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Scar-mediated inhibition and CSPG receptors in the CNS

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Abstract

Severed axons in adult mammals do not regenerate appreciably after central nervous system (CNS) injury due to developmentally determined reductions in neuron-intrinsic growth capacity and extracellular environment for axon elongation. Chondroitin sulfate proteoglycans (CSPGs), which are generated by reactive scar tissues, are particularly potent contributors to the growth-limiting environment in mature CNS. Thus, surmounting the strong inhibition by CSPG-rich scar is an important therapeutic goal for achieving functional recovery after CNS injuries. As of now, the main *in vivo* approach to overcoming inhibition by CSPGs is enzymatic digestion with locally applied chondroitinase ABC (ChABC), but several disadvantages may prevent using this bacterial enzyme as a therapeutic option for patients. A better understanding of the molecular mechanisms underlying CSPG action is needed in order to develop more effective therapies to overcome CSPG-mediated inhibition of axon regeneration and/or sprouting. Because of their large size and dense negative charges, CSPGs were thought to act by non-specifically hindering the binding of matrix molecules to their cell surface receptors through steric interactions. Although this may be true, recent studies indicate that two members of the leukocyte common antigen related (LAR) phosphatase subfamily, protein tyrosine phosphatase σ (PTP σ) and LAR, are functional receptors that bind CSPGs with high affinity and mediate CSPG inhibitory effects. CSPGs also may act by binding to two receptors for myelin-associated growth inhibitors, Nogo receptors 1 and 3 (NgR1 and NgR3). If confirmed, it would suggest that CSPGs have multiple mechanisms by which they inhibit axon growth, making them especially potent and difficult therapeutic targets. Identification of CSPG receptors is not only important for understanding the scar-mediated growth suppression, but also for developing novel and selective therapies to promote axon sprouting and/or regeneration after CNS injuries, including spinal cord injury (SCI).

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Keywords

Axon regeneration; axon injury; scar tissue; CSPG receptor; axon growth inhibitor; spinal cord injury; LAR; PTP σ ; Nogo receptor

Introduction

During the course of embryonic development, axons elongate and form synaptic connections with target neurons, and these connections are essential for maintaining nervous system function. Interruption of immature axons may be followed by regeneration and resumption of normal development (Bernstein-Goral and Bregman 1993; Coumans et al. 2001; Nicholls and Saunders 1996). However, in the mature animal and even in the newborn, axon injury leads to axon degeneration, signal conduction failure and persistent, severe functional deficits, as seen in patients with white matter stroke, progressive multiple sclerosis, traumatic brain injury and SCI. The failure of severed axons to regenerate after CNS injury in adult mammals has been ascribed to both a developmental reduction in the intrinsic growth capacity of mature neurons and to environmental factors (Chen et al. 1995; David and Aguayo 1981; Goldberg et al. 2002; McGee and Strittmatter 2003). These two classes of determinants are not independent of one another. It is now clear that the intrinsic growth capacity is based on expression, availability and activation of a large number of cell-autonomous molecules that are involved in signaling effects of several classes of growth-inhibitory and growth-promoting environmental factors. Thus, to make sense of the cell-autonomous molecules, they should be placed in the context of parallel, counter balancing and possibly inter-digitating signaling pathways for mediating the effects of extrinsic factors for axon growth and inhibition (Fig. 1) (Park et al. 2010).

An important class of extrinsic growth-inhibitory factors is the CSPGs, which are large and highly negatively-charged molecules. CSPGs were found to bind and block the normal growth-promoting properties of laminin (Muir et al. 1989; Zhou et al. 2006; Zuo et al. 1998). Thus, it was widely believed that CSPGs inhibit axon growth by non-specific steric hindrance of the binding of laminin and other adhesive matrix molecules to their transmembrane receptors. However, more recent evidence suggests that CSPGs also may bind to members of at least two distinct classes of growth-inhibitory receptors expressed on the surfaces of axons and thereby activate specific growth-inhibitory pathways. If so, then CSPGs appear to be such robust inhibitors of repair mechanisms in the CNS and could be the multiplicity of mechanisms by which they restrict axon growth. Development of therapies to counteract CSPG effects would then require either enzymatic digestion, which is highly invasive and has other limitations, or a combination of molecular and/or pharmacological approaches, based on a detailed understanding of the signaling pathways activated by CSPGs and how they interact with the cell-autonomous factors involved in axon sprouting and/or regeneration. Understanding of CSPG receptor signaling may help make sense of the myriad cell-autonomous factors previously ascribed roles in axon growth and develop therapies to promote neural repair and functional recovery after CNS injury. This review will focus on the CSPG-mediated inhibition and CSPG receptors identified recently.

