS) and central (CNS) nervous system.

Nervous system injury	PNS	CNS
Spontaneous axonal regeneration	Good: substantial regeneration often occurs following compressive injury	Poor: very limited and mostly incomplete regeneration of severed fibers; typically results in permanent functional deficits distal to the injury
Environment	Growth permissive	Nonpermissive
-myelin	Contains inhibitory molecules, however some of the known CNS myelin inhibitors are less abundant or not present in PNS myelin.	Contains multiple inhibitory factors, including MAG, Nogo, OMgp, netrin-1, Sema4D, and ephrinB3 among others.
-Wallerian degeneration	Occurs rapidly following injury, myelin debris is cleared from distal stump of nerve by Schwann cells and activated macrophages	Occurs slowly, protracted and incomplete clearance of myelin debris from distal portion of injured fiber tracts
-glial scar	No glial scar is formed at the injury site, however CSPGs are present	Forms within days of injury and may inhibit axonal regeneration, rich in inhibitory CSPGs
-growth factors	abundant, up-regulated following injury	not expressed in temporal or spatial gradients supportive of regeneration
Immune system response	Supports clearance of myelin debris in distal nerve stump	Prolonged, with recruitment of innate and possibly adaptive immune cells
Cell intrinsic mechanisms		
-rate of axonal regrowth	Possess the ability to regenerate axons throughout adulthood at a rate of ~1 mm per day.	Some neurons possess the ability to regrow axons throughout adulthood if provided with a growth permissive environment.
- form of process growth	Capability for long-distance axonal growth.	Injury-induced neuronal plasticity results in reactive sprouting of processes from injured and noninjured neurons. Long-distance axonal regeneration does not occur.
Secondary damage	Minimal	Extensive degeneration after the injury that substantially contributes to parenchymal destruction
Cell-intrinsic growth programs	Activated and efficient	Deficient in various CNS populations, esp. in upper motoneurons that form the corticospinal tract
"Bridges" for regeneration in lesion site	Spontaneously formed by Schwann cells, macrophages and fibroblasts	Absent, resulting in failure of axonal attachment and extension

about the mechanisms that regulate neuronal growth and plasticity following injury to the adult nervous system. A number of axon guidance molecules are expressed in the adult CNS and their expression is regulated following injury. When coupled with the observation that many CNS neurons continue to express guidance receptors, this implies that adult CNS neurons remain responsive to guidance cues throughout life. Ironically, the picture emerging from these studies is that the inability of severed axons to undergo spontaneous repair in the adult CNS is, at least in part, attributable to the presence of the very same molecules that were so important during development in establishing the network. Below we summarize experimental evidence for a role of axon guidance molecules in CNS regeneration and provide specific examples for some of the best characterized guidance cues.

SEMAPHORINS

Many semaphorins function as inhibitory or repulsive guidance cues, and the presence of these proteins in the mature brain and spinal cord suggests roles in network stabilization by limiting neuronal growth. Indeed, similar to embryonic DRG neurons, adult DRG neurons show growth cone collapse in the presence of acutely applied Sema3A in vitro (Reza et al. 1999), and preconditioned adult DRG axons stop growing on encountering cells in SCI lesion sites that express *Sema3A* (Pasterkamp et al. 2001). Class 3 semaphorins (Sema3s) are expressed by glial scar-associated meningeal cells and have been proposed to contribute to the growth inhibitory nature of injured CNS tissue (Pasterkamp and Verhaagen, 2006). Interfering with the interaction between Sema3s and CSPGs blocks Sema3A repulsion in vitro, raising the possibility that Sema3s secreted by meningeal cells augment inhibition by glial scar tissue in a CSPG-dependent manner (Pasterkamp and Verhaagen 2006).

Recently, a small molecule agent (SM-216289) was found to block binding of Sema3A to the neuropilin-1/plexinA receptor complex, attenuating Sema3A repulsion of DRG neurons in vitro (Kikuchi et al. 2003). Further, SM-216289 accelerates axon regeneration in a rat model of olfactory nerve axotomy (Kikuchi et al. 2003), and it has been reported to enhance growth of neuropilin-1-expressing serotonergic axons after SCI in rats (Kaneko et al. 2006). In the same injury model, blocking Sema3A signaling does not lead to enhanced regeneration of corticospinal axons or ascending sensory axons (Kaneko et al. 2006), suggesting that blocking Sema3A function enhances growth of a subset of axons. In organotypic brain slices, transection of the entorhinal-hippocampal pathway (EHP) leads to up-regulation of Sema3A and neuropilin-1 expression in the hippocampus and entorhinal cortex. No spontaneous regeneration of severed EHP axons is observed. In the presence of a peptoid inhibitor that selectively blocks the Sema3A-neuropilin-1 interaction, the number of EHP axons that grows into the denervated hippocampus increases significantly (Montolio et al. 2009). Together these studies support the idea that Sema3A inhibits regenerative axonal growth in vitro and in vivo. As Sema3A (and other class 3 semas) not only regulate neuronal growth but also play important roles in vascular remodeling (Wang et al. 2005), immune system function (Suzuki et al. 2008) (Mizui et al. 2009), and cell death (Bagnard et al. 2004; Giraudon et al. 2004; Ben-Zvi et al. 2008; Moretti et al. 2008), any of these activities could influence outcomes after SCI. Additional studies are needed to more precisely define the mechanism(s) and role of Sema3s in the injured CNS.

