

Sugar-dependent modulation of neuronal development, regeneration, and plasticity by chondroitin sulfate proteoglycans



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ABSTRACT

Chondroitin sulfate proteoglycans (CSPGs) play important roles in the developing and mature nervous system, where they guide axons, maintain stable connections, restrict synaptic plasticity, and prevent axon regeneration following CNS injury. The chondroitin sulfate glycosaminoglycan (CS GAG) chains that decorate CSPGs are essential for their functions. Through these sugar chains, CSPGs are able to bind and regulate the activity of a diverse range of proteins. CSPGs have been found both to promote and inhibit neuronal growth. They can promote neurite outgrowth by binding to various growth factors such as midkine (MK), pleiotrophin (PTN), brain-derived neurotrophic factor (BDNF) and other neurotrophin family members. CSPGs can also inhibit neuronal growth and limit plasticity by interacting with transmembrane receptors such as protein tyrosine phosphatase σ (PTP σ), leukocyte common antigen-related (LAR) receptor protein tyrosine phosphatase, and the Nogo receptors 1 and 3 (NgR1 and NgR3). These CS–protein interactions depend on specific sulfation patterns within the CS GAG chains, and accordingly, particular CS sulfation motifs are upregulated during development, in the mature nervous system, and in response to CNS injury. Thus, spatiotemporal regulation of CS GAG biosynthesis may provide an important mechanism to control the functions of CSPGs and to modulate intracellular signaling pathways. Here, we will discuss these sulfation-dependent processes and highlight how the CS sugars on CSPGs contribute to neuronal growth, axon guidance, and plasticity in the nervous system.

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1. Introduction

Chondroitin sulfate proteoglycans (CSPGs) play critical roles in the developing central nervous system (CNS) and in response to adult CNS injury. During embryonic development, axons must elongate, navigate specific paths, and form synapses with their target neurons. To establish precise patterns of connectivity, a range of attractive or repulsive cues guide axons to their proper targets. Several families of extracellular receptors and their ligands are known to attract or repel growth cones, including netrins, ephrins, semaphorins, and slits (Bashaw and Klein, 2010; Dickson, 2002; Tessier-Lavigne and Goodman, 1996). In addition to these prototypical axon guidance molecules, increasing evidence suggests that the chondroitin sulfate (CS) sugars on CSPGs can serve as guidance cues for growth cones and contribute to the formation of neuronal boundaries in the developing CNS (Brittis et al., 1992; Carulli et al., 2005; Snow et al., 1990). CSPGs are also major components of perineuronal nets (PNNs), where they play crucial roles in the maturation of synapses and the closure of critical periods by limiting synaptic plasticity (Berardi et al., 2003; Dityatev et al., 2010; Kwok et al., 2011; Miyata and Kitagawa, 2015). In the adult CNS, CSPGs are dramatically upregulated in the glial scar around injury sites, where they

restrict synaptic and anatomical plasticity, neuronal regeneration, and repair (Bartus et al., 2012; Galtrey and Fawcett, 2007; Silver and Miller, 2004; Yiu and He, 2006). Enzymatic digestion of the CS GAG chains on CSPGs can promote axon regeneration, sprouting, and functional recovery in *in vivo* models of CNS injury, underscoring again critical roles for CS sugars. In this review, we will highlight the various functions of the CS sugars on CSPGs and how they contribute to neuronal growth, axon guidance, and plasticity in the nervous system. We will also discuss strong evidence that specific sulfated motifs within CS chains can serve as ligands for extracellular receptors, thereby enabling CSPGs to activate key signaling pathways important for neuronal development and function.

1.1. Structure of chondroitin sulfate proteoglycans

CSPGs are composed of a core protein with one or more covalently attached CS GAG chains (Galtrey and Fawcett, 2007; Kjellén and Lindahl, 1991). They are major components of the extracellular matrix (ECM), where they provide structural support and modulate neuronal activity (Busch and Silver, 2007; Galtrey and Fawcett, 2007; Kwok et al., 2011). In addition, some CSPGs are inserted into the membrane via a single membrane-spanning domain or a glycosylphosphatidylinositol (GPI) anchor, or are localized to secretory granules. The most abundant CSPGs in the CNS are members of the lectican family, which is comprised of

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aggrecan, brevican, neurocan, and versican (Yamaguchi, 2000). Lecticans contain an N-terminal G1 domain, C-terminal G3 domain, and a central region decorated with varying numbers of CS chains (ranging from 1 to > 100). Unlike other lecticans, aggrecan also contains a G2 domain following the N-terminal G1 domain (Yamaguchi, 2000). The phosphacan or receptor-type protein-tyrosine phosphatase zeta (PTP ζ /RPTP β) family, which consists of both transmembrane and soluble secreted forms, is expressed predominantly in the CNS and is found in neurons and astrocytes throughout development and adulthood (Hayashi et al., 2005; Maurel et al., 1994). The small leucine-rich proteoglycans (SLRPs) such as decorin and biglycan have N-terminal binding sites for 1 or 2 CS chains and leucine-rich repeats flanked by cysteine residues in their central domain (Hocking et al., 1998). Another prominent CSPG in the nervous system is NG2, a transmembrane proteoglycan with a CS chain attached to its large extracellular domain (Stallcup, 2002).