Three classes of extrinsic factors inhibit axon growth

The non-permissiveness of the injured adult CNS to axon growth has been ascribed to three classes of molecules: 1) Myelin-associated growth inhibitors, 2) chemorepulsive guidance molecules, and 3) highly sulfated proteoglycans, especially CSPGs (He and Koprivica 2004; Liu et al. 2006; McGee and Strittmatter 2003), which are components of the extracellular matrix (ECM) comprising the perineuronal nets. CNS myelin-forming oligodendrocytes inhibit axonal regeneration *via* a neuronal membrane protein Nogo-66 receptor (NgR1) (Fournier et al. 2001; GrandPre et al. 2002; Kim et al. 2004). At least three distinct proteins expressed by oligodendrocytes have been shown to inhibit axonal extension, including myelin-associated glycoprotein (MAG), oligodendrocyte-myelin glycoprotein (OMgp) and Nogo (Chen et al. 2000; Domeniconi et al. 2002; Domeniconi et al. 2005; GrandPre et al. 2000; Liu et al. 2002; Mikami et al. 2009; Wang et al. 2002b). Although they are molecularly unrelated, these molecules inhibit axon extension by binding to the same GPI-linked membrane protein NgR1, with signal transduction occurring *via* the co-receptor complexes that includes the low-affinity neurotrophin receptor p75 and Lingo1, or TROY and Lingo1 (Mi et al. 2004; Park et al. 2005; Shao et al. 2005; Wang et al. 2002a). Moreover, several molecules that serve as repulsive axon-guidance cues during development, *e.g.*, semaphorin 3A and 5A, Ephrin-B3 and Ephrin-A4, may limit axonal elongation in the injured CNS (Kaneko et al. 2006; Kantor et al. 2004; Pasterkamp and Verhaagen 2006). Neither myelin-associated growth inhibitors nor most guidance molecules explain why the glial scar represents such a strong barrier to axon regeneration, although class 3 semaphorins are expressed by fibroblasts contributing to the scar after SCI (De Winter et al. 2002; Kaneko et al. 2006; Niclou et al. 2003). On the other hand, CSPGs are secreted by reactive astroglia, are prominent chemical constituents of scar tissues, and play an important role in restricting neural repair by suppressing axon extension across the lesion and/or inhibiting collateral sprouting by spared axons near the lesion (Bradbury et al. 2002; Silver and Miller 2004). Remarkably, the intracellular effects of most axon growth-suppressing proteins are mediated by activation of the small GTP-binding protein RhoA (Fig. 1) (Luo 2000; Mueller et al. 2005; Walker and Olson 2005), a signaling molecule that regulates neuronal morphogenesis via interaction with a number of other molecules, including serine/threonine kinases, tyrosine kinases, lipid kinases, lipases, oxidases and scaffold proteins. In particular, the activated (GTP-bound) form of Rho can bind and directly activate Rho kinase (ROCK). ROCK activation leads to phosphorylation of several target proteins, including myosin light chain, and mediating cytoskeletal rearrangements and disassembly in neurons and collapse of growth cones. Thus, an alternative strategy to overcome growth inhibition from multiple extracellular factors is to influence the common downstream pathway including RhoA and ROCK (Fu et al. 2007; Luo 2000; Mueller et al. 2005). Suppressing these signals with pharmacological inhibitors and nonsteroidal anti-inflammatory drugs has been shown to stimulate axon growth⁴² and improve behavioral recovery following SCI in rodents (Dergham et al. 2002; Dill et al. 2010; Fournier et al. 2003; Fu et al. 2007; Xing et al.). A phase I/IIa clinical trial of an inhibitor of RhoA has been completed, with results suggesting that the treatment is safe and possibly beneficial (Fehlings et al. 2011).

