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Molecular mechanisms of scar-sourced axon growth inhibitors

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Abstract

Astrogliosis is a defense response of the CNS to minimize primary damage and to repair injured tissues, but it ultimately generates harmful effects by upregulating inhibitory molecules to suppress neuronal elongation and forming potent barriers to axon regeneration. Chondroitin sulfate proteoglycans (CSPGs) are highly expressed by reactive scars and are potent contributors to the non-permissive environment in mature CNS. Surmounting strong inhibition by CSPG-rich scar is an important therapeutic goal for achieving functional recovery after CNS injuries. Currently, enzymatic digestion of CSPGs with locally applied chondroitinase ABC is the main *in vivo* approach to overcome scar inhibition, but several disadvantages may prevent using this bacterial enzyme as a therapeutic option for patients. A better understanding of molecular mechanisms underlying CSPG function may facilitate development of new effective therapies to overcome scar-mediated inhibition. Previous studies support that CSPGs act by non-specifically hindering the binding of matrix molecules to their cell surface receptors through steric interactions, but two members of the leukocyte common antigen related (LAR) phosphatase subfamily, protein tyrosine phosphatase σ and LAR, are functional receptors that bind CSPGs with high affinity and mediate CSPG inhibition. CSPGs may also act by binding two receptors for myelin-associated growth inhibitors, Nogo receptors 1 and 3. Thus, CSPGs inhibit axon growth through multiple mechanisms, 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.

Keywords

Astrogliosis; axon regeneration; CNS injury; reactive astrocyte; scar; CSPG receptor; LAR; PTP σ ; Nogo receptor

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1. Introduction

Reactive astrogliosis occurs in response to CNS injuries and is characterized by proliferation of astrocytes, glial precursors, microglia and fibroblasts. Lesioned CNS usually induces formation of marked hypertrophy and proliferation of astrocytes along with significant overlap of astrocytic domains (Karimi-Abdolrezaee and Billakanti, 2012). Pronounced astrocytic proliferation occurs with overlapped and densely-packed astrocytic processes and overlapping astrocytes usually intertwine with fibromeningeal cells and NG2 positive glia to form dense scar tissues. In addition to the featured hypertrophic astrocytes in penumbra, the lesion core consists of NG2-expressing glia, including oligodendrocyte precursor cells (OPCs), meningeal and/or vascular derived fibroblasts, pericytes, ependymal cells and phagocytic macrophages (Cregg et al., 2014). Unlike other glia, fibroblasts invade the lesion site from adjacent meningeal and perivascular cells. After CNS injury with intact dura mater, perivascular collagen1 α 1 cells appear the main source of fibrotic scar (Soderblom et al., 2013). Reactive astrocytes express high levels of a great number of molecules, including intermediate filaments glial fibrillary acidic proteins (GFAP), nestin and vimentin (Ridet et al., 1997; Sofroniew, 2009). Astrogliosis is a defense response of CNS to minimize and repair primary damage, including isolation of intact tissue from secondary lesion, maintenance of favorable environment for surviving neurons, preservation of the blood brain barrier (BBB), generation of permissive substrates for neurite elongation and other protective effects (Karimi-Abdolrezaee and Billakanti, 2012; Sofroniew, 2009). However, reactive glial scar eventually generates harmful effects due to forming both physical and chemical barriers to axon regeneration, including producing high levels of inhibitory molecules to suppress neuronal elongation. An important class of molecules in the scar extracellular matrix (ECM) is chondroitin sulfate proteoglycans (CSPGs), which are highly upregulated after CNS injury and predominantly responsible for the non-permissive nature of glial scar. In this review, we will focus on recent progress in scar-rich axonal growth inhibitors and their molecular mechanisms.

2. Functions of reactive scar tissue after CNS injury

Astrocytes are actively involved in synthesis and maintenance of ECM molecules in intact CNS. Following CNS injury, reactive astrocytes remarkably change the ECM composition by highly expressing some ECM components, including CSPGs and tenascins (Carulli et al., 2005; Galtrey and Fawcett, 2007; Kwok et al., 2011). Numerous transgenic studies demonstrate that ablation of reactive astrocytes or interfering with their activation exacerbates tissue damage after spinal cord injury (SCI) by increasing tissue degeneration and failure to reconstruct BBB (Faulkner et al., 2004; Sofroniew, 2009). Astrocyte reactivity has beneficial effects in the early stage by limiting tissue damage to certain areas and preventing extension of injury into adjacent domains. Scar tissues produce a number of ECM components with growth-promoting properties, such as fibronectin and laminin, indicating possible repairing role of astrogliosis after CNS damage (Silver and Miller, 2004). However, migration of a large number of astrocytes into and around the lesion areas and formation of glial scar tissues also constitute physical barrier of axon regeneration. More importantly, upregulation of suppressing substances, particularly CSPGs, potentially

impedes neural repair and regeneration and the inhibitory properties of reactive astrocytes evolve with time after injury.