1.2. Structure of chondroitin sulfate sugars

Proteoglycan core proteins are decorated at certain serine residues with CS GAG chains via a tetrasaccharide linker. CS GAGs are linear polysaccharides comprised of a repeating disaccharide unit containing *N*-acetyl-D-galactosamine (GalNAc) and D-glucuronic acid (GlcA) (Gama and Hsieh-Wilson, 2005; Sugahara et al., 2003). Each chain contains up to 100 disaccharide units and undergoes extensive sulfation in the Golgi by chondroitin sulfotransferases (Kusche-Gullberg and Kjellen, 2003; Mikami and Kitagawa, 2013). The commonly occurring CS disaccharide units are characterized by the number and position of their sulfate modifications (Fig. 1A). For instance, the monosulfated CS-A and CS-C motifs contain sulfate groups at the 4-O and 6-O positions of the GalNAc residue, respectively, and are generated by the sulfotransferases chondroitin 4-O-sulfotransferase (C4ST) and chondroitin 6-O-sulfotransferase (C6ST), respectively (Fig. 1B). The disulfated CS-D unit is synthesized from the CS-C precursor via 2-O sulfation of the GlcA residue by uronyl 2-O-sulfotransferase (UST), while the CS-E unit is generated from a CS-A unit by 6-O sulfation of the GalNAc residue by the sulfotransferase *N*-acetylgalactosamine 4-sulfate 6-O-sulfotransferase (GalNAc4S-6ST). Thus, a suite of sulfotransferase enzymes works in concert to produce a structurally complex, heterogeneously sulfated polysaccharide. This non-template driven process results in diverse patterns of sulfation that allow CS GAGs to interact with a wide range of proteins, including different growth factors, cytokines, and transmembrane receptors.

1.3. CSPG receptors

Many of the diverse functions of CSPGs arise from their ability to bind a large number of protein partners. CSPGs interact with various proteins in the ECM, including fibronectin, laminin, neural cell adhesion molecule (NCAM), and neural glial cell adhesion molecule (NgCAM) (Friedlander et al., 1994; Grumet et al., 1993; Wu et al., 2005b). Through these interactions, CSPGs can block laminin-mediated integrin activation, as well as cell adhesion molecules important for promoting neuronal migration and growth (Muir et al., 1989; Tan et al., 2011; Zuo et al., 1998). CSPGs are also known to interact with a variety of growth factors, such as midkine (MK), pleiotrophin (PTN), nerve growth factor (NGF), and brain-derived neurotrophic factor (BDNF), and in some cases, can help assemble growth factor-receptor complexes (Deepa et al., 2002; Gama et al., 2006; Maeda et al., 2003; Rogers et al., 2011; Zou et al., 2003). In this way, CSPGs can modulate growth factor signaling pathways by presenting soluble factors to their cell surface receptors and/or potentially sequestering them from their cell surface receptors. It is becoming increasingly clear that CSPGs can also interact with and modulate the activity of many membrane-associated proteins, including the protein tyrosine phosphatases PTP α and leukocyte common antigen-related (LAR) and the Nogo receptors NgR1 and NgR3 (Brown et al., 2012; Dickendesher et al., 2012; Fisher et al., 2011; Shen et al., 2009).

For example, the interaction of CS and heparan sulfate (HS) GAGs with the thrombospondin repeats of semaphorin 5A (Sema5A) guides neurons in the developing diencephalon fiber tract, with each interaction resulting in different functional outcomes. While HS is required for Sema5A-mediated attraction, the interaction with CS converts Sema5A to a repulsive guidance cue, suggesting that neuronal responses to axon guidance cues can depend on their GAG binding status (Kantor et al., 2004). We will highlight additional examples below where the CS sugar chains mediate the interactions and activity of CSPGs toward their protein partners.

1.4. Regulation of the 'sulfation code'

The sulfation profiles of CS are spatially and temporally regulated in a tissue-specific and cell type-specific manner. In the nervous system, particular CS sulfation motifs are upregulated during early and postnatal development (Fernaund-Espinosa et al., 1996; Ishii and Maeda, 2008a, 2008b; Kitagawa et al., 1997; Maeda et al., 2003; Mitsunaga et al., 2006). For example, distinct immunohistochemical patterns are exhibited by monoclonal antibodies that recognize sulfated CS chains (antibodies CS-56, 2H6 and MO-225). Although these antibodies recognized a varied set of partially overlapping epitopes, each revealed distinct CS expression patterns in the brain (Sugahara and Mikami, 2007). For example the CS-56 and 2H6 epitopes were highly expressed in the postnatal day 7 (P7) mouse cortex and showed decreased expression in P12 and P20 cortical tissue (Maeda et al., 2003; Sugahara et al., 2003). In the cerebellum, CS-56 immunoreactivity was detected at P7 and P12 but was absent at P20. Interestingly, the MO-225 epitope was not observed in the cortex but showed strong expression in the cerebellum at P7, P12, and P22 (Maeda et al., 2003; Sugahara and Mikami, 2007).

Additional insights into the expression dynamics of CS motifs was obtained from high-performance liquid chromatography (HPLC) analysis of CS disaccharides following digestion of the GAG chains. The monosulfated CS-A motif was the most abundant motif in the embryonic mouse cortex and cerebellum, and its levels increased as development progressed (Ishii and Maeda, 2008a, 2008b; Mitsunaga et al., 2006). Expression of the CS-C motif was highest during embryonic development and steadily decreased through development, but it remained the second most abundant CS motif (Ishii and Maeda, 2008a, 2008b; Mitsunaga et al., 2006). Interestingly, the disulfated CS-E motif exhibited its highest expression in the embryonic mouse cortex, and its levels decreased into adulthood but remained higher in the cortex than in the cerebellum (Ishii and Maeda, 2008a, 2008b; Mitsunaga et al., 2006). In contrast, expression of the disulfated CS-D motif was highest in the cerebellum and peaked around P10 (Ishii and Maeda, 2008a, 2008b). Notably, deletion of these highly sulfated motifs by genetic knockdown of the sulfotransferases UST and GalNAc4S-6ST via *in utero* electroporation resulted in impaired migration of cortical neurons *in vivo* (Ishii and Maeda, 2008a). Electroporated neurons accumulated in the lower intermediate zone and the subventricular zone and did not migrate radially in the neocortex.