CSPG-rich scar tissue mediates inhibition on neuronal growth

CSPGs are a family of ECM molecules characterized by a core protein and a variable number of highly sulfated glycosaminoglycan (GAG) side chains. The major sulfate proteoglycans found in the CNS include lecticans (neurocan, versican, aggrecan and brevican), phosphacan and NG2. The spatiotemporal distribution of CSPGs in the developing CNS is related to glial boundaries, including in the optic tectum, spinal cord roof plate and dorsal root entry zones. CSPGs are highly expressed in adult CNS and form the extracellular matrix together with other proteoglycans including hyaluronan and tenascins (Carulli et al. 2005; Galtrey and Fawcett 2007; Kwok et al. 2008). CSPGs are concentrated into perineuronal nets and may attach to the cell membrane (Carulli et al. 2005; Galtrey and Fawcett 2007; Kwok et al. 2011). The postnatal development of perineuronal nets may underlie some of the age-related loss of “plasticity” associated with the closing of neurological “critical periods” in different parts of the CNS (Fawcett 2009). After CNS injury, CSPGs are rapidly upregulated at the lesion site by reactive astrocytes in the glial scar tissues. Spinal cord trauma usually induces over-expression of CSPGs in the lesion penumbra with higher levels in the epicenter of scar tissues (Davies et al. 1997). In addition to the physical barrier of scar tissues including reactive astrocytes, meningeal cells, fibroblasts and microglia, the greatly increased levels of CSPGs form a potent chemical barrier for axon regeneration by preventing elongation (Bradbury et al. 2002; Faulkner et al. 2004; Jones et al. 2003; McKeon et al. 1991). Thus, surmounting strong suppression of CSPG inhibitors in glial scars is a major target for therapeutic intervention following CNS injuries including SCI.

Evidence for the inhibitory nature of CSPGs on axon regeneration comes largely from studies on digestion of the CSPG GAG side chains with the bacterial enzyme ChABC. Although CSPG core proteins appear to be inhibitory by themselves (Oohira et al. 1991; Tan et al. 2006), removal of GAG side chains with ChABC makes the ECM environment much more permissive to axon outgrowth (Crespo et al. 2007) and may promote axon regeneration after CNS injury, suggesting that the inhibitory properties of CSPGs principally depend on the sulfated sugar GAG chains.

Overcoming the inhibitory effect of CSPGs on neuronal growth by digestion with ChABC

A great number of studies have shown that local application of ChABC to injured CNS *in vivo* either enhances regeneration of lesioned axons or increases collateral sprouting by spared axons (Bradbury et al. 2002; Crespo et al. 2007; Davies et al. 1999; Fawcett 2006; Jefferson et al. 2011; Smith-Thomas et al. 1995). ChABC treatment enhanced neurite outgrowth in neuronal cultures grown on CSPG-containing substrates (Busch et al. 2009; Kigerl et al. 2009) and also enhanced *in vivo* axon regeneration or sprouting of injured projection tracts in the CNS (Krekoski et al. 2001; Moon et al. 2001; Yick et al. 2000). By using different injury models, several groups have demonstrated that digestion of CSPGs with ChABC promotes regrowth of axons and formation of synaptic contacts along different axonal pathways, including nigrostriatal, corticospinal, serotonergic, reticulospinal,

ascending dorsal column axons and Clarke's nucleus neurons (Barritt et al. 2006; Bradbury et al. 2002; Fouad et al. 2005; Garcia-alias et al. 2009; Garcia-alias et al. 2011; Moon et al. 2001; Tom et al. 2009; Yick et al. 2000). In transgenic mice using a glial fibrillary acidic acid protein promoter to express ChABC in reactive astrocytes, growth of descending corticospinal tracts into the injury site after spinal cord dorsal over-hemisection was increased, as was regeneration of ascending sensory fibers into the spinal cord following dorsal root crush injury (Cafferty et al. 2007).

Local ChABC treatment shows synergistic effects when combined with other regenerative strategies, such as transplants of different types of cells or biomaterials, neurotrophic factors, agents that block myelin inhibitors, and other effective approaches (Alilain et al. 2011; Bradbury and Carter 2011; Chau et al. 2004; Crespo et al. 2007; Fouad et al. 2005; Garcia-alias et al. 2009; Garcia-alias et al. 2011; Houle et al. 2006; Ikegami et al. 2005; Mingorance et al. 2006; Tom et al. 2009). Numerous experiments indicate that CSPG digestion with ChABC at the edge of cellular transplants enhances axonal exit from the grafts into the spinal cord caudal to the lesion site (Alilain et al. 2011; Fouad et al. 2005; Tom et al. 2009). Most studies on SCI repair have been performed using anterograde tracing in animals with incomplete injuries. In such experiments it is difficult to differentiate regenerating axons from sprouting of undamaged fibers. In these reports it may be that both axon regeneration in disconnected tracts and sprouting from spared axons contributed to the enhanced behavioral recovery and plasticity in these reports. However, some studies reported limited axon regeneration and functional recovery in rodents with complete spinal cord transections when local ChABC treatment was combined with other strategies (Bai et al. 2010; Fouad et al. 2009; Fouad et al. 2005).