Growing evidence suggests that in addition to Sema3s, membrane-associated semaphorins contribute to the regenerative failure of injured CNS axons. Sema4D, expressed by oligodendrocytes and transiently up-regulated near sites of spinal cord injury, inhibits outgrowth of postnatal cerebellar and sensory neurites in vitro (Moreau-Fauvarque et al. 2003). Similarly, Sema7A is expressed by oligodendrocytes in spinal cord white matter (Pasterkamp et al. 2007) and Sema6B is strongly up-regulated near the lesion site following transection of the fornix in the adult rat (Kury et al. 2004). Whether targeting of membrane-bound semaphorins will

influence outcomes following CNS injury is an important question for future studies.

EPHRINS

Similar to the semaphorins, the predominant neuronal response to ephrins is repulsive. Ephrins bind to members of the EphA and EphB receptor tyrosine kinase families. Expression of several ephrins and Eph receptors continues beyond nervous system development and remains robust in the mature rodent (Liebl et al. 2003) and human (Sobel, 2005) CNS. Of interest for nervous system regeneration is the strong expression of ephrinB3 in CNS myelin, the injury-induced up-regulation of ephrinB2 in reactive astrocytes, and the increase in ephrinA5 expression around ischemic cortical lesions (Bundesen et al. 2003; Benson et al. 2005; Carmichael et al. 2005). In vitro, ephrinB3 is a strong inhibitor of neurite outgrowth for postnatal cortical neurons and functions in a EphA4-dependent manner (Benson et al. 2005). In spinal cord injured rats, EphA4 protein accumulates in severed CST axons, suggesting that they are responsive to ephrin ligands present in myelin (ephrinB3) and scar tissue (ephrinB2) (Fabes et al. 2006). It was found that blocking of EphA4 with an infused peptide agonist enhances sprouting of CST axons rostral to the injury site but fails to promote axonal regeneration across the lesion into the distal portion of the spinal cord (Fabes et al. 2007). Although a regeneration phenotype was reported for spinal cord injured *EphA4* null mice through a spinal cord hemisection lesion site (Goldshmit et al. 2004), lesion completeness could not be determined with confidence in this report. The exact mechanism by which loss of *EphA4* may influence axonal growth in the adult CNS is complicated by the observation that the protein serves ligand as well as receptor functions in neurons and glia. EphA4 expressed by CST axons may function as an inhibitory receptor for ephrinB3 and ephrinB2. Moreover, EphA4 is up-regulated by reactive astrocytes and strongly inhibits neurite outgrowth through a reverse signaling mechanism (Goldshmit et al. 2004). In *EphA4* mutant mice, reactive gliosis and expression of CSPGs is reportedly reduced compared with wild-type mice (Goldshmit et al. 2004). Additional evidence that ephrin-Eph signaling may play a role in CNS response to injury stems from reports showing up-regulation of EphA3 in astrocytes after SCI in rats (Irizarry-Ramirez et al. 2005). Similarly, EphA7 is up-regulated by SCI and is thought to be a regulator of apoptosis in rat astrocytes (Figueroa et al. 2006). Thus, in addition to their role as growth inhibitory cues, Eph-ephrin signaling also influences formation of the glial scar and apoptosis.

WNTS

Another set of developmental guidance molecules implicated in SCI is the Wnt family. Decreasing anterior to posterior gradients of Wnts mediate both anterior growth of post-crossing commissural axons as well as posterior growth of descending corticospinal axons (Lyuksyutova et al. 2003; Liu et al. 2005). The differential response of ascending and descending axons to Wnt stimulation is thought to be mediated by differential receptor expression, with Frizzled receptors mediating Wnt-4 attraction and the atypical receptor tyrosine kinase Ryk promoting Wnt-5a-mediated posterior growth of the corticospinal tract through repulsion (Lyuksyutova et al. 2003; Liu et al. 2005). Little is known of the signaling cascades downstream of the Wnt-Ryk interaction, although there is evidence to suggest that Ryk and Frizzled act as function-modulating Wnt coreceptors (Lu et al. 2004b; Li et al. 2009). Following dorsal column injury, re-induction of Wnt-5a surrounding the lesion correlates with Ryk induction in corticospinal axons and axonal die-back (Liu et al. 2008; Miyashita et al. 2009). Infusion of functional blocking Ryk antibodies appears to either reduce axonal die-back or promote sprouting of lesioned corticospinal axons (Liu et al. 2008). Similar to re-induction of Ryk in injured corticospinal neurons, Ryk expression is up-regulated in DRG neurons following peripheral nerve injury (Song et al. 2008), although DRG neurons nonetheless show an extensive capacity for regeneration.