2.1 Positive roles of reactive glial scar

Glial scar is well known as an important impediment to axonal regeneration, but it is a defense mechanism of the CNS to injury and has multiple protective and repair functions. Many *in vivo* genetic studies strongly supported the protective roles of glial scar (Burda and Sofroniew, 2014). Conditional ablation of reactive astrocytes in transgenic mice increased vasogenic edema, tissue destruction, inflammation response, demyelination, oligodendrocyte death and worsen functional outcome following CNS injury (Faulkner et al., 2004; Sofroniew, 2009). Lack of scar formation in the suppressor of cytokine signaling 3 (SOCS3) or signal transducers and activators of transcription 3 (STAT3) deletion mice has similar effects by inducing widespread lesion and increasing neuronal and oligodendroglial cell death and locomotor deficits after SCI (Herrmann et al., 2008; Okada et al., 2006). The reactive glial responses generally have the following positive functions after CNS injury: 1) separate the lesion area from normal CNS tissue and protect intact CNS tissue from further damage; 2) limit vasogenic edema by upregulating aquaporin 4 channels, modulate blood flow by regulating release of vasoconstrictors and blood vessel diameter, repair damaged BBB, and reduce infiltration of peripheral leukocytes and activation of resident microglia, 3) protect neurons and oligodendrocytes from excitotoxicity by upregulating glutamate transporter and taking up extracellular glutamate and reduce oxidative stress by increasing glutathione production and NH_4^+ -mediated toxicity, 4) provide trophic and metabolic support to surviving neurons by producing glucose and various growth factors (insulin-like growth factor, Neu differentiation factor, brain-derived neurotrophic factor, neurotrophin 3, fibroblast growth factor 2, and S100 β) and stabilizing extracellular fluid ion balance, 5) promote recruitment of endothelial cells and fibroblasts and ultimately induce vascularization at injury site by scar matrix components, and 6) restrict inflammation by turning monocytes into resolving phenotype and enhancing production of anti-inflammatory cytokines, such as interleukin-10, by the scar matrix components, especially CSPGs. In addition, CSPGs have anti-oxidative and anti-excitotoxic effects and contribute to cellular water homeostasis due to the hydrophilic properties of some proteoglycans. Anionic CSPGs also help stabilize ionic microenvironment by restricting diffusion of cations, such as calcium, potassium and sodium (Kwok et al., 2011) and this function might be related to the fast firing of parvalbumin-expressing GABAergic interneurons. Some studies indicate certain CSPGs and their core proteins promote rather than inhibit neurite outgrowth (Bandtlow and Zimmermann, 2000).

2.2 The Inhibitory properties of scar-rich proteoglycans on neuronal growth

During development and following CNS injury, the integrations between growth-promoting ECM molecules and their receptors (such as laminin, fibronectin and certain integrins) and growth-suppressing molecules are critical for determining elongating, turning and terminating behavior of axons. Neurons that express high levels of ECM adhesion receptors, such as integrins, and low levels of repulsive molecules (such as CSPG receptors) should grow axons readily. Alternatively, the territories containing high positive molecules and low repulsive cues should provide a permissive environment and favor axon elongation. Glial

scar is a major detriment to regeneration of severed axons by upregulating a great number of molecules around the lesion and preventing regrowth of injured axons at the lesion area, including CSPGs, tenascin, semaphorin 3A, keratan sulfate proteoglycans (KSPGs), myelin-associated inhibitors and ephrins/Eph receptors. These molecules have repelling function on axonal growth and also play important role in CNS axon guidance during development. Among them, CSPGs are an extremely important class of growth inhibitors that are highly upregulated by scar tissues.

CSPGs are a family of molecules characterized by a core protein to which the large and highly sulfated glycosaminoglycan (GAG) chains are attached. The major CSPGs found in the CNS include lecticans (neurocan, versican, aggrecan and brevican), phosphacan (6B4 proteoglycan) and NG2 (Fig. 1). Lecticans share similar N-terminal hyaluronan-binding domains and C-terminal globular domains with a unique lectin domain. The core proteins of lecticans (97-262 kD) are linked by a central CS-GAG anchoring backbone bound to one or multiple long-chain CS-GAG polysaccharide. Lecticans interact with carbohydrate and protein ligands in the ECM and act as linkers of the ECM molecules, including hyaluronan and tenascin-R (Yamaguchi, 2000). Phosphacan represents the extracellular domain of transmembrane receptor-type protein tyrosine phosphatase (PTP) β . NG2, a transmembrane CSPG, exhibits no significant homologies to other proteins.

CSPGs are concentrated into perineuronal nets (PNNs) and may attach to cell membrane (Fig. 1). PNNs are mainly composed of hyaluronan, CSPGs, tenascin R and link proteins. Interactions between these molecules form a stable pericellular complex around synapses. CNS starts to form PNNs during its later development, which appear to play a crucial role in controlling reduced plasticity of developed neurons (Kwok et al., 2011). The spatiotemporal expression of CSPGs correlates with glial boundaries during development, such as in the spinal cord roof plate, optic tectum and dorsal root entry zone. Postnatal development of PNNs appears to underlie age-related loss of plasticity in some CNS locations.