Currently, local application of ChABC is the main *in vivo* approach to surmounting the strong growth-inhibitory effect of CSPGs. This could have several disadvantages if applied to SCI patients. ChABC does not completely digest GAG chains from the core protein and may leave undigested carbohydrate side chains on the molecules, which though less potent are still inhibitory (Lemons et al. 2003). ChABC has a short period of enzymatic activity at body temperature and cannot cross the blood–brain barrier. A single local application may not be sufficient to overcome inhibition due to continuous generation of CSPGs after injury. Recently, one group generated thermostabilized ChABC, which remained active at 37°C *in vitro* for several weeks (Lee et al. 2010). Bacterial ChABC may also induce immune reactions after repeated injections. Thus, new strategies to overcome inhibition of axon growth by CSPGs are required to facilitate research on CNS axon regeneration. Recently, several groups reported other strategies to attenuate CSPG-mediated inhibition, including: a) suppressing CSPG expression with a small proteoglycan decorin, b) disrupting assembly of CSPG GAG chains with a DNA enzyme that targets exylosyltransferase-1, or c) knocking down the synthetic enzyme chondroitin polymerizing factor with an siRNA (Davies et al. 2004; Grimpe and Silver 2004; Laabs et al. 2007). Decorin has been reported to suppress the levels of several CSPGs and to promote axon growth across lesions after SCI (Davies et al. 2004; Minor et al. 2008). Treatments with antibodies against chondroitin sulfate-E or NG2 and competitive inhibition of CSPGs with oligosaccharides have also been shown to promote recovery after CNS injury (Brown et al. 2012; Tan et al. 2006). However, the *in vivo* significance of these promising approaches has not yet been determined.

Molecular mechanisms of CSPG inhibition

Although the inhibitory effect of CSPGs on neuronal regeneration and plasticity has been known for many years (McKeon et al. 1991; Snow et al. 1990; Snow et al. 1991), the underlying molecular mechanisms are still not well understood. The inhibitory actions of CSPGs seem to be dependent on the sulfation pattern of GAG chains, since preventing GAG sulfation eliminates much of the inhibitory activity on axon growth *in vitro* (Gilbert et al. 2005; Sherman and Back 2008; Wang et al. 2008). Several general mechanisms have been proposed, including binding to functional CSPG receptors on the neuronal membrane, formation of a non-permissive perineuronal net that causes steric hindrance of growth-promoting adhesion molecules such as laminin and integrins, and facilitation of the inhibitory effects of some chemo-repulsive molecules (Fig.2). So far, several receptors for CSPGs have been reported, including PTP σ , LAR phosphatase, NgR1 and NgR3 (Dickendesher et al. 2012; Fisher et al. 2011; Shen et al. 2009).

Integrins are important regulators of neuronal adhesion and growth. Their growth-promoting function derives from their role as the transmembrane receptors for ECM molecules, such as laminin, and cell surface adhesion molecules, linking them to actin cytoskeleton. CSPGs can suppress neurite growth by attenuating integrin activation and conversely, high levels of integrins can surmount CSPG inhibition of neurite growth (Afshari et al. 2010; Condic et al. 1999; Tan et al. 2011). CSPGs appear to contribute to inhibitory functions of some chemo-repulsive proteins. The thrombospondin repeats of Sema5A interact physically with the GAGs of both CSPGs and heparan sulfate proteoglycans (HSPGs). The CSPG binding may convert Sema5A from an attractive to an inhibitory guidance cue (Kantor et al. 2004). Sema3A may interact with chondroitin sulfate-E enriched in the perineuronal nets and this interaction may mediate the repulsive function of Sema3A (De Wit et al. 2005; Deepa et al. 2006; Kwok et al. 2011). In addition, the GAGs of CSPGs may bind to extracellular calcium or its channels and regulate neuronal growth by affecting calcium availability and entry into neurons (Hrabetova et al. 2009).