PROTOTYPIC MYELIN INHIBITORS: NOGO, MAG, AND OMgp

Growth inhibitory molecules that do not belong to any of the known families of axon guidance molecules have been identified. These include the myelin-associated inhibitors Nogo-A, MAG, and OMgp, hereafter called the prototypic myelin inhibitors (Fig. 3). Nogo-A (RTN4a) is a member of the reticulon (RTN) family of membrane associated proteins (Chen et al. 2000; GrandPre et al. 2000; Prinjha et al. 2000) and is comprised of two distinct growth inhibitory domains: 1) Amino-Nogo, an activity that inhibits both neurite outgrowth and the adhesion of several nonneuronal cell types, and 2) Nogo66, a 66-amino acid residue hydrophilic loop (Fig. 3) (Fournier et al. 2001; Oertle et al. 2003). Antibody blocking of Nogo-A in rats has been reported to facilitate long-distance

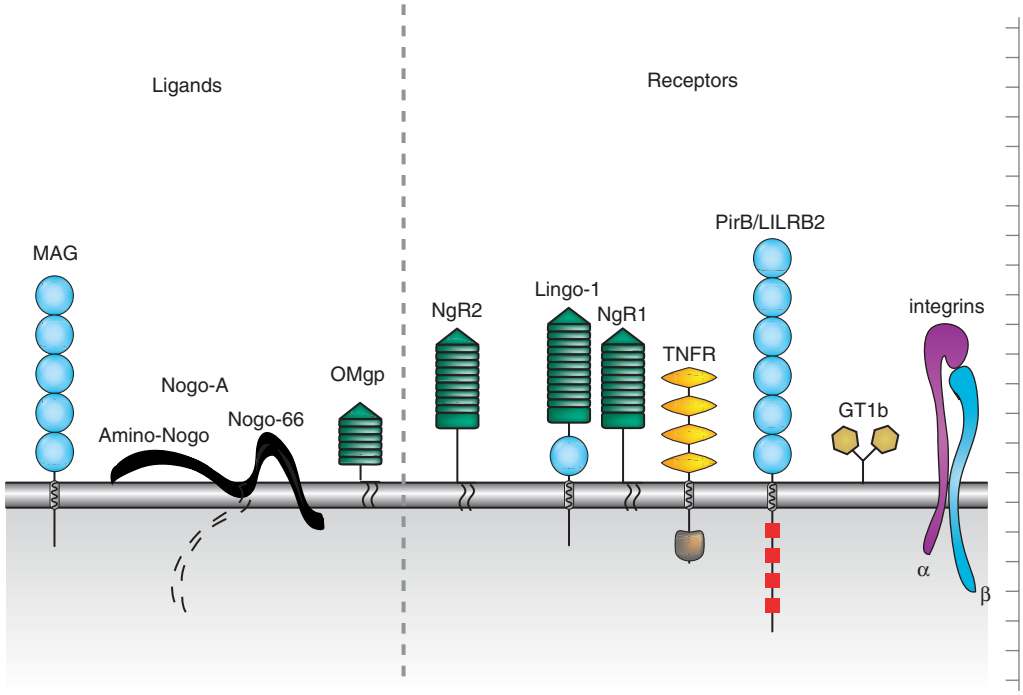

Figure 3. The prototypic myelin inhibitors MAG, Nogo-A, and OMgp and their receptors. MAG (Siglec 4a) is a type-1 transmembrane protein with an ectodomain composed of five Ig-like domains. Nogo-A (RTN4A) has two inhibitory domains: Amino-Nogo and Nogo-66. The membrane topology of Nogo-A appears to be variable. Although Amino-Nogo can be detected extracellulary, a significant portion of Amino-Nogo is thought to have a cytoplasmic orientation (dotted line). OMgp is a member of the large family of leucine-rich repeat (LRR) proteins and linked to the cell surface by a GPI anchor.

Several receptors for prototypic myelin inhibitors have been identified: PirB (and its human homolog LILRB2) binds to MAG, Nogo-66, and OMgp and signals neuronal growth cone collapse and neurite outgrowth inhibition in vitro. The ectodomain of PirB is composed of Ig-like domains and the cytoplamsic portion contains four immunoreceptor tyrosine-based inhibitory motifs (ITIM). The Nogo-66 receptor 1 (NgR1) binds directly to Nogo-66, MAG, and OMgp and is important for growth cone collapse responses toward acutely presented inhibitors. In some neurons, NgR1 is thought to form a tripartite receptor complex with Lingo-1 and the death domain containing TNFR superfamily members p75 or its functional substitute TROY (TNFRSF19). NgR2 is a receptor selective for MAG, functionally redundant with NgR1. The lectin-activity of MAG forms a complex with select gangliosides, including GT1b. Integrins, a family of heterodimeric cell surface receptors composed of α- and β-subunits, have been found to participate in Nogo-A and MAG inhibition in vitro.

regeneration and sprouting of corticospinal axons (Schnell and Schwab, 1990; Bregman et al. 1995; Thallmair et al. 1998). Growth of serotonergic fibers in the presence of anti-Nogo-A has also been reported (Gonzenbach and Schwab, 2008). However, regeneration studies in mice null for *Nogo* did not show enhanced longitudinal growth of severed CST axons (Zheng et al. 2003; Lee et al. 2009b); similar to wild-type mice, CST axons of *Nogo* null mice fail to extend past the injury site. Together with other studies (GrandPre et al. 2002; Kim et al. 2003; Simonen et al. 2003; Dimou et al. 2006; Steward et al. 2008), the overall effectiveness of Nogo-neutralizing approaches for SCI remains the subject of some debate: It is clear that Nogo inhibits axonal growth, but the ability of Nogo neutralization alone to facilitate axonal sprouting or regeneration, in the presence of a number of other myelin and ECM-associated inhibitors, remains uncertain (Zheng et al. 2005; Lee et al. 2009b). Nonetheless, Nogo neutralizing antibody infusions are now undergoing translational human testing in acute SCI.