In addition to dynamic changes in CS sulfation, the expression of specific CS sulfotransferases is spatially and temporally regulated (Ishii and Maeda, 2008a, 2008b; Kitagawa et al., 1997; Maeda, 2010; Purushothaman et al., 2007; Sugahara and Mikami, 2007). Widespread mRNA expression of *C4ST* and *GalNAc4S-6ST* was observed in the cortex, hippocampus, cerebellum, striatum, and the olfactory bulb during postnatal development and into adulthood, while *UST* was preferentially expressed in the developing cerebellum (Mitsunaga et al., 2006; Purushothaman et al., 2007). In the cerebellum, expression of *GalNAc4S-6ST* shifted during postnatal development from the external to the internal granular layer (Ishii and Maeda, 2008b; Purushothaman et al., 2007). This change in sulfotransferase mRNA expression profile matched the levels of CS-E expression observed by immunostaining and coincided with the migration and maturation of granular cells (Purushothaman et al., 2007).

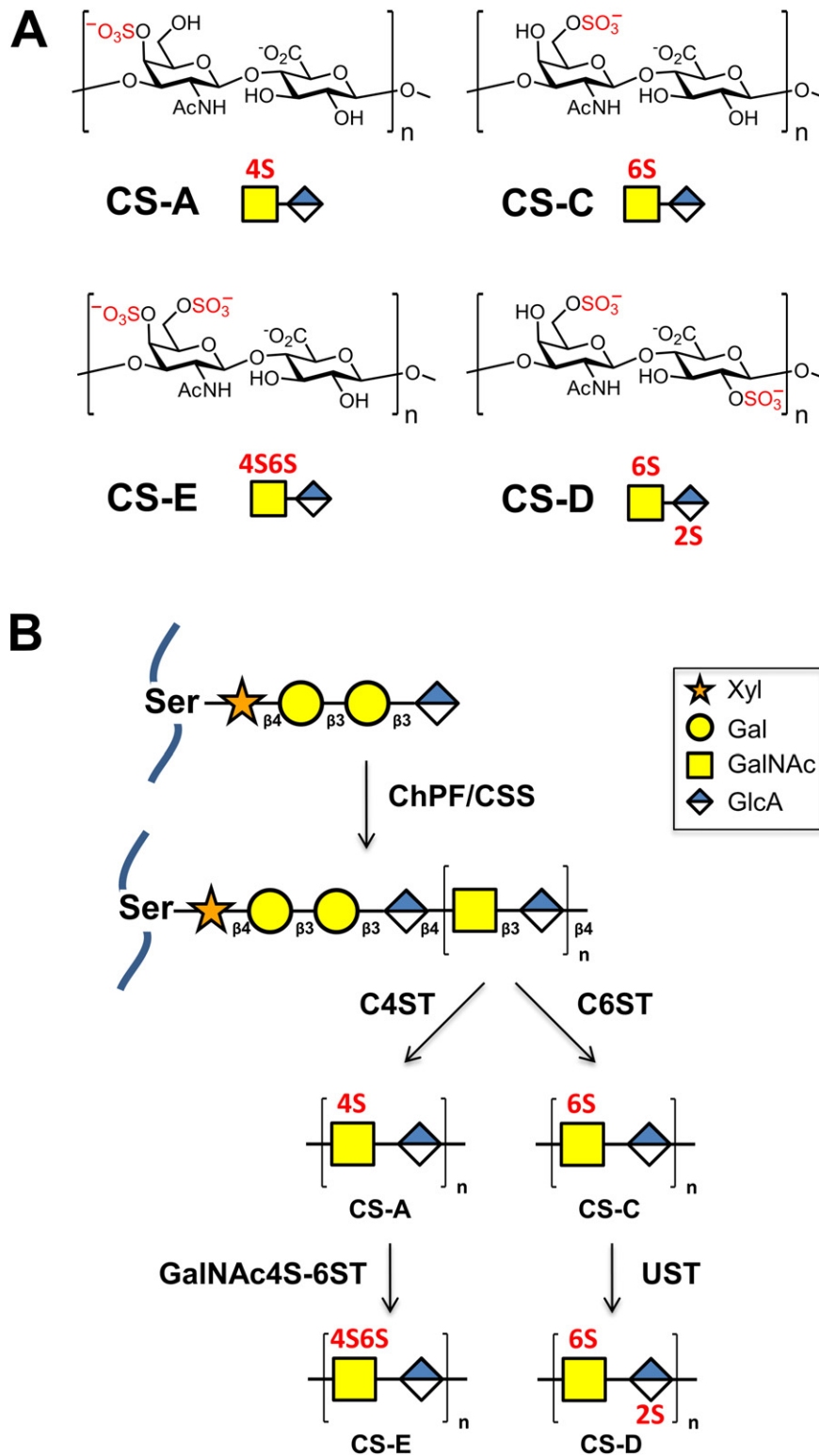


Fig. 1. (A) Common sulfation motifs of chondroitin sulfate, which consists of the repeating disaccharide *N*-acetyl-*D*-galactosamine- β (1,3)-*D*-glucuronic acid. $n = 20$ –100. CS-A and CS-C are monosulfated at the 4-*O* and 6-*O* positions of GalNAc, respectively. CS-D is sulfated at the 2-*O* position of GlcA and 6-*O* position of GalNAc. CS-E is sulfated at the 4-*O* and 6-*O* positions of GalNAc. (B) Biosynthesis of chondroitin sulfate. A core tetrasaccharide (xylose (Xyl)-galactose (Gal)-galactose (Gal)-glucuronic acid (GlcA)) is appended to serine residues of the core proteoglycan. Chain extension is performed by chondroitin sulfate synthase (CSS) and chondroitin polymerizing factor (ChPF). The polysaccharide chains are then elaborated through sulfation by C4ST to generate CS-A or C6ST to generate CS-C, followed by GalNAc4S-6ST or UST to form CS-E or CS-D, respectively.