LAR subfamily of phosphatases as the functional receptors for CSPGs

Most axon growth inhibitors in the CNS restrict axon extension by binding to specific receptor proteins in the membrane. It is very likely that CSPGs mediate growth suppression of neurons primarily through binding and activating functional receptors on neurons. An important advance in recent years has been the discovery that two members of the LAR subfamily of PTPs are the functional receptors for CSPG molecules (Fisher et al. 2011; Shen et al. 2009). The PTP family plays a vital role in modulating the levels of intracellular tyrosine phosphorylation in various types of cells. During development, PTPs exhibit a distinct spatial pattern of expression and are implicated in axon growth and guidance in the CNS (Bixby 2000; Stoker 2001). For example, a number of PTPs displayed a distinct spatiotemporal regulation in the pre- and postnatal superior colliculus, which appears to correlate with neuronal proliferation, differentiation, axon innervation and arborisation (Reinhard et al. 2009). The LAR subfamily is composed of three vertebrate homologs: LAR, PTP σ , and PTP δ , which share 66% amino acid identity in the full-length proteins and 84% identity in the catalytic domains. Mice lacking the LAR subfamily

proteins have various morphological and functional deficiencies. The number of progeny in LAR $-/-$ mice is significantly lower than in LAR $+/+$ mice (17 vs. 25%) (Yeo et al. 1997), but LAR $-/-$ and $+/-$ mice are viable and grossly normal in appearance. Morphologically, LAR $-/-$ mice have significantly smaller basal forebrain cholinergic neurons and reduced cholinergic innervation of their target neurons in the dentate gyrus (Yeo et al. 1997). Mice lacking LAR phosphatase domains exhibit spatial learning impairment and hyperactivity (Kolkman et al. 2004). PTP σ -deficient mice exhibit severe growth retardation, high neonatal mortality and neurological defects, including motor dysfunction, defective proprioception, hippocampal dysgenesis, abnormal pituitary development, and thinning of the corpus callosum and cerebral cortex (Meathrel et al. 2002; Uetani et al. 2006). PTP6 knockout mice also exhibit marked motor dysfunction and impaired visuospatial processing with low survival rates (Uetani et al. 2006; Uetani et al. 2000).

A number of previous studies indicate strong chemical interactions between PTPs and the GAG chains of some proteoglycans. The first Ig-like domain of PTP σ homologs bound to the heparan sulfate GAG chains of agrin and collagen XVIII and promoted retinal axon growth (Aricescu et al. 2002; Ledig et al. 1999). *Drosophila* LAR binds to HSPGs syndecan and dallylike with high affinity and regulates synaptic function (Fox and Zinn 2005; Johnson et al. 2006). Thus, it was reasonable to expect that PTPs might interact with the GAGs of CSPGs. Indeed, recent studies indicate that acting as the functional receptors, PTP σ and LAR are responsible for much of CSPGs' inhibition of axon growth.

PTP σ mediates CSPG inhibition of axonal growth

Recently, the groups of Flanagan and Silver showed that PTP σ is one of the functional receptors for CSPG-mediated inhibition (Shen et al. 2009). Their co-immunoprecipitation experiments and binding assays using PTP σ fusion protein and astrocytic cultures indicate the interaction of CSPG neurocan with PTP σ . The GAG chains of CSPG and a number of positively charged amino acids in the first Ig-like domain of PTP σ significantly contribute to the binding between the ligands and receptors (Aricescu et al. 2002; Shen et al. 2009). Given the high level of PTP σ expression in dorsal root ganglion (DRG) neurons, they showed that DRGs derived from PTP σ $-/-$ mice have increased neurite outgrowth on CSPG substrate, suggesting partial reversal of CSPG-mediated inhibition. The effect was specific to CSPG since PTP σ deletion did not overcome the inhibition of growth imposed by MAG.

To study the *in vivo* significance of PTP σ , these groups examined regrowth of lesioned ascending sensory axons in the fasciculus gracilis following a dorsal column crush injury using PTP σ deficient mice. They detected significantly increased growth of tracer-labeled axons into the CSPG-rich lesion area in PTP σ $-/-$ mice although the regenerating axons failed to pass the injury site (Shen et al. 2009). Similarly, growth of corticospinal tract axons into the spinal cord 3–7 mm distal to a T9 hemisection was reported in adult PTP σ $-/-$ mice (Fry et al. 2010). Moreover, previous studies indicated enhanced regeneration of injured optic nerve and peripheral nerves in PTP σ knockout mice (Fry et al. 2010; McLean et al. 2002; Sapieha et al. 2005; Thompson et al. 2003). Together, these studies support that PTP σ is a functional receptor for CSPG inhibitors.