Myelin-associated glycoprotein (MAG) is a sialic acid-binding Ig-superfamily lectin (siglec4a) composed of 5 Ig-like domains, a single transmembrane domain and a short cytoplasmic domain (Fig. 3) (Filbin, 2003). In vitro, MAG regulates neurite outgrowth in an age-dependent manner. MAG promotes growth of many types of embryonic and neonatal neurons (Johnson et al. 1989) and, at more mature stages, inhibits neurite outgrowth from a broad spectrum of primary neurons (DeBellard et al. 1996). The MAG lectin activity, located within the first two Ig-like domains, binds to a broad range of sialoglycans, including ganglioside GT1b, and has been found to augment the neurite outgrowth inhibitory activity of soluble MAG in some neuronal populations in vitro (Vinson et al. 2001; Vyas et al. 2002). When presented in membrane bound form, the MAG lectin activity is largely dispensable for neurite outgrowth inhibition (Tang et al. 1997) and can be dissociated from the MAG growth inhibitory site (Cao et al. 2007; Robak et al. 2009; Worter et al. 2009). Although the growth inhibitory nature of MAG is well established, mice carrying a null allele for *MAG* do not show enhanced growth of injured corticospinal or optic nerve axons when compared with wild-type controls (Bartsch et al. 1995).

The third molecule of the prototypic myelin inhibitors is OMgp, a member of the leucine-rich repeat (LRR) protein family (Wang et al. 2002b). OMgp is linked via a glycosylphosphatidylinositol (GPI) anchor to the cell surface and is expressed by myelinating glia in the CNS but not the PNS (Mikol and Stefansson, 1988). In addition, OMgp is strongly expressed by many types of neurons in the mature CNS (Habib et al. 1998; Lee et al. 2009a). Although *OMgp* null mice do not show detectably enhanced growth of axotomized corticospinal axons, increased sprouting of serotonergic axons has been reported (Ji et al. 2008).

Taken together, deletion or neutralization of Nogo, MAG, or OMgp alone results in limited or no regeneration of corticospinal axons, the most important system for voluntary motor control in humans. Other axonal systems, including descending serotonergic or raphespinal axons, may show apparent genetic-background dependent increases in axonal growth in mutants compared with wild-type controls.

MECHANISMS OF MYELIN-ASSOCIATED GROWTH INHIBITION

The first mechanistic clue regarding the function of the prototypic myelin inhibitors stemmed from the identification of the Nogo66 receptor 1 (NgR1) as a high affinity receptor for the Nogo inhibitory peptide Nogo66, MAG and OMgp (Fig. 3) (Fournier et al. 2001; Domeniconi et al. 2002; Liu et al. 2002a; Wang et al. 2002b). The NgR1 related molecule NgR2, supports binding of MAG, but unlike NgR1, does not associate with Nogo66 or OMgp (Venkatesh et al. 2005). Functional studies with primary neurons obtained from *NgR1* null mice revealed that *NgR1* is necessary for collapse of growth cones in postnatal dorsal root ganglion neurons following acute presentation of soluble Nogo66, MAG, or OMgp (Kim et al. 2004; Chivatakarn et al. 2007). When plated on substrate bound Nogo66, MAG, or OMgp, however,

various types of primary neurons null for *NgR1* are strongly inhibited, and growth inhibition is comparable to wild-type neurons from littermate controls (Zheng et al. 2005; Chivatakarn et al. 2007; Venkatesh et al. 2007). The combined loss of *NgR1* and *NgR2* leads to a partial disinhibition of DRG neurons cultured on fibroblasts stably expressing MAG (Worter et al. 2009). NgR1 has been reported to complex with Lingo-1 and select members of the tumor necrosis factor receptor (TNFR) superfamily to signal growth inhibition in vitro (Fig. 3) (Wang et al. 2002a; Mi et al. 2004; Park et al. 2005; Shao et al. 2005). Growth inhibitory responses to Amino-Nogo are not well understood; indirect and integrin-dependent mechanisms for Amino-Nogo mediated inhibition have been reported (Hu and Strittmatter, 2008).

More recently, paired immunoglobulin-like receptor B (PirB), and its human homolog (LILRB2), were identified as novel receptors for Nogo-66, OMgp, and MAG (Atwal et al. 2008). PirB, a member of the leukocyte immunoglobulin receptor (LIR) subfamily, is comprised of six Ig-like domains, a transmembrane segment, and a cytoplasmic region harboring immunoreceptor tyrosine-based inhibitory motifs (Fig. 3). Antibody blocking or genetic ablation of PirB renders primary neurons more resistant to inhibition by substrate bound Nogo66, OMgp, MAG, or CNS myelin. The combined blockade of NgR1 and PirB largely abolishes neurite outgrowth inhibition on substrate bound CNS myelin (Atwal et al. 2008). These experiments reveal a significant degree of functional redundancy for the mechanisms used by prototypic myelin inhibitors. Furthermore, PirB and NgR1 are functionally linked and collaborate in signaling neurite outgrowth inhibition (Atwal et al. 2008).