The levels of CSPGs increase dramatically following various CNS injuries, including lesions in the spinal cord, cortex, fornix and nigrostriatal area. CSPGs are primarily generated by reactive astrocytes and to a lesser extent by oligodendrocytes and monocytes. The lesion penumbra significantly expresses CSPGs with higher levels in the epicenter of scar tissues (Davies et al., 1997). In addition to the physical barrier of scar tissues, 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., 2003a; McKeon et al., 1991). Thus, surmounting strong suppression of CSPG inhibitors in glial scar is a major target for therapeutic intervention following CNS injuries. Evidence for the inhibitory nature of CSPGs on axon regeneration came largely from studies on digestion of GAG side chains of CSPGs with the bacterial enzyme chondroitinase ABC (ChABC). Although CSPG core proteins are 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 promotes axon sprouting/regeneration after CNS injury. Application of chemically-synthesized specific oligosaccharide chondroitin sulfate-E (CS-E), a sugar epitope on CSPGs, potently inhibited axon growth and removal of CS-E motif reduced inhibitory activity of CSPGs. An antibody

against CS-E motif reversed CSPG inhibition and stimulated axon regeneration in vivo (Brown et al., 2012).

The core proteins of CSPGs have also been shown to inhibit axonal growth. Degradation of CSPG core proteins by matrix metalloproteinases, including a disintegrin and metalloproteinase with thrombospondin motifs 4 (ADAMTS-4), overcomes CSPG inhibition on neurite growth in culture (Cua et al., 2013). Local administration of ADAMTS-4 enhanced axonal regeneration/sprouting and promoted motor function recovery after SCI (Tauchi et al., 2012). Inhibition of agrin, a large proteoglycan that contributes to development of neuromuscular junction, is confined to its N-terminal segment called N150. GAGs are required for inhibition by N-terminal portion of N150, but the core protein is necessary of inhibition by the C-terminal portion of N150 (Baerwald-de la Torre et al., 2004). Matrigel loaded with either intact aggrecan or purified core glycoprotein of aggrecan is inhibitory on axon growth in rats with spinal cord hemisection (Lemons et al., 2003).

CNS injury increases the levels of NG2 mainly due to rapid accumulation of OPCs, but function of NG2-expressing OPCs on axon growth and neural repair is controversy (Busch and Silver, 2007). Some in vitro and in vivo studies support inhibitory activity of NG2. When mixed with laminin or L1, NG2 was inhibitory for neurite growth of cultured cerebellar neurons. It also reduced dorsal root ganglion (DRG) growth in a laminin mixture despite lack of inhibition to DRGs when mixed with the adhesion protein L1. The surface of OPCs appears inhibitory for neurite outgrowth and treatment with NG2 antibodies reversed OPC inhibition (Chen et al., 2002). Neu7 cells, a cell line sourced from reactive astrocytes, produces NG2, versican and CS-56 antigen and antibodies against NG2 or CS-56 increase growth of DRG and cortical neurons over Neu7 cells (Fidler et al., 1999). The core protein of NG2 appears mainly responsible for its inhibition because digestion with ChABC did not reduce NG2 inhibitory activity (Dou and Levine, 1994). Studies with NG2 domain-specific fusion proteins and antibodies indicate that an N-terminal globular domain and a juxtamembrane domain independently suppress neurite elongation in vitro (Ughrin et al., 2003). NG2 knockout mice exhibited greater growth of serotonergic axons into scar tissue after a transection SCI (de Castro et al., 2005). Treatment with a NG2 antibody partially promotes synaptic and anatomical plasticity and functional recovery after hemitransection SCI (Petrosyan et al., 2013). Combination of peripheral nerve conditioning lesion with treatment of NG2 antibodies demonstrates regeneration of sensory axons beyond glial scar and into the rostral spinal cord compared to axon growth into the lesion in conditional lesioned controls (Tan et al., 2006). In addition, local application of NG2 into the spinal cord dose-dependently attenuates axon signal conduction through unknown mechanisms (Hunanyan et al., 2010).

Other studies, however, showed controversial results for function of NG2+ cells on neuronal growth. Instead of suppressing neuronal growth, NG2 positive cells have been reported to promote axon growth of hippocampal and cortical neurons in vitro. Axonal growth cones formed extensive contacts with NG2+ cells in vitro as well as in developing corpus callosum in vivo (Yang et al., 2006b). Interactions between dystrophic adult sensory neurons and NG2+ cells derived from adult spinal cord can stabilize dystrophic growth cones during macrophage attack and high levels of laminin and fibronectin expressed by NG2+ cells

promote neurite outgrowth. NG2+ cells also promote axon growth by generating matrix metalloproteases to digest CSPGs and providing a permissive bridge for growing axons (Busch et al., 2010). Some descending and ascending axons extended into NG2-rich substrates in injured rat spinal cord transplanted with fibroblast bridges (Jones et al., 2003b). Thus, a number of studies support the growth-promoting effect of NG2+ cells in the CNS (Busch and Silver, 2007).

CSPG upregulation also controls the properties of OPCs and remyelination after CNS injury (Siebert and Osterhout, 2011). CSPGs, especially phosphocan and neurocan, inhibited elongation of OPC processes and differentiation of OPCs into mature oligodendrocytes and myelination (Siebert and Osterhout, 2011). ChABC treatment enhanced migration and differentiation of OPCs after SCI (Siebert and Osterhout, 2011). Consistently, reactive scars that upregulate and activate bone morphogenetic proteins suppressed OPC differentiation into oligodendrocytes and impaired functional recovery after contusive SCI (Wang et al., 2011). Treatment with bone morphogenetic protein receptor antagonists promoted OPC differentiation into myelinating oligodendrocytes in addition to reducing astrocyte differentiation.