LAR functions as a receptor for CSPG-mediated inhibition of axon growth

Because *Drosophila* LAR binds to the GAG chains of HSPGs with high affinity and regulates neuronal functions (Fox and Zinn 2005; Johnson et al. 2006) and several PTPs can regulate neurite outgrowth *in vitro* and nerve regeneration (Stepanek et al. 2005; Sun et al. 2000; Wang and Bixby 1999; Wills et al. 1999; Xie et al. 2001; Yang et al. 2003; Yang et al. 2006; Yang et al. 2005), we studied whether LAR phosphatase is able to bind CSPGs and function as a transmembrane receptor for these growth inhibitors. LAR is widely expressed in neurons of the adult brain and spinal cord, including axon cylinders along the white matter tracts (Fisher et al. 2011). Using co-immunoprecipitation with different types of tissues and binding assays in transfected COS-7 cells, we found the high affinity binding of purified CSPGs to LAR in a dose-dependent manner. By removal of the GAG chains with ChABC digestion and generation of point mutations in the first Ig domain of LAR, we demonstrated that the CSPG GAGs and the first Ig-like domain of LAR contribute to CSPG-LAR interaction. Moreover, by measuring PTP activity in COS-7 cells transfected with either wild type LAR or LAR mutation, we found that CSPG stimulation could activate LAR phosphatase *in vitro* (Fisher et al. 2011).

To determine whether LAR deletion overcomes the restriction of neuronal growth by CSPGs, we studied LAR deletion on neurite outgrowth in DRGs cultured from LAR-deficient mice. LAR $-/-$ DRGs significantly increased neurite length in the presence of CSPGs although the recovery did not reach to the normal control level (Fisher et al. 2011). To confirm that LAR inhibition attenuates suppression of neurite growth by CSPGs, we also examined DRG neurite growth following treatments with the extracellular and intracellular LAR peptides (ELP and ILP). Both blocking peptides at low micromolar levels remarkably increased neurite length on CSPGs, but did not significantly increase neurite growth in the absence of axon growth inhibitors or in the presence of CNS myelin inhibitors. These *in vitro* experiments suggest that LAR activation contributes to neurite growth suppression induced by CSPGs. The remaining suppression by CSPGs after LAR deletion or inhibition is probably regulated by other receptors or receptor-independent mechanisms (Carulli et al. 2005; Kwok et al. 2011; Shen et al. 2009).

To study the *in vivo* significance of LAR inhibition after CNS injury, we applied ELP and ILP systemically to adult mice with a T7 dorsal transection lesion at a post-injury time frame. Compared to SCI controls, we detected increased density of 5-HT fibers in transverse sections of the spinal cord 5–7 mm caudal to the lesion at the upper lumbar levels and a greater number of 5-HT axons into the CSPG-rich scar tissues around the lesion and the caudal spinal cord from longitudinal sections containing the lesion site in the peptide-treated mice. Moreover, by performing several behavioral tests, we observed the enhanced locomotor Basso Mouse Scale (BMS) scores and reduced grid walk errors of the hindpaws in both peptide-treated mice several weeks after injury. Thus, systemic treatment with LAR-targeting peptides significantly improves axonal growth and behavioral recovery in adult rodents with a dorsal transection SCI. These results suggest that LAR peptides penetrated into the lesioned spinal cord efficiently following systemic application.

NgR1 and NgR3, two receptors for myelin-associated inhibitors, mediate CSPG suppression on neuronal growth

Nogo receptors NgR1-3 are the GPI-linked membrane proteins and share similar structures, including eight leucine-rich repeats (LRRs) flanked by N-terminal and C-terminal LRR-capping domains. NgR1 binds to three myelin inhibitors Nogo, OMgp and MAG (Fournier et al. 2002; Fournier et al. 2001; Liu et al. 2006; McGee and Strittmatter 2003), whereas NgR2 interacts with MAG (Venkatesh et al. 2005). The ligands that bind to NgR3 are less clear. Recently, Dickendesher TL et al. reported that NgR1 and NgR3 bound to the GAGs of CSPGs with high affinity and participated in CSPG inhibition on neuronal growth (Dickendesher et al. 2012). Combined deletion of NgR1 and NgR3, but not NgR1 and NgR2, was able to overcome CSPG-mediated inhibition of neurite elongation and promoted regeneration of injured optic nerves in adult mutant mice. Simultaneous ablation of PTP σ with NgR1 and NgR3 further promoted regrowth of lesioned optic nerves in mutant mice. Thus, NgR1 and NgR3 may function as CSPG receptors and mediate at least some of the axon growth-inhibiting effects of two completely different groups of inhibitors generated by oligodendrocytes and reactive astrocytes.

A recent study indicates that chondroitin sulfate E polysaccharide regulates neurite growth via interaction with the cell adhesion molecule contactin-1, a GPI-anchored neuronal membrane protein, in the neuroblastoma cell line and primary hippocampal neurons (Mikami et al. 2009). This raises the question whether contactin-1 serves as a functional receptor for CSPGs, regulating axonal growth *in vitro* and *in vivo*.