Interestingly, studies suggest that proteoglycans possess well-defined GAG sequences. For example, early studies demonstrated that the CS chains of phosphacan purified from P20 mouse brains contained a higher abundance of the CS-D motif compared to those of phosphacan purified from P7 and P12 brains, and accordingly, they

exhibited higher binding affinity for the growth factor PTN (Maeda et al., 2003). More recently, GAG fragments of similar size and charge were isolated from the heparan sulfate proteoglycan bikunin, and mass spectrometry sequencing revealed a single, defined sequence motif (Ly et al., 2011).

Together, multiple lines of evidence indicate that the sulfation patterns on CS chains are specific and highly controlled. Spatiotemporal regulation of CS GAG biosynthesis could provide an important mechanism to modulate the interactions and functions of CSPGs and activate intracellular signaling pathways. Moreover, the concerted expression of particular sulfation motifs on different proteoglycan core proteins could provide an elegant mechanism to coordinate the activities of various CSPGs. In this review, we will discuss current hypotheses regarding CSPGs and evidence supporting an important role for the sulfated CS chains in mediating the diverse activities of CSPGs toward neurons.

2. CSPGs as stimulatory cues for neuronal growth

Although CSPGs are traditionally considered inhibitory molecules, they are sometimes expressed in growth-permissive regions, such as the neocortical subplate where thalamocortical afferent axons grow (Bicknese et al., 1994; Sheppard et al., 1991). As such, CSPGs do not always constitute a barrier to axon initiation or outgrowth and may also participate in axon extension and pathfinding. In fact, numerous studies have established the ability of CSPGs to stimulate neurite outgrowth *in vitro*, and their growth-promoting activity depends on the CS sugar chains.

2.1. Role of CS sulfation patterns

The stimulatory effects of CSPGs on cultured neurons vary with the CS sulfation pattern and neuron type. CS chains enriched in highly sulfated motifs have been shown to promote neurite outgrowth of embryonic neurons *in vitro* (Bao et al., 2005; Clement et al., 1998, 1999; Faissner et al., 1994; Garwood et al., 1999; Hikino et al., 2003; Nadanaka et al., 1998; Nandini et al., 2004). For example, the phosphacan variant DSD-1-PG contains a specific sugar epitope, DSD-1, that stimulates the outgrowth of embryonic day 18 (E18) rat hippocampal neurons and E14 mesencephalic neurons (Faissner et al., 1994; Garwood et al., 1999). The growth-promoting effects of DSD-1-PG were blocked by removal of the sugar chains or by the monoclonal antibody (mAb) 473HD, which recognizes the DSD-1 sugar epitope (Clement et al., 1998; Faissner et al., 1994; Hikino et al., 2003). Polysaccharides enriched in the disulfated CS-D sulfation motif inhibited the interaction of mAb 473HD and DSD-1-PG, suggesting that CS-D may comprise part of the DSD-1 epitope (Clement et al., 1998, 1999; Nadanaka et al., 1998). Further characterization of mAb 473HD demonstrated that it recognized the tetrasaccharide sequence A-D or D-A (Ito et al., 2005). Both CS-D and DSD-1 interacted strongly with PTN (also known as HB-GAM) and promoted neurite outgrowth, at least in part, through those interactions (Bao et al., 2005; Maeda et al., 2003). PTN induced cortical cell migration *in vitro* through the proteoglycan receptor RPTP β , and this activity was blocked by the addition of RPTP β antibodies, the tyrosine phosphatase inhibitor sodium vanadate, or exogenous CS (Maeda and Noda, 1998).

Interestingly, while CS-D-enriched polysaccharides promoted the outgrowth of dendrite-like extensions in cultured embryonic hippocampal neurons, polysaccharides enriched in the disulfated CS-E motif promoted the outgrowth of a single, axon-like extension (Hikino et al., 2003). Moreover, the activity of CS-E was not blocked by mAb 473HD, suggesting that a structurally distinct epitope from DSD-1 is responsible for the growth-promoting effects (Clement et al., 1999; Nadanaka et al., 1998). Both CS-D- and CS-E-enriched polysaccharides bound preferentially to a variety of growth factors. MK and PTN appeared to have similar affinities for DSD-1, CS-D, and CS-E but did not interact strongly with CS-A- or CS-C-enriched polysaccharides (Deepa et al., 2002; Maeda et al., 2003; Ueoka et al., 2000). Similarly, other neurotrophic factors such as NGF, BDNF, neurotrophin-3 (NT-3), and neurotrophin-4/5 (NT-4/5) showed preferential binding to CS-E compared to CS-A or CS-C polysaccharides (Gama et al., 2006; Rogers et al., 2011).