3. Traditional notion of axon growth suppression by CSPGs

Prior to identification of functional CSPG receptors, several mechanisms for CSPG inhibition of axonal growth had been suggested. Given the large molecular mass of CSPGs and their involvement in formation of non-permissive PNNs, CSPGs were thought to cause steric hindrance of growth-promoting adhesion molecules including laminin and integrins. 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 as cell surface adhesion molecules, linking them to actin cytoskeleton. Through their highly charged GAG moieties, CSPGs can interact with ECM molecules and 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). Over-expression integrin by viral infection is sufficient to eradicate aggrecan inhibition on neuronal growth (Condic et al., 1999). Analyses of growth cone dynamics on different concentrations of CSPGs and laminin suggest that neuronal growth is guided by the ratio between growth-promoting and growth-inhibiting molecules present in the environment (Snow et al., 2002).

CSPGs inactivate integrin signaling pathway and integrin over-activation overcomes inhibition by CSPGs. Activation of integrin signaling by manganese or an activating antibody surmounts aggrecan inhibition on axon growth of cultured neurons. Aggrecan impairs integrin signaling by reducing levels of phosphorylated focal adhesion kinase and Src and suppresses laminin-mediated growth of cultured rat sensory neurons without altering surface integrin levels (Tan et al., 2011). Activation of integrin signaling by overexpression of kindlin-1, a phosphoprotein involved in attachment of actin cytoskeleton to plasma membrane and integrin-mediated function, enhanced growth of sensory neurons cultured on aggrecan and regeneration of injured sensory axons across the dorsal root entry zone and into the spinal cord (Tan et al., 2012). Compared to cortical neurons, serotonergic axons

could partly regenerate on high amounts of CSPG probably due to high expression of growth-associated protein-43 and/or $\beta 1$ integrin. Blockade of $\beta 1$ integrin reduced serotonergic and cortical outgrowth on laminin (Hawthorne et al., 2011). Because integrin activation also reversed growth suppression on neuronal growth by other inhibitors, such as Nogo-A (Tan et al., 2011), the functional link between laminin/integrins and CSPGs appears not specific to CSPGs.

CSPGs have been shown to contribute to inhibitory function of some chemo-repulsive proteins. The thrombospondin repeats of Sema5A, an axon guidance cue, 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, a repulsive guidance molecule, may interact with CS-E enriched in the PNNs and this interaction could 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).

4. Receptor-mediated inhibition by CSPGs

Inhibition of CSPGs on neuronal regeneration and plasticity has been known for over two decades (McKeon et al., 1991; Snow et al., 1990; Snow et al., 1991), but the molecular mechanisms for CSPG function are not well understood. Sulfation pattern of GAG chains is important for CSPG inhibition 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 suggested, including binding to functional CSPG receptors on neuronal membrane, formation of a non-permissive PNNs that causes steric hindrance of growth-promoting adhesion molecules (such as laminin and integrins) and facilitating function of some chemo-repulsive molecules (Fig.2). Although CSPGs may non-specifically hinder binding of matrix molecules to their cell surface receptors through steric interactions, recent studies indicate that two members of the leukocyte common antigen-related (LAR) phosphatase subfamily, the transmembrane proteins of PTP σ and LAR phosphatase, are functional receptors that bind CSPGs with high affinity and mediate CSPG inhibitory effects (Fig. 2) (Fisher et al., 2011; Shen et al., 2009). CSPGs also may act by binding to two receptors for myelin-associated inhibitors, Nogo receptor 1 (NgR1) and NgR3 (Dickendesher et al., 2012). Thus, CSPGs inhibit axon growth likely by multiple mechanisms, making them especially potent and difficult therapeutic targets.

4.1 LAR subfamily of phosphatases as CSPGs receptors

Like most other axon growth inhibitors in the CNS, CSPGs may mediate growth suppression of neurons primarily through binding and activating functional receptors on neurons. An important advance in recent years is the discovery that two members of the LAR subfamily of PTPs are functional receptors for CSPGs (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 CNS

(Bixby, 2000; Stoker, 2001). A number of PTPs display a distinct spatiotemporal regulation in the pre- and postnatal superior colliculus, which appears to correlate with neuronal proliferation, differentiation, axon innervation and arborization (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 LAR subfamily proteins have various morphological and functional deficiencies. The number of progeny in LAR $-/-$ mice is lower than in wild type mice (17 vs. 25%) (Yeo et al., 1997), but LAR $-/-$ and $+/-$ mice are viable and grossly normal in appearance. LAR $-/-$ mice have 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). PTP δ knockout mice also exhibit marked motor dysfunction and impaired visuospatial processing with low survival rates (Uetani et al., 2000; Uetani et al., 2006).

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 bound to HSPGs syndecan and Dallylike with high affinity and regulated synaptic function (Fox and Zinn, 2005; Johnson et al., 2006). Thus, it was reasonable to expect that two groups studied the interactions of PTPs with the GAGs of CSPGs and identified PTP σ and LAR as functional receptors of CSPGs (Fisher et al., 2011; Sharma et al., 2012; Shen et al., 2009).