Downstream signaling pathways to convey growth inhibition by CSPGs and regeneration of axons

Several intracellular signals have been reported to mediate CSPG inhibition on neuronal growth, including Akt/PKB (protein kinase B), glycogen synthase kinase 3 β (GSK-3 β), RhoA, protein kinase C and others (Dill et al. 2008; Fu et al. 2007; Monnier et al. 2003; Powell et al. 2001; Sivasankaran et al. 2004). We recently determined the downstream signaling pathways that mediate CSPG-LAR interactions by measuring activities of Akt, RhoA and CRMP2 in cerebellar granule neurons derived from postnatal LAR $-/-$ or $+/+$ mice. CSPG stimulation induced significant reduction of phosphorylated Akt at Ser473 and enhancement of active RhoA signals in neurons derived from LAR $+/+$ mice, but did not result in significant changes of these signaling proteins in LAR $-/-$ neurons. In contrast, CSPG incubation failed to cause significant alteration of phosphorylated CRMP-2 at Thr514 in neurons cultured from either LAR $+/+$ or $-/-$ mice. Thus, CSPG-LAR interaction mediates growth inhibition of neurons partly via inactivating Akt and activating RhoA signals, but not via CRMP2. Although the possible downstream signals to regulate CSPG-PTP σ interaction have not been studied, the scar-sourced and myelin-derived growth inhibitors share certain downstream signals to regulate neuronal growth, such as activation of RhoA and inactivation of Akt signaling (Fig. 1 and Fig. 2) (Dill et al. 2010; Dill et al. 2008; Etienne-Manneville and Hall 2002; Fisher et al. 2011; Fu et al. 2007; Luo 2000; McGee and Strittmatter 2003; Mueller et al. 2005).

Intracellular signaling pathways for true axon regeneration may be different from those that mediate axon guidance and outgrowth during early embryonic development. The latter are dependent on F-actin-mediated filopodial outgrowth. Collateral sprouting may employ similar mechanisms. In more mature neurons, regeneration of axons does not require F-actin polymerization (Jin et al. 2009; Jones et al. 2006; Marsh and Letourneau 1984), but may depend on internal protrusive forces generated by assembly of microtubules (Jones et al. 2006) or neurofilaments (Jacobs et al. 1997) in the axon tips and may involve activation of mammalian target of rapamycin (mTOR) pathway (Park et al. 2010). This further suggests that CSPGs are potent inhibitors of axonal growth in the injured CNS by binding multiple specific receptors and activating growth cone collapsing activity. CSPGs inactivate Akt (Dill et al. 2008; Fisher et al. 2011), a key signaling molecule in the mTOR activation pathway⁹³. Whether this is an independent action or is secondary to activation of RhoA and ROCK is not known, but in macrophages and neutrophils, ROCK1 activates PTEN (Vemula et al. 2010), which inactivates Akt. Therefore, under some circumstances, growth cone collapsing activity is probably linked functionally to inhibition of mTOR (reviewed by Ferguson T, Selzer ME and He Z, in press).

Conclusion

Growing axon tips is subjected to both positive and negative environmental influences, which seem to affect neuronal survival and axon elongation signaling through predominantly phosphorylation-activated molecules. Neurotrophins bind to receptor tyrosine kinases, which phosphorylate and thereby activate phosphoinositide 2 kinase (PI2K) to form PI3K. This in turn activates Akt, leading ultimately to activation of mTOR, an upstream activator of protein synthesis implicated in axon regeneration (Liu et al. 2010; Park et al. 2010; Park et al. 2008). On the other hand, many inhibitory cues act by binding to specific receptors that either directly or through receptor complex partners activate the small GTPase RhoA, which leads to cytoskeletal disaggregation and inhibition of axon growth through activation of ROCK (Gross et al. 2007). While myelin-derived axon growth inhibitors function by binding to a unique receptor NgR1 and activating its downstream intracellular signaling pathways, CSPGs appear to be mechanistically highly promiscuous. As highly charged elements of the ECM, CSPGs screen chemical interactions between growth-promoting matrix and cell adhesion molecules. Through their highly charged GAG moieties, CSPGs may act as steric inhibitors of the ECM and cell adhesion molecule receptors such as integrins. CSPGs have at least two PTP receptors and also may bind to two NgRs at the sites remote from the binding domains for myelin-associated inhibitors. Of all the inhibitory cues in the CNS environment, CSPGs are particularly vicious at blocking regeneration. Thus, glial scar becomes a formidable barrier to axon regeneration.