Consistent with the idea that there is significant functional redundancy among the receptor systems used by myelin inhibitors, CST axons in spinal cord injured *NgR1* null mice fail to grow past the lesion site (Kim et al. 2004; Zheng et al. 2005). Because of its direct interaction with MAG, Nogo-66 and OMgp, a soluble peptide of the NgR1 ligand binding domain was developed (NgR1(310)-Fc) and used to antagonize myelin inhibition. NgR1(310)-Fc complexes with NgR1 ligands and competes for ligand binding to neuronal cell surface receptors, including NgR1. In vitro, NgR1(310)-Fc overcomes CNS myelin inhibition and promotes neurite outgrowth of different types of neurons plated on substrate bound CNS myelin or individual inhibitors (Fournier et al. 2002; He et al. 2003; Zheng et al. 2005; Peng et al. 2009). Interpretation of in vivo effects of NgR1(310)-Fc administration is more complicated: intrathecally administrated NgR1(310)-Fc was reported to increase growth of corticospinal and raphaespinal axons and to improve functional outcome after spinal cord injury (Li et al. 2004; Wang et al. 2006). However, at least some of the reported effects of NgR1(310)-Fc may be an artifact of axon labeling methods (Steward et al. 2007). In an independent study, NgR1(310)-Fc was reported to stimulate regrowth of myelinated sensory axons into the dorsal root entry zone, spinal cord white and grey matter following dorsal root crush injury (Harvey et al. 2009; Peng et al. 2009).

EXPERIENCE-DEPENDENT AND INJURY-INDUCED NEURONAL PLASTICITY ARE REGULATED BY RELATED MECHANISMS

In the immune system, PirB and its close relative PirA are receptors for major histocompatibility complex (MHC) class I molecule(s) (Takai 2005). MHC class I proteins are ubiquitously expressed and include classical and nonclassical molecules essential for adaptive and innate immune responses. In the developing and mature CNS select MHC class I molecules show distinct neuronal distribution patterns, and expression is regulated by neuronal activity (Corriveau et al. 1998). In the hippocampus and visual system, MHC class I molecules regulate activity-dependent changes in synaptic connectivity (Huh et al. 2000). Perturbations of MHC class I function in the hippocampus enhance long-term potentiation (LTP) at Schaffer collateral-CA1 synapses (Huh et al. 2000). Similar to MHC class 1 molecules, PirB is expressed in CNS neurons and participates

in limiting neuronal plasticity. In mice lacking transmembrane anchored PirB (called *PirBTM* mice), cortical ocular dominance (OD) plasticity is more robust at all ages compared with wild-type controls (Syken et al. 2006). Interestingly, defects observed in the visual system of *PirBTM, NgR1, and Nogo-A/B* mutant mice are very similar: connectivity in the visual cortex of mutant mice is not consolidated at the end of the critical period and OD plasticity is more robust in adulthood (McGee et al. 2005; Syken et al. 2006). In the mature hippocampus, NgR1 is found at synapses and influences dendritic spine morphology in vivo (Lee et al. 2008). *NgR1* not only influences neuronal structure but also modulates synaptic strength at Schaffer collateral-CA1 synapses (Lee et al. 2008). Interestingly, some of the synaptic defects reported for *NgR1* mutants resemble those reported in mice deficient for MHC class I molecules (Huh et al. 2000). Together, these findings reveal insights into the physiological function of myelin inhibitors and also suggest that mechanisms influencing neuronal growth following CNS injury and also synaptic plasticity in the intact CNS are related.

Because of the growing complexity of molecular players contributing to the growth inhibitory milieu of injured CNS tissue, plasticity from spared fiber tracts is a potentially more tractable target for improving functional outcome after SCI than true axonal regeneration. Indeed, SCI results in collateral sprouting of corticospinal axons with formation of novel connections to intraspinal neurons (Bareyre et al. 2004). Similarly, injury-induced sprouting of spared reticulospinal axons, another modulator of motor function, has been described (Ballermann and Fouad 2006). Most recently, extensive synaptic rearrangements of propriospinal projections after spinal cord injury, leading to improvement in locomotion, has been reported (Courtine et al. 2008). When coupled with the fact that many humans with complete loss of function below a site of SCI nonetheless show spared axons in rims of peripheral white matter (Tuszynski et al. 1999), enhancement of plasticity from spared axons is an alternative and compelling target for SCI therapy.

EXTRACELLULAR MATRIX MOLECULES AND AXONAL GROWTH AFTER INJURY

An important class of inhibitory ECM molecules are the chondroitin sulfate proteoglycans (CSPGs), a diverse group of glycoproteins composed of a core protein covalently linked to specific types of glycosaminoglyan (GAG) side chains (Galtrey and Fawcett, 2007). Several types of CSPGs are found in adult CNS tissue, many of which are expressed throughout the brain and spinal cord and condensed into perineuronal nets surrounding the somata and dendrites of various types of CNS neurons (Bruckner et al. 2000). CSPGs are up-regulated following CNS injury (McKeon et al. 1995; Davies et al. 1996; Fitch et al. 1999; Jones et al. 2002; Morgenstern et al. 2002; Jones et al. 2003b) and appear to inhibit neurite outgrowth from adult neurons (Snow et al. 1990; Davies et al. 1999). Enzymatic degradation of the GAG side chains of CSPGs, using chondroitinase ABC (ChABC), largely abrogates the neurite outgrowth inhibitory action of substrate bound CSPGs in vitro (McKeon et al. 1995; Zuo et al. 1998; Grimpe et al. 2005). In the rat visual cortex, local delivery of ChABC allows experience-dependent neuronal plasticity beyond the critical period (Pizzorusso et al. 2002) and reportedly enhances axonal growth following CNS injury (Moon et al. 2001; Bradbury et al. 2002; Tester and Howland 2008). More recent work shows that ChABC treatment in rats after SCI opens a window during which rehabilitative training supports functional improvement (Garcia-Alias et al. 2009). Importantly, only the trained skills are improved in injured animals, suggesting that during the window of enhanced plasticity the formation of new and appropriate connections may be driven by task-specific training. Rehabilitation only enhances functions that are trained and may come at a high cost, as other tasks that are not trained are worsened compared with animals that receive no training at all (Garcia-Alias et al. 2009). ChABC treatment reportedly also has neuroprotective effects: following SCI, administration of ChABC near the injury site prevents atrophy of axotomized corticospinal projection neurons following dorsal

column lesion and activates growth promoting intracellular signaling pathways (Carter et al. 2008)