4.1.1 PTP σ mediates CSPG inhibition of axonal growth—PTP σ has been reported to be one of the functional receptors for CSPGs (Shen et al., 2009). CSPG neurocan binds and interacts with PTP σ through CSPG GAG chains and a number of positively-charged amino acids in the first Ig-like domain of PTP σ (Aricescu et al., 2002; Shen et al., 2009). DRGs derived from PTP σ $-/-$ mice have increased neurite outgrowth on CSPG substrate and the effect was specific to CSPG since PTP σ deletion did not overcome growth inhibition imposed by myelin associated glycoprotein (MAG). The in vivo study using PTP σ mutant mice indicates regrowth of lesioned ascending sensory axons in the fasciculus gracilis into the CSPG-rich lesion area in PTP σ $-/-$ mice although regenerating axons failed to pass injury area (Shen et al., 2009). Another group reported regrowth of corticospinal tract (CST) axons into the spinal cord 3-7 mm distal to a T9 hemisection in adult PTP σ $-/-$ mice (Fry et al., 2010). Consistently, PTP σ knockout mice exhibited enhanced regeneration of injured optic nerve and peripheral nerves after injury (Fry et al., 2010; McLean et al., 2002; Sapieha et al., 2005; Thompson et al., 2003). Thus, these studies support that PTP σ is a functional receptor that partially mediates CSPG-mediated inhibition.

4.1.2 LAR functions as a receptor for CSPGs—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., 2005; Yang et al., 2006a), we studied whether LAR phosphatase bound CSPGs and function as a transmembrane CSPG receptor. LAR is widely expressed in neurons of the adult brain and spinal cord, including axon cylinders in the white matter (Fisher et al., 2011). Purified CSPGs bind LAR with high affinity in a dose-dependent manner and the CSPG GAGs and the first Ig-like domain of LAR are critical for CSPG-LAR interaction. CSPG stimulation activates LAR phosphatase *in vitro* (Fisher et al., 2011). LAR deletion or LAR blockade with sequence-targeting blocking peptides partly increased neurite length of DRGs cultured on CSPGs (Fisher et al., 2011), but did not enhance 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 partly 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).

We employed two LAR blocking peptides to study *in vivo* significance of LAR inhibition on CNS regeneration. Systemic application of extracellular LAR peptide (ELP) or intracellular LAR peptide (ILP) increased the density of 5-HT fibers in the spinal cord 5-7 mm caudal to the lesion (Fisher et al., 2011). Longitudinal sections containing lesion site indicated a greater number of 5-HT axons into the CSPG-rich scar tissues and caudal spinal cord in LAR peptide-treated mice. ELP and ILP treated mice also performed better in several behavioral tests, including enhanced locomotor Basso Mouse Scale scores and reduced grid walk errors of the hindpaws several weeks after injury. Thus, LAR is essential for mediating CSPG inhibition as a functional receptor and LAR blockade improves axonal growth and behavioral recovery in adult rodents with SCI.

More recent studies further support the role of PTP σ and LAR in mediating CSPG function. Newly-generated neurons from neuronal restricted precursors express low levels of PTP σ and LAR proteins and are intrinsically insensitive to CSPG substrates. Secreted factors by cultured neuronal and glial restricted precursors reduce CSPG inhibition and promote axonal growth *in vitro* (Ketschek et al., 2012). Lamprey, a type of jawless fish, exhibits heterogeneous neuronal regenerative capacities after CNS injury and only a portion of descending reticulospinal neurons regenerate after SCI. Both CSPG receptors, PTP σ and LAR, are selectively expressed in the bad-regenerating neurons and have overlapping cellular distributions, suggesting potential link between CSPG receptors and poor intrinsic regenerative ability of bad-regenerating neurons in non-mammals (Zhang et al., 2013). Consistently, CSPGs are upregulated in the lesioned spinal cord of lamprey.

4.2 Other receptors that mediate CSPG suppression

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, MAG and oligodendrocyte myelin glycoprotein (Fournier et al., 2001; Fournier et al., 2002; 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. NgR1 and NgR3 appear to bind CSPG GAGs and mediate CSPG inhibition on neuronal growth (Dickendesher et al., 2012). Combined deletion of NgR1 and NgR3, but not NgR1 and NgR2, overcame CSPG inhibition and enhanced regeneration of injured optic nerves in double knockout mice. Thus, NgR1 and NgR3 may function as CSPG receptors and mediate some suppression by two completely different groups of inhibitors generated by oligodendrocytes and reactive astrocytes. Moreover, NgR2 specifically interacts with the C-terminal G3 domain of versican and nociceptive nonpeptidergic sensory neurons with NgR2 deletion becomes insensitive to inhibition by skin-derived versican. The interactions between versican and NgR2 at dermo-epidermal junction seem to control the plasticity of peripheral sensory fibers (Baumer et al., 2014).