Identification of CSPG receptors is an important advance for better understanding the scar-mediated suppression, but many questions remain unanswered regarding the CSPG receptor-mediated suppression on neuronal growth. Do the reported receptors PTP σ , LAR, NgR1 and NgR3 completely convey the growth inhibition of different CSPGs molecules? Are there additional receptors that mediate CSPG suppressive function, such as the other PTP members? How much of the CSPG inhibitory activity is accounted for by the binding to receptors *vs.* by the steric hindrance of ECM integrin function? Do they use distinct or

redundant downstream signaling pathways to control the growth failure of neurons? Since both CSPGs and HSPGs are able to bind to and interact with LAR subfamily PTPs, do these ligands compete for the binding to the receptor proteins? What is the functional significance of different types of sulfate proteoglycan binding to the same receptor? Given that the GAGs of sulfate proteoglycans are critical for binding to receptors, are the core proteins required for the suppressive function of CSPGs?

Because CSPGs may be involved in regulating so many aspects of neurite growth, elucidating the mechanisms by which CSPG acts will lead to a more profound understanding of CNS axon regeneration, and ultimately may be an important key to developing therapies that restore function to persons disabled by brain and spinal cord injuries. By targeting critical binding or functional domains of CSPG receptors, such as LAR and PTP σ , we may design more specific, potent and feasible compounds, such as blocking antibodies or peptides, for promoting CNS axonal growth than the bacterial enzyme ChABC. For example, systemic application of LAR antagonistic peptides initiated at a post-injury time window may provide a basis for achieving effective axonal regeneration and locomotor recovery in adult mammals with CNS injury (Fisher et al. 2011) given the obvious advantages of peptides over ChABC and the wide application of FDA-approved peptide drugs in humans. Since multiple factors contribute to neuronal growth failure in adult CNS, more successful regenerative strategies appear to require combinatorial treatments targeting different mechanisms for neuronal repair failure. It is very interesting to target both extracellular inhibitory and neuron-intrinsic factors to promote more robust axon regeneration and neuronal plasticity. Several intracellular molecules, including PTEN, mTOR and Krüppel-like factors, appear to be important targets for regulating the intrinsic growth ability of mature neurons (Liu et al. 2010; Moore et al. 2009; Park et al. 2008; Sun et al. 2011). Combinations of diverse effective compounds with other successful strategies, such as cell transplants to overcome physical barrier of lesion areas and task-specific rehabilitative training to reinforce functionally meaningful synaptic reconnections, may help achieve more successful functional regeneration after CNS injury.

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Fig. 1. Schematic of the major factors to regulate neuronal growth

The myelin-associated inhibitors, such as Nogo, and scar-rich CSPGs activate intracellular RhoA to collapse axon growth cones. Cyclic AMP surmounts the suppression of these inhibitory signals and promotes axon growth. Intracellular PTEN phosphatase blocks axon growth by inactivation of mTOR pathway. Cytokine signaling, such as STAT3, may promote neuronal growth by alternating the expression of some regeneration-associated genes. RhoA might also modulate PTEN activity and suppress neuronal growth via inactivation of mTOR signaling. (Adaptation from a book chapter by Toby Ferguson, Michael Selzer and Zhigang He).

Fig. 2. Schematic of the molecular mechanisms of CSPG inhibition on neuronal growth
 CSPGs induce growth inhibition via binding and activating several receptor proteins, including PTP σ , LAR (A), NgR1 and NgR3 (B). CSPGs contribute to inhibitory properties of Sema 5A by converting it from an attractive to an inhibitory cue (C). CSPGs may suppress axon growth via blocking functions of growth-promoting molecules, such as laminin and its receptor integrins (D). CSPGs involve the formation of the perineuronal nets with several other extracellular matrix molecules, including hyaluronan (HA), HA receptor, tenascin R and phosphacan (PPC, E). The CSPG-rich pericellular matrix wraps around neurons in the CNS and contributes to various neuronal activities including axonal growth. In addition, CSPGs may regulate neuronal growth by altering calcium influx into cells. Intracellularly, several signaling pathways mediate the activity of CSPG inhibitors, including RhoA, Rock, Akt, GSK-3 β and other signaling proteins. Ig-like: Immunoglobulin-like domains; FN-III: Fibronectin Type III domains; D1: D1 domain; D2: D2 domain; HA: Hyaluronan; PPC: phosphacans.