In addition to CS, GAGs such as HS and dermatan sulfate (DS) can also bind to growth factors, including MK, PTN, FGF, and BDNF, and promote neurite outgrowth (Häcker et al., 2005; Nandini et al., 2005; Shipp and Hsieh-Wilson, 2007; Sugahara and Mikami, 2007). Heparan sulfate proteoglycans (HSPGs) stimulated neurite outgrowth of cultured rat sympathetic neurons, spinal cord neurons, chick retinal neurons, and motor neurons in a GAG-dependent manner (Beller and Snow, 2014; Hantaz-Ambroise et al., 1987; Jung Kim et al., 2003; Lander et al., 1982). Hybrid CS/DS chains, which are synthesized from CS through the action of chondroitin-glucuronate 5-epimerase to convert GlcA to L-iduronic acid (IdoA) (Sugahara et al., 2003), have been shown to promote the outgrowth of embryonic hippocampal neurons (Bao et al., 2004; Hikino et al., 2003; Li et al., 2007; Nandini et al., 2005). Moreover, DS chains enriched in the disulfated DS-D and DS-E motif, but not the disulfated DS-B motif (sulfated at the 2-O position of IdoA and 4-O position of GalNAc), stimulated neurite outgrowth, highlighting the importance of the sulfate group position for the activity of GAGs (Hikino et al., 2003; Li et al., 2007). Interestingly, the DS-D and DS-E motifs also promoted the formation of an increased number of neurites per cell compared to CS-D and CS-E, respectively (Hikino et al., 2003; Nandini et al., 2005). Thus, CS and DS structures with the same sulfation patterns can exhibit different activities toward developing neurons. In the future, comparative analyses of various GAG families and their distinct functions or mechanisms will be important for understanding the physiological roles of GAGs during development and in the mature nervous system.

2.2. Pure synthetic molecules for assessing structure–function relationships

The structural complexity of CS GAGs renders it difficult to purify well-characterized, homogeneous oligosaccharides and polysaccharides from natural sources. For instance, the CS-D polysaccharide used in the literature contains only ~20% of the CS-D sulfation motif, with the CS-C motif constituting the majority of the polysaccharide (Hikino et al., 2003). Thus, from a purity and selectivity standpoint, chemically-synthesized GAG structures with defined sulfation motifs are crucial for studying the structure–function relationships of CS GAGs (Gama and Hsieh-Wilson, 2005). To determine whether the growth-promoting activity of CS-E-enriched polysaccharides (which contain only ~60% CS-E) was due to the CS-E sulfation motif, defined tetrasaccharides were chemically synthesized (Gama et al., 2006; Tully et al., 2004). Interestingly, a tetrasaccharide structure was sufficient to promote neurite outgrowth, and only a tetrasaccharide containing the CS-E motif (E-E), not the CS-A (A-A) or CS-C motifs (C-C), strongly promoted the outgrowth of embryonic hippocampal, cortical, and dopaminergic neurons (Gama et al., 2006; Sotogaku et al., 2007; Tully et al., 2004). A different CS tetrasaccharide containing the same number of sulfate groups as CS-E had no appreciable effect on neuronal growth, indicating that the precise position of the sulfate groups was important (Gama et al., 2006). The CS-E tetrasaccharide bound preferentially to specific growth factors, including BDNF and MK, and the growth-promoting activity of the tetrasaccharide was abolished by the addition of function-blocking antibodies against these growth factors or their cell surface receptors (TrkB and RPTP β / ζ , respectively) (Gama et al., 2006; Rogers et al., 2011). Together, these results demonstrate that the specific sequence of the sulfate groups along the sugar backbone, rather than electrostatics alone, modulates GAG–protein interactions and can direct important neuronal signaling events.

2.3. Mechanisms of growth promotion

Studies suggest that the CS chains on CSPGs promote neuronal growth *in vitro* by recruiting growth factors to the cell surface and facilitating interactions with their cell surface receptors (Fig. 2). CS-E polysaccharides can enhance the formation of neurotrophin–Trk complexes in a sulfation-dependent manner (Rogers et al., 2011). For

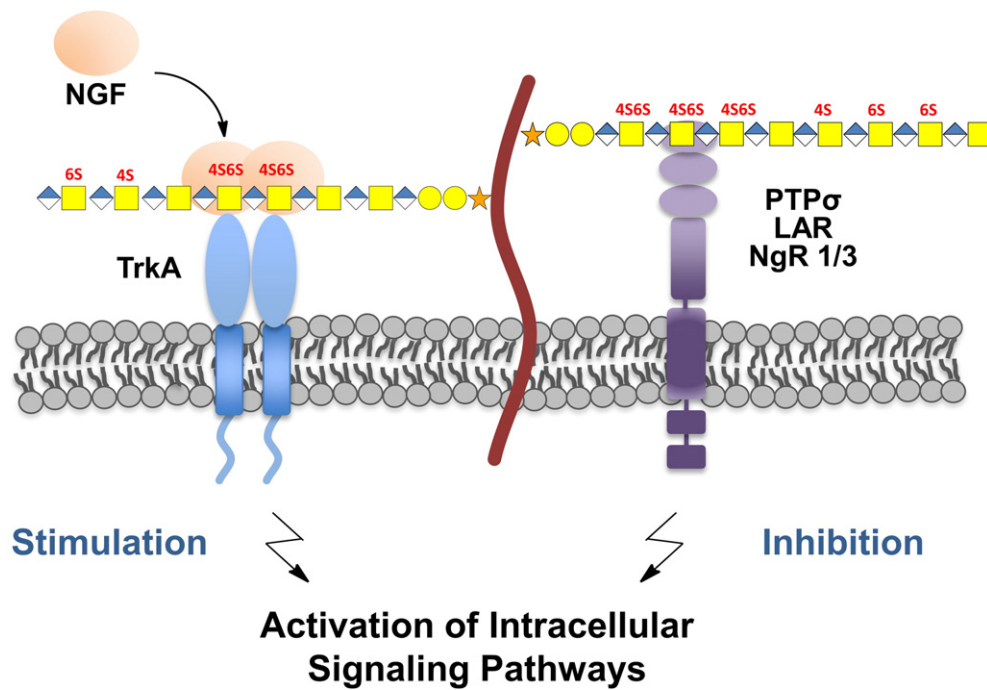


Fig. 2. Modulation of intracellular signaling pathways by chondroitin sulfate. (left) CS chains can localize soluble ligands such as growth factors to the cell surface and facilitate interactions with their cognate receptors. For example, nerve growth factor (NGF) signaling and neurite outgrowth are enhanced when CS-E is presented on the cell surface. (right) Alternatively, CS chains can interact directly with transmembrane receptors, such as PTP σ , LAR, NgR1 and NgR3, and affect intracellular signaling. For example, CS binds to lysine-rich IgG domains of PTP σ and thereby inhibits neurite outgrowth.