Lecticans were frequently used to evaluate interactions between CSPGs and the receptors described above. It is unclear whether CSPGs phosphacan and NG2 and other sulfate proteoglycans (KSPGs and HSPGs) share the same and/or employ distinct receptors with lecticans. Both PTP σ and LAR have been reported to interact with HSPGs and regulate their function (Coles et al., 2011; Wang et al., 2012). Moreover, CS-E polysaccharides regulate neurite growth by interaction with the cell adhesion molecule contactin-1, a GPI-anchored neuronal membrane protein, in neuroblastoma cell line and primary hippocampal neurons (Mikami et al., 2009). Whether contactin-1 serves as a functional receptor for CSPGs to regulate axonal growth is unknown.

5. Downstream signaling pathways that convey growth inhibition by CSPGs

Several intracellular signals have been implicated to mediate CSPG inhibition on neuronal growth, including Akt, glycogen synthase kinase 3 β (GSK-3 β), RhoA, protein kinase C (PKC) and others (Fig. 2) (Dill et al., 2008; Fu et al., 2007; Monnier et al., 2003; Powell et al., 2001; Sivasankaran et al., 2004). PTP σ and LAR bind CSPG with high affinity as the CSPG receptors (Fisher et al., 2011; Shen et al., 2009), but their downstream signaling pathways to mediate neuron growth failure are less clear. We measured activities of Akt, RhoA and collapsin response mediator protein 2 (CRMP2) in cerebellar granule neurons cultured from postnatal LAR $-/-$ or $+/+$ mice. CSPG stimulation induced significant reduction of phosphorylated Akt at Ser473 and enhancements 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 CRMP2 at Thr514 in neurons cultured from either LAR $+/+$ or $-/-$ mice. Thus, CSPG-LAR interaction mediates growth inhibition of neurons partly by inactivating Akt and activating RhoA signals, but not by CRMP2. Local translation of RhoA in axons appears to contribute to CSPG inhibition (Walker et al., 2012). Axons of cultured DRGs contain transcripts encoding RhoA. Applying CSPGs to axonal compartment increased intra-axonal RhoA synthesis and depletion of RhoA transcripts in axons enhanced their growth in the presence of CSPGs. Moreover, treatment with a LAR peptide and PTP σ deficiency elevated both Erk and Akt activities in neurons (Sapieha et al., 2005; Xie et al., 2006).

Myosin II, an ATP-dependent motor protein, appears to mediate CSPG inhibition on neuronal growth (Hur et al., 2011; Yu et al., 2012). CSPGs increase phosphorylation of nonmuscle myosin II regulatory light chains and pharmacological or genetic inhibition of myosin II promotes axon growth on inhibitory substrates including CSPGs. NG2 appears to regulate axon growth by activating PKC ζ and Cdc42 and increasing association of PKC ζ with Par6 (Lee et al., 2013). Although the downstream signals to regulate CSPG-PTP σ interactions 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 (Fig. 2) (Dill et al., 2008; Dill et al., 2010; Etienne-Manneville and Hall, 2002; Fisher et al., 2011; Fu et al., 2007; Luo, 2000; McGee and Strittmatter, 2003; Mueller et al., 2005). It is likely that PTP σ and LAR share certain pathways to mediate CSPG function.

6. CSPG inhibition and axon regeneration

Surmounting strong inhibition by the CSPG-rich scar is an important therapeutic goal for achieving functional recovery after CNS injuries. As of now, the main *in vivo* approach to overcome inhibition by CSPGs is enzymatic digestion with locally applied ChABC, but some disadvantages may prevent using this bacterial enzyme as a therapeutic option for patients. Recent identification of CSPG receptors and better understanding of signaling pathways activated by CSPGs 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.

6.1 Removal of sulfated GAG chains by digesting CSPGs with ChABC

Many groups 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; Fawcett, 2006; Jefferson et al., 2011). Digestion of proteoglycans with ChABC *in vitro* could remove up to 88% of sulfated GAGs from the ligands (Henninger et al., 2010). ChABC treatment enhanced neurite outgrowth in neuronal cultures grown on CSPG-containing substrates (Busch et al., 2009; Kigerl et al., 2009) and *in vivo* axon regeneration or sprouting of injured CNS projection tracts (Krekoski et al., 2001; Moon et al., 2001; Yick et al., 2000). With different injury models, a number of groups demonstrate that ChABC application 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). Transgenic expression of ChABC in reactive astrocytes increased growth of descending CSTs into the injury site after dorsal over-hemisection SCI and regeneration of ascending sensory fibers into the spinal cord after dorsal root crush injury (Cafferty et al., 2007).

Application of ChABC may also facilitate recovery after CNS injury through other mechanisms. ChABC treatment upregulated tissue plasminogen activator (tPA) and plasmin around lesioned spinal cord and contributed to neuronal plasticity by degrading CSPG core proteins (Bukhari et al., 2011). tPA deletion attenuated neurite outgrowth and sensory/motor

functional recovery due to ChABC treatment and coadministration of ChABC and plasmin reversed the effects of tPA deficiency. ChABC treatment induced an extended period of astrocyte remodeling (up to 4 weeks) and a favored orientation of astrocytic processes directed toward injury area, which might become guidance bridges for regenerating axons (Milbreta et al., 2014). ChABC treatment affected morphology of laminin-positive blood vessel basement membranes and vessel-independent laminin deposits. Delivering an engineered ChABC by a lentiviral vector was also neuroprotective by facilitating generation of activated M2 macrophages (such as upregulation of CD68 and CD206), remodelling of specific CSPGs and enhancing vascularity (Bartus et al., 2014).