Members of the receptor protein tyrosine phosphatases (RPTPs) of the leukocyte antigen-related (LAR) subfamily have previously been shown to associate with heparan sulfate proteoglycans (HSPGs) (Aricescu et al. 2002; Fox and Zinn, 2005; Johnson et al. 2006). The family member RPTPσ was recently identified as a receptor that directly interacts with growth inhibitory CSPGs and signals neuronal inhibition toward neurocan, aggrecan, or CSPGs expressed on the surface of astrocytes in vitro (Fig. 4) (Shen et al. 2009). In the mature mouse PNS, loss of *RPTPσ* enhances regenerative axonal growth following injury to the sciatic or facial nerve (McLean et al. 2002; Thompson et al. 2003). In the CNS, loss of *RPTPσ* has been reported to promote axon regeneration in the injured optic nerve (Sapieha et al. 2005) and to markedly improve axon extension into the lesion

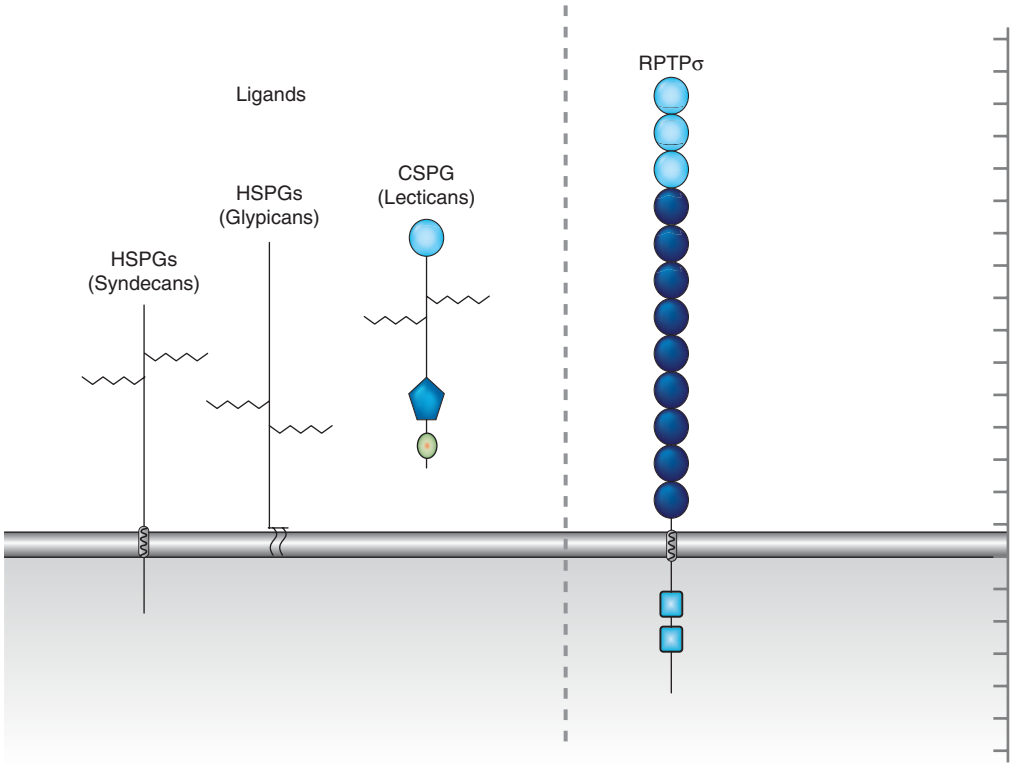

Figure 4. Mechanisms for CSPG inhibition. Proteoglycans are a heterogenous class of extracellular proteins with distinct protein core structures bearing covalently attached sulfated glycosaminoglycan (GAG) side chains. Prominent members of neural proteoglycans bearing heparan sulfate GAG chains (HSPGs) include the syndecans, glycpicans and agrin. Chondroitin sulfate GAG bearing proteoglycans (CSPGs) include members of the lectican family (neurocan, aggrecan, versican, and brevican). The leukocyte antigen-related (LAR) subfamily of receptor protein tyrosine phosphatases (RPTPs) includes the mammalian members LAR, RPTPσ and RPTPδ. LAR type RPTPs are transmembrane proteins with cell adhesion molecule-like ectodomains and a large cytoplasmic region with two conserved phosphatase domains. The *Drosophila* homolog of LAR-RPTPσ-RPTPδ is called DLAR and is a functional receptor for the fly HSPGs syndecan and glypican (dally-like). Avian RPTPσ binds directly to the HSPGs agrin and collagen XVIII. In addition, CSPGs belonging to the lectican family (including neurocan and aggrecan) bind to the first Ig-like domain of mouse RPTPσ to signal neuronal growth inhibition. The interaction between lecticans and RPTPσ is direct, depends on the presence of CS-GAG chains, and is sensitive to chondroitinase ABC treatment.

penumbra following dorsal column crush injury (Shen et al. 2009). Effects of loss of *RPTPσ* on corticospinal axon regeneration reported in a recent study seem to be very robust but are more difficult to interpret, as lesions may have been incomplete (Fry et al. 2009).