instance, CS-E-enriched polysaccharides presented on a substratum increased NT-4/5-mediated TrkA activation in PC12 cells, while removal of endogenous CS chains by chondroitinase ABC (ChABC) reduced TrkA activation. A complex of CS-E with neurotrophins and their cognate Trk receptors was observed on CS GAG microarrays, and the colocalization of CS-E and TrkA increased in PC12 cells upon treatment with the neurotrophin NGF. On the other hand, the addition of exogenous CS-E polysaccharides into the culture medium inhibited NT-4/5- and NGF-mediated TrkA activation, consistent with the mechanism that CS-E polysaccharides in solution can sequester neurotrophins away from the cell surface and prevent them from activating TrkA receptors. In addition to neurotrophin-Trk complexes, contactin-1 (CNTN-1) has been identified as a receptor for CS-E (Mikami et al., 2009). CNTN-1 regulates neurite outgrowth through the non-receptor-type tyrosine kinase Fyn. Phosphorylation and activation of Fyn was induced upon treatment with CS-E, but not CS-A or CS-C, polysaccharides. Antibodies against CNTN-1 inhibited CS-E-induced neurite outgrowth of embryonic hippocampal neurons. This stimulation was further inhibited when MK or BDNF antibodies were used in combination with CNTN-1 antibodies. Although it remains to be determined whether CSPGs modulate neurotrophin and CNTN-1 signaling *in vivo*, heparan sulfate proteoglycans are well established to utilize similar mechanisms for the regulation of numerous growth factors such as fibroblast growth factors, Sonic Hedgehog, and epidermal growth factor (Bernfield et al., 1999; Chan et al., 2009; Xu and Esko, 2014).

As CS GAGs can modulate growth factor activation at the cell surface, the ability to engineer cells to express specific GAG structures would provide a novel means to control cell growth pathways. Recent studies have explored the ability to promote neuronal growth through the cell surface presentation of particular sulfated GAGs (Pulsipher et al., 2014). For example, liposomal-mediated delivery of CS-E, but not CS-A or CS-C, polysaccharides to cultured cortical neurons enhanced neurite outgrowth mediated by NGF (Pulsipher et al., 2014). The presentation of CS-E at neuronal cell membranes also stimulated NGF-mediated phosphorylation of Akt. The observed sulfation-dependent results are consistent with the high affinity of NGF for the disulfated CS-E motif

(Rogers et al., 2011). In other studies, pluripotent embryonic stem cells engineered to display highly sulfated heparan sulfate underwent accelerated exit from self-renewal and differentiation into mature neuronal populations through increased activation of fibroblast growth factor/extracellular signal-regulated kinase (FGF/ERK)-mediated signaling pathways (Pulsipher et al., 2015). These studies lend strong support to the mechanism that specific sulfation motifs on GAG chains can recruit soluble factors to the cell surface and facilitate activation of their receptors (Fig. 2). Thus, controlled expression of GAG sulfation patterns at the cell surface may afford regulation of growth factor binding sites and tune intracellular signaling in a cell-type specific manner.

3. CSPGs as inhibitory cues for neuronal growth

CSPGs have been well documented to act as inhibitory cues in a variety of neuronal contexts. During development, CSPGs delineate boundaries that prevent extending axons from crossing (Brittis et al., 1992; Carulli et al., 2005; Snow et al., 1990). In the mature system, CSPGs have been shown to restrict synaptic plasticity and help stabilize existing connections (Bartus et al., 2012; Miyata and Kitagawa, 2015). Following CNS injury, CSPGs prevent axons from regenerating past the injury site to form functional connections (Bartus et al., 2012; Galtrey and Fawcett, 2007; Silver and Miller, 2004; Yiu and He, 2006). Although CSPGs were originally thought to function as non-specific, electrostatic or physical barriers to neuronal growth, it is becoming evident that the CS sugar chains on CSPGs can engage and modulate the activity of many inhibitory cell surface receptors. We will highlight recent studies that have demonstrated the importance of specific sulfation motifs in these systems.

3.1. Inhibition of neurite growth and axon elongation

While many studies have noted the stimulatory effects of CSPGs on embryonic hippocampal neurons, other studies have reported that CSPGs can inhibit the outgrowth of dorsal root ganglion (DRG), retinal ganglion cell (RGC) and cerebellar granule neurons (CGN) (Brown

et al., 2012; Dou and Levine, 1994; Garwood et al., 1999; Koprivica et al., 2005; Monnier et al., 2003; Snow et al., 1990; Snow and Letourneau, 1992; Ughrin et al., 2003). The seemingly paradoxical activity of CSPGs appears to depend on the neuronal lineage, neuronal age, and expression of specific CSPG receptors. For example, certain CSPGs exhibit both stimulatory and inhibitory effects on neurons, depending on the neuron type. Whereas DSD-1-PG promoted the outgrowth of embryonic hippocampal neurons as described above, it potently inhibited the outgrowth of neonatal DRG explants (Garwood et al., 1999). In addition to their effects on neurite outgrowth, CSPGs also form an inhibitory barrier to elongating DRG, RGC, and CGN axons *in vitro* (Brown et al., 2012; Laabs et al., 2007; Snow et al., 1990). This activity depends on the CSPG concentration: neurons grown on a step gradient of immobilized CSPGs extended their axons at a reduced rate for each successively increasing CSPG concentration (Snow and Letourneau, 1992). Interestingly, RGC neurons extended axons further than DRG neurons on these CSPG step gradients, highlighting cell type-specific responses.