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 lesion site (Alilain et al., 2011; Fouad et al., 2005; Tom et al., 2009). ChABC combined with PNS autograft induced long regeneration of serotonin and other bulbospinal fibers and recovery of diaphragmatic function after cervical SCI. Transplanted Schwann cells genetically modified to secrete a bifunctional neurotrophin and ChABC into a subacute contusion injury in rats promoted regrowth of multiple types of axons (propriospinal, CST, 5-HT and other brainstem projecting fibers) into and caudal to the grafts, the number of myelinated axons and recovery of locomotion and sensory functions (Kanno et al., 2014).

Most studies on SCI repair have been performed using anterograde tracing or immunostaining in animals with incomplete injuries. In such experiments it is difficult to differentiate regenerating axons from sprouting of undamaged fibers. It is most likely that both axon regeneration in disconnected tracts and sprouting from spared axons contributed to enhanced behavioral recovery and plasticity in these reports. However, some studies reported limited axon regeneration and functional recovery in rodents with complete spinal cord transection when local ChABC treatment was combined with other strategies (Bai et al., 2010; Fouad et al., 2005; Fouad et al., 2009).

6.2 Inhibition of CSPG receptors and intracellular signaling pathways

Local application of ChABC could have several disadvantages if applied to SCI patients. ChABC does not completely digest GAG chains from the core proteins 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 BBB. A thermostabilized ChABC has been generated, which appears active at 37°C *in vitro* for several weeks (Lee et al., 2010). A single local application may not be sufficient to overcome inhibition due to continuous generation of CSPGs after injury. Bacterial ChABC may also induce immune reactions after repeated injections. Thus, new strategies to overcome inhibition by CSPGs are required to facilitate

CNS axon regeneration. An alternative approach to surmount scar-mediated inhibition is to design novel compounds to block function of CSPGs or their receptors PTP σ , LAR and NgRs. Peptide antagonists for LAR receptor reduced CSPG inhibition in vitro and subcutaneous administration of these peptides at a post-trauma time frame increased descending raphespinal axon growth and promoted sustained locomotor recovery in adult mice with SCI (Fisher et al., 2011). Systemic administration of peptides could efficiently block CSPG inhibition in contrast to the highly invasive approach of applying ChABC locally. Receptor blockade should also circumvent the issues of incomplete digestion of CSPGs and digestion of other sulfated proteoglycans that have beneficial roles for recovery. Given that multiple factors contribute to repair failure after CNS injury, combining CSPG receptor blockade with other strategies, such as cell transplants, is likely to be more effective.

A number of axon growth inhibitors including CSPGs are intracellularly mediated by activating the small GTP-binding signaling protein RhoA (Fig. 2) (Luo, 2000; Mueller et al., 2005; Walker and Olson, 2005), which regulates neuronal morphogenesis by interaction with a number of other molecules, including serine/threonine kinases, tyrosine kinases, lipid kinases, lipases, oxidases and scaffold proteins. GTP-bound Rho (active form) 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. An alternative strategy to overcome growth inhibition from extracellular factors is to influence the common downstream pathway including RhoA and ROCK (Fu et al., 2007; Luo, 2000; Mueller et al., 2005). Pharmacological inhibitors, including C3 transferase and some non-steroidal anti-inflammatory drugs, stimulate axon growth and improve behavioral recovery in SCI in rodents (Dergham et al., 2002; Dill et al., 2010; Fournier et al., 2003; Fu et al., 2007; Xing et al., 2011). 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). In addition, GSK-3 β signal partially mediates CSPG inhibition on neuronal growth and GSK-3 β suppression overcomes CSPG inhibition of neuronal growth (Dill et al., 2008; Fisher et al., 2011). GSK-3 β inhibitors, particularly the clinical drug lithium, have been reported to be beneficial after CNS injuries. Lithium has been studied in phase I/II clinical trials to evaluate its efficacy on chronic SCI patients (Yang et al., 2012).

6.3 Other approaches to surmount scar inhibition

Several other strategies have been reported to attenuate CSPG-mediated inhibition and stimulate axon regeneration. Decorin treatment markedly increases neurite growth in vitro on CSPGs or myelin membranes, especially on the former (Minor et al., 2008). Decorin reduced expression of CSPGs and promoted axon growth across lesion after SCI (Ahmed et al., 2014; Davies et al., 2004; Minor et al., 2008; Minor et al., 2011). Disrupting assembly of CSPG GAG chains, such as by knocking down xylosyltransferase-1 with deoxyribozyme, overcomes CSPG inhibition (Grimpe and Silver, 2004; Hurtado et al., 2008; Oudega et al., 2012). After CNS injury, reactive astrocytes generate high levels of old astrocyte specifically induced substance (OASIS), which upregulates chondroitin 6-O-sulfotransferase 1 (C6ST1), a major enzyme involved in CSPG sulfation (Okuda et al., 2014). Suppression of

OASIS and C6ST1 may attenuate sulfation and inhibition of CSPGs. Also, knockdown of chondroitin polymerizing factor, a major synthetic enzyme for CSPG GAGs, with an siRNA, may reduce generation of GAGs and CSPG suppression (Laabs et al., 2007).