Counter-intuitively, axons regenerating into cellular substrates placed in a spinal cord lesion site have also been observed to specifically associate with cells expressing CSPGs (Jones et al. 2003a; Lu et al. 2007). These CSPG-expressing cells in the lesion site are host Schwann cells that have migrated into the site of injury (Jones et al. 2003a). Notably, these Schwann cells simultaneously express NCAM, L1, and possibly other classically "permissive" ECM and cell adhesion molecules (Jones et al. 2003a). These findings reflect the complexity of the injured in vivo CNS environment: most likely, the success or failure of axonal regeneration in vivo represents the summation of various inhibitory and permissive factors. If the amount of inhibition present in the extracellular matrix and on myelin exceeds the stimulation derived from growth factors and permissive substrates in the environment, then growth will fail, as observed in the mature CNS. On the other hand, regeneration may succeed if the balance tips in favor of growth, as observed in the injured peripheral nerve. The activation and persistence of an active growth state in the injured neuron further contributes to the success or failure of adult axonal regeneration.

GROWTH FACTORS AS GROWTH AND GUIDANCE SIGNALS IN THE INJURED CNS

Neurotrophic factors contribute to growth, guidance and survival of several neuronal populations during development. Neurotrophins, including nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), and neurotrophin-4 (NT-4) have unique functions in the CNS and are differentially regulated during developmental CNS maturation. Neurotrophin expression is regulated both spatially and temporally, generally decreasing as development proceeds. However, neurotrophin expression persists widely in adulthood in many CNS regions associated with functional plasticity (Maisonpierre et al. 1990).

Notably, successful axonal regeneration in the adult PNS is accompanied by rapid production of several growth factors by Schwann cells, including NGF, BDNF, IGF, CNTF, GDNF and others (Meyer et al. 1992; Sendtner et al. 1992; Curtis et al. 1993; Funakoshi et al. 1993; Glazner et al. 1994; Naveilhan et al. 1997; Shen et al. 1999; Höke et al. 2000; Costigan et al. 2002). Depletion of Schwann cell-derived BDNF reduces regeneration and remyelination of both sensory and motor neurons (Zhang et al. 2000; Song et al. 2008; Geremia et al. 2009). Motor neuron death following root avulsion in the adult is also ameliorated by growth factors (Novikov et al. 1995; Kishino et al. 1997; Novikov et al. 1997).

Growth factors elicit extensive growth of several axonal populations after SCI. BDNF elicits growth of lesioned raphaespinal, cerulospinal, rubrospinal, and reticulospinal motor axons into permissive growth matrices placed in sites of SCI (Liu et al. 1999; Jin et al. 2002; Lu et al. 2005). NT-3 promotes regeneration of lesioned dorsal column proprioceptive sensory axons (Zhang et al. 1998; Bradbury et al. 1999; Oudega and Hagg, 1999; Ramer et al. 2000; Lu et al. 2004a; Taylor et al. 2006; Alto et al. 2009). NGF promotes growth of nociceptive axons (Tuszynski et al. 1996; Grill et al. 1997) and may contribute to the spontaneous development of dysfunctional pain after SCI.

Notably, the corticospinal projection appears to be among the most refractory axonal systems from which to elicit experimental regeneration after SCI (Blesch and Tuszynski, 2009). For example, whereas insulin-like growth factor-I (IGF-I) promotes regeneration of coerulospinal and raphaespinal axons, and prevents axotomy-induced death of cortiocspinal motor neurons, it does not promote regeneration of corticospinal axons into a lesion cavity filled with a substrate that supports growth of other axonal systems (Hollis II et al. 2009a). Similarly, BDNF prevents axotomy-induced atrophy of rubrospinal neurons and promotes their regeneration into sites of SCI (Kobayashi et al. 1997; Jin et al. 2002; Kwon et al. 2002; Liu et al. 2002b; Kwon et al. 2007), and BDNF prevents corticospinal

neuronal death after axotomy (Giehl and Tetzlaff 1996; Giehl et al. 2001; Lu et al. 2001); yet BDNF does not promote corticospinal axonal regeneration into a spinal cord or cortical lesion site (Lu et al. 2001). If, however, corticospinal neurons are genetically modified to overexpress the BDNF receptor trkB at the same time that BDNF is expressed in a cortical lesion site, corticospinal axons can be induced to regenerate (Fig. 5) (Hollis II et al. 2009b). These findings indicate that different populations of axons in the CNS show distinct patterns of growth factor sensitivity. Moreover, patterns of growth factor sensitivity can be modified by altering the neuron's repertoire of growth factor receptor expression.