Many studies have established that CSPG-mediated inhibition occurs through activation of the small GTPase RhoA and its effector protein, Rho-associated, coiled-coil containing protein kinase ROCK (Borisoff et al., 2003; Brown et al., 2012; Monnier et al., 2003). Activation of the Rho/ROCK pathway leads to phosphorylation of LIM domain kinase 2 (LIMK2), myosin light chain (MLC), and other downstream proteins that induce cytoskeletal rearrangements such as neurite retraction and growth cone collapse (Maekawa et al., 1999; Wu et al., 2005a). CSPGs are also known to activate epidermal growth factor receptor (EGFR) pathways, and blocking the kinase activity of EGFR or mitogen-activated protein kinase (MAPK) reversed the inhibition by CSPGs (Brown et al., 2012; Kaneko et al., 2007; Koprivica et al., 2005).

The CS GAG chains on CSPGs are essential for the inhibitory activity of CSPGs. Enzymatic removal of the CS chains with ChABC rendered CSPGs significantly less inhibitory toward cultured DRG, RGC, and CGN neurons in neurite outgrowth and boundary assays (Dou and Levine, 1994; Laabs et al., 2007; Ughrin et al., 2003; Wang et al., 2008). Genetic disruption of CS biosynthesis by deletion of chondroitin polymerizing factor (ChPF) also reduced the inhibitory activity of CSPGs (Laabs et al., 2007). Specifically, CSPGs isolated from ChPF-deficient astrocytes failed to repel cultured CGN axons. Notably, decreasing the sulfation levels of CSPGs using the general sulfotransferase inhibitor chlorate reduced the ability of CSPGs to inhibit neurite outgrowth of DRG neurons (Smith-Thomas et al., 1995). Although the inhibitory activity of CSPGs resides primarily in their sugar chains, some core proteins also exhibit inhibitory properties (Beller and Snow, 2014; Inatani et al., 2001; Lemons et al., 2003; Schmalfeldt et al., 2000). For instance, the proteoglycan NG2 inhibited the outgrowth of neonatal CGN and embryonic DRG neurons even after ChABC treatment (Dou and Levine, 1994; Ughrin et al., 2003). Three independent domains within the extracellular portion of NG2 each exhibited comparable inhibitory activity as the full extracellular domain of NG2 (Ughrin et al., 2003). Notably, only the activity of the central domain containing CS attachment sites was affected by enzymatic digestion with ChABC. In some cases, the interaction of CSPGs with cell surface receptors does not require the GAG chains. For example, the binding of phosphacan to N-CAM and Ng-CAM was not significantly reduced by ChABC treatment, and phosphacan lacking GAG chains still bound to neurons and inhibited neurite growth comparable to intact phosphacan (Maeda and Noda, 1996; Milev et al., 1994). Thus, the core protein, in addition to the GAG chains, can contribute to the inhibitory functions of certain CSPGs. Below, we will focus on the importance of the CS chains and specific sulfation motifs in mediating the inhibitory effects of CSPGs in the context of visual plasticity and CNS injury.

3.2. CSPGs and visual plasticity

During the critical period of development, the brain is most plastic, and experience-dependent activity shapes neuronal connections

(Berardi et al., 2003; Hensch, 2005; Morishita and Hensch, 2008). Sensory input during the critical period is required for the formation of functional neural circuits. In the mouse visual cortex, monocular deprivation during the critical period leads to a shift in the responsiveness of neurons toward the non-deprived eye (Berardi et al., 2003; Morishita and Hensch, 2008). This shift in ocular dominance (OD) is not observed if monocular deprivation is performed after the close of the critical period, which is accompanied by a marked reduction in plasticity and the formation of perineuronal net (PNN) structures (Pizzorusso et al., 2002, 2006). PNNs, which consist of CSPGs, tenascin, link-proteins and hyaluronic acid, surround the cell body and extend along the dendrites of inhibitory neurons expressing the calcium-binding protein parvalbumin (PV). They serve to restrict synaptic plasticity and stabilize the network of existing neuronal connections (Berardi et al., 2003; Dityatev et al., 2010; Kwok et al., 2011; Miyata and Kitagawa, 2015). The CSPGs in PNNs, and in particular, their CS sugar chains, are essential to the structure and function of PNNs. Digestion of the CS sugars by ChABC in the visual cortex reactivated critical period plasticity following monocular deprivation in adult mice (Pizzorusso et al., 2002, 2006). ChABC treatment also increased dendritic spine dynamics and density in the visual cortex *in vivo* and in hippocampal organotypic slices (de Vivo et al., 2013; Orlando et al., 2012; Pizzorusso et al., 2006). These effects were prevented by pretreatment with β 1-integrin blocking antibodies, suggesting that the increase in dendritic spine dynamics occurred through disruption of CSPG interactions with integrin and activation of β 1-integrin signaling pathways (Orlando et al., 2012). Interestingly, CSPG interactions with hyaluronic acid (HA) chains are also required for the formation and stabilization of PNN structures. The N-terminal and C-terminal domains of lecticans allow these CSPGs to interact with HA and tenascins, two key components of PNNs (Grumet et al., 1994; Matsumoto et al., 2003). CSPG-HA interactions are stabilized by link protein, and genetic deletion of link protein or enzymatic digestion of HA or CS resulted in the loss of PNNs (Carulli et al., 2010; Galtrey and Fawcett, 2007; Koppe et al., 1997; Pizzorusso et al., 2002).