Treatment with Taxol, a mitotic inhibitor clinically used for cancer chemotherapy, decreased scarring and promoted 5-HT axon growth and functional recovery after SCI by suppressing transforming growth factor- β signaling (Hellal et al., 2011). Treatment with interferon gamma (IFN γ), a dimerized soluble cytokine, inhibited neurocan production by activated astrocytes in vitro and enhanced the number of 5-HT fibers and myelinated axons in contused spinal cord probably by decreasing neurocan accumulation and upregulating glial cell-derived neurotrophic factor and insulin-like growth factor-1 (Fujiyoshi et al., 2010). Expression of R-Ras GTPase, an upstream positive regulator of PI3K signaling, promoted axon extension and growth cone elaboration on CSPGs and permissive substrates (Silver et al., 2014), suggesting that activation of R-Ras-PI3K signaling surmounts CSPG inhibition. In spite of controversy, NG2 appears inhibitory and its blockade (such as with antibodies) may promote axon growth and recovery after CNS injury (Brown et al., 2012; Tan et al., 2006). Simultaneous interruption of multiple components of PNNs, such as deletion of four PNN components (brevican, neurocan, tenascin-C and tenascin-R) using quadruple knockout mouse (Geissler et al., 2013), may further overcome scar-sourced inhibition.

7. Conclusions

Astrogliosis is able to minimize and repair the initial damage after CNS injuries, but it produces high levels of inhibitory components (particularly CSPGs) and forms chemical and physical barriers to axon elongation. Although CSPGs may act as steric inhibitors of the ECM and cell adhesion molecule receptors (such as laminin and integrins), CSPGs have at least two PTP receptors and may also bind NgRs at the sites remote from the binding domains for myelin-associated inhibitors. Of all the inhibitors present in the CNS, CSPGs are particularly vicious and form a formidable barrier to axon regeneration. Identification of CSPG receptors is an important advance for better understanding the scar-mediated suppression and for developing novel therapies, but many issues remain unknown regarding scar-mediated suppression. How much of CSPG inhibition is accounted for by binding receptors vs. steric hindrance of ECM molecules? Which receptor is most critical for mediating scar inhibition? Are expression and function of CSPG receptors neuron-type dependent? Does PTP σ inhibition have any functional significance after CNS injury? Do the identified receptors completely convey inhibition by different CSPG molecules or is there other unidentified crucial receptor(s)? Do they employ distinct or redundant downstream signaling pathways to convey inhibition? Do CSPG receptors regulate functions of glial cells? Further illumination of the mechanisms by which CSPG acts is extremely important for developing therapies that maximally overcome scar-mediated suppression and promote robust regeneration after CNS injuries.

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Highlights

- As a defense response of the CNS to injury, astrogliosis suppresses neuronal regeneration.
- Chondroitin sulfate proteoglycans expressed by reactive scar contribute to regeneration failure.
- Protein tyrosine phosphatase σ and LAR are functional receptors of CSPG inhibitors.
- Overcoming scar-mediated growth suppression may promote CNS regeneration and repair.

Fig. 1. Schematic of CSPG molecules and perineuronal nets

A: All lecticans have a core protein with the globular G1 (N-terminal) and G3 (C-terminal) domains connected by a central domain to CS-GAG chains. AggreCAN, but not other lecticans, also contains G2 domain linked to G1 by an interglobular domain. G1 domain binds hyaluronan and link proteins and G3 domain binds tenascins and glycolipids through lectin-like region. Phosphacan is a secreted splice variant of transmembrane receptor-type PTP β . NG2 is a transmembrane proteoglycan and also has a soluble form after its proteolytic cleavage. **B:** CSPGs involve formation of perineuronal nets with several other extracellular matrix molecules, including hyaluronan (HA), HA receptor, tenascin R and phosphacan (PPC). The CSPG-rich pericellular matrix wraps around neurons in the CNS and contributes to various neuronal activities including axon growth. In addition, CSPGs may regulate neuronal growth by altering calcium influx into cells.

Fig. 2. Schematic of the molecular mechanisms for CSPG inhibition on neuronal growth and the downstream signaling pathways

CSPGs induce growth inhibition by binding and activating several receptor proteins, including PTP σ , LAR (A), NgR1 and NgR3. CSPGs contribute to inhibitory properties of Sema 5A by converting it from an attractive to an inhibitory cue. CSPGs may suppress axon growth by blocking function of growth-promoting molecules, such as laminin and its receptor integrins. CSPGs might also suppress neuronal growth through other unidentified transmembrane receptors. Intracellularly, interactions between CSPGs and receptors/other proteins activate RhoA-Rho kinase signaling and inactivate Akt and Erk pathways. Activation and/or inactivation of these signaling pathways mediate suppression of CSPGs through other downstream signals, including GSK-3 β and mTor. RhoA might also modulate PTEN activity and suppress neuronal growth by inactivation of mTOR signaling. Ig-like: immunoglobulin-like domains; FN-III: fibronectin Type III domains; D1: D1 domain; D2: D2 domain.