Recent findings indicate that axons can be induced to regenerate not just into, but *beyond*, sites of SCI when multiple mechanisms influencing axonal growth are experimentally manipulated (Lu et al. 2004a; Pearse et al. 2004; Fouad et al. 2005; Houle et al. 2006; Lu and Tuszynski 2008; Alto et al. 2009; Kadoya et al. 2009). In these studies, the environment of the lesioned CNS is rendered more permissive to growth by cell grafting or peripheral nerve grafting into the central lesion site. In addition, the endogenous growth state of the neuron in several studies must be activated using either a conditioning lesion or cAMP administration to the injured neuronal soma (Neumann et al. 2002; Qiu et al. 2002; Lu et al. 2004a; Pearse et al. 2004; Alto et al. 2009; Kadoya et al. 2009). Importantly, additional chemoattractive growth signals must be provided *beyond* the lesion site (Lu et al. 2004a; Alto et al. 2009; Kadoya et al. 2009). Under the latter circumstances, axons will regenerate into host spinal cord beyond the lesion site (Lu et al. 2004a; Alto et al. 2009). The chemotropic gradient can, over distances of several millimeters, guide regenerating axons to appropriate preinjury targets. Once in the target, regenerating axons form phenotypically correct synapses at the ultrastructural level, complete with presynaptic elements containing clusters of synaptic vesicles that are indistinguishable from the prelesioned state. Yet, the reconstituted neural circuitry is not electrophysiologically active, likely because of the absence of remyelination following injury (Alto et al. 2009). These

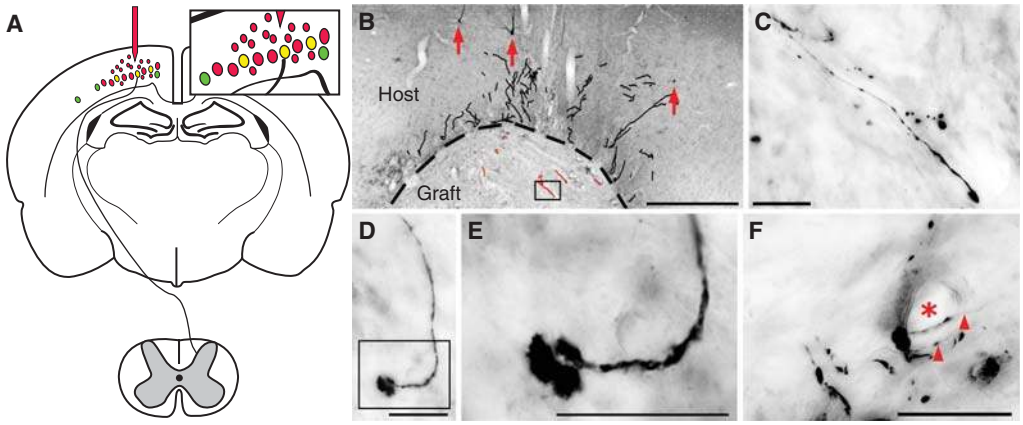

Figure 5. Corticospinal regeneration induced by over-expression of the high-affinity BDNF receptor trkB. (*A*) Corticospinal motor neurons are retrogradely infected with adeno-associated virus expressing GFP (green), whereas lentivirus encoding trkB (red) is delivered directly to the motor cortex. Some cells will coexpress both GFP and trkB (yellow). (*B*) Traced GFP-immunoreactive corticospinal axons demonstrate regeneration into a subcortical lesion grafted with BDNF-secreting syngeneic fibroblast substrate (red outlines). (*C*) Regenerated corticospinal axon within the subcortical graft. (*D–F*) Other examples of regenerated corticospinal axons within subcortical BDNF-secreting grats after trkB over-expression demonstrate growth cone-like morphology (*D, E*) and associations with the vasculature; axons indicated with arrowheads in (*F*) wrap around a blood vessel (asterisk). Scale bars, 500um (*A*), 25um (*C–F*). Adapted from Hollis *et al.* PNAS (2009).

findings remind us that the reconstitution of functional activity in mature neural systems after injury is a highly challenging endeavor. Developmental patterning of the nervous system occurs as a result of a delicately orchestrated series of genetic and epigenetic events coordinated between neurons and their environment, and over very short distances. Recapitulating this series of events in the large, injured adult CNS is, to say the least, challenging.

SUMMARY

Depending on severity and location, injury to the adult spinal cord causes substantial damage to neural tissue and is typically associated with permanent functional deficits. Over the past two decades enormous progress has been made in our understanding of the molecular and cellular events triggered by injury, providing insights into key mechanisms that contribute to tissue damage and regenerative failure of injured CNS neurons. One major goal of SCI research is to reestablish neuronal connectivity lost as a consequence of injury. In patients with incomplete SCI, reinnervation may be established by short distance axonal sprouting and formation of new synaptic contacts with neurons that bypass the injury site. When coupled with sprouting of spared axons beyond the injury site, this may allow reinnervation of preinjury targets. Neuronal growth and axonal sprouting of injured and noninjured CNS neurons may be achieved by lowering environmental growth inhibitory signals, enhancing growth promoting signals, activation of intrinsic growth programs, or some combination thereof. It is likely that treatments that enhance neuronal growth and plasticity need to be combined with task-specific rehabilitative training to strengthen and consolidate functionally meaningful connections in an activity-dependent manner. Indeed, experimentally enhanced neuronal plasticity combined with task-specific training regimes following SCI are reminiscent of activity-dependent refinement processes that occur in the visual system during development. During a temporally restricted window, enhanced plasticity combined with activity shapes neuronal structure. At the end of this critical period, appropriate connections are established and stabilized. The similarity between restricted neuronal growth following CNS injury and experience-dependent plasticity at the end of the critical period is further underscored by a striking convergence of molecular mechanisms that limit neuronal plasticity in both processes. These recent advances in the field of SCI research are based on rodent SCI models. Critical next steps are likely to include SCI experiments in larger animal models to further develop treatment strategies and if successful, develop protocols for human clinical trials.

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Guidance Molecules in Axon Regeneration

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