Specific sulfation motifs on CSPGs have been shown to regulate PNN formation and critical period plasticity (Miyata and Kitagawa, 2015). The sulfation patterns of CSPGs are tightly regulated during postnatal development in the mouse visual cortex. While 6-O sulfation of CS (CS-C) decreases, 4-O sulfation (CS-A) increases as the critical period comes to a close, resulting in an increase in the 4-O to 6-O sulfate (4S/6S) ratio (Miyata et al., 2012). Transgenic mice overexpressing C6ST-1 retain a low 4S/6S ratio and develop fewer PNNs around PV neurons (Miyata et al., 2012). Their PNNs are rich in CS-C and display a diffuse structure that is unable to tightly enwrap thalamocortical synaptic contacts. Importantly, the mice also exhibit persistent cortical plasticity into adulthood. When subjected to monocular deprivation, adult mice overexpressing C6ST-1 show ocular dominance plasticity similar to juvenile wild-type mice. Thus, the change from low to high 4S/6S sulfation ratio on CSPGs coincides with the close of the critical period when plasticity is restricted, and reducing this ratio can modulate PNN structure and enhance cortical plasticity.

CSPGs are believed to regulate plasticity in a sulfation-dependent manner by localizing plasticity-restricting factors to PNNs (Miyata and Kitagawa, 2015; Sugiyama et al., 2009). The transcription factor orthodenticle homeobox 2 (Otx2) is a key regulator of visual cortex plasticity (Spatazza et al., 2013; Sugiyama et al., 2008). The localization of Otx2 to PV neurons occurs through binding of Otx2 to sulfated CS GAG chains. In transgenic mice overexpressing C6ST-1, Otx2 was not incorporated in PV neurons surrounded by PNNs enriched in CS-C (Miyata et al., 2012). Otx2 fragments interact preferentially with highly sulfated CS-D- and CS-E-enriched polysaccharides (Beurdeley et al., 2012). The localization of Otx2 to PV neurons was disrupted by blocking Otx2-CS interactions through intracortical infusion of a CS-E hexasaccharide or an arginine-lysine rich N-terminal Otx2 fragment that interacts with CS-D and CS-E polysaccharides (Beurdeley et al., 2012; Despras et al.,

2013). Infusion of this N-terminal Otx2 fragment, but not an alanine-containing mutant, also reopened critical period plasticity in adult mice (Beurdeley et al., 2012). Thus, the accumulation of Otx2 within PV neurons is sulfation pattern-dependent and appears to be mediated by highly sulfated CS sequences. Interestingly, the CS-E motif was also recently shown to interact with semaphorin 3A (Sema3A), a chemorepulsive guidance protein (Dick et al., 2013). Sema3A is enriched in PNNs, and ChABC treatment disrupted the localization of Sema3A to PNNs (Vo et al., 2013). Notably, the abundance of Sema3A was reduced in PNNs during periods of enhanced synaptic remodeling in regions undergoing structural reorganization (Carulli et al., 2013). These examples suggest important roles for CS sugars in the regulation of synaptic plasticity and highlight the potential to modulate plasticity by altering the sulfation patterns of CSPGs and their sulfation-dependent interactions.

3.3. CSPGs and CNS injury

CSPGs are dramatically upregulated in the glial scar, which forms in response to CNS injury (Cregg et al., 2014; Silver and Miller, 2004; Yiu and He, 2006). Comprised of reactive astrocytes, microglia, and ECM molecules, the glial scar serves as a major barrier to regenerating axons (Cregg et al., 2014; Galtrey and Fawcett, 2007; Silver and Miller, 2004; Yiu and He, 2006). It also functions to restrict anatomical plasticity by inhibiting collateral sprouting and synaptic reorganization (Bartus et al., 2012; Galtrey and Fawcett, 2007; Silver and Miller, 2004). Within 24 h after injury, reactive astrocytes begin to synthesize and secrete CSPGs in high concentrations into the glial scar, where they persist for months (Jones et al., 2003; McKeon et al., 1999; Tang et al., 2003). One well-established strategy for overcoming the inhibition of the glial scar is localized delivery of the enzyme ChABC (Bradbury and Carter, 2011). ChABC digestion of the CS sugars on CSPGs induced axon regeneration following injury to the nigrostriatal, serotonergic, or reticulospinal axon pathways (Fouad et al., 2005; García-Alías et al., 2011; Moon et al., 2001). ChABC treatment also resulted in improved regeneration and functional recovery in several models of spinal cord injury (Alilain et al., 2011; Barritt et al., 2006; Bartus et al., 2014; Bradbury et al., 2002; García-Alías et al., 2009). For example, mice treated with ChABC after a dorsal column crush injury exhibited growth of ascending sensory projections and descending corticospinal tract axons (Barritt et al., 2006). Enhanced functional recovery of locomotor and proprioceptive behaviors was also observed. In other studies, ChABC treatment increased conduction through intact fibers in the ventrolateral funiculus following unilateral hemisection of adult rat spinal cord (Hunanyan et al., 2010). The remarkable effects of ChABC have been proposed to occur both through regeneration of corticospinal tract axons, as well as enhanced plasticity and sprouting of spared axons.

Interestingly, enzymatic digestion of keratan sulfate (KS) GAGs using keratanase II (K-II) also led to axon regeneration and improved recovery of motor and sensory function following injury (Imagama et al., 2011; Ishikawa et al., 2015). KS, another component of the glial scar, is comprised of repeating disaccharide units of galactose (Gal) and *N*-acetyl-*D*-glucosamine (GlcNAc), and it can be sulfated at the 6-*O* position of Gal and GlcNAc residues (Funderburgh, 2000; Kadomatsu and Sakamoto, 2014). KS digestion by K-II led to comparable effects on axon regeneration and sprouting as CS digestion by ChABC (Imagama et al., 2011; Ishikawa et al., 2015). The effects of K-II and ChABC on axon regeneration were not additive, as treatment with both K-II and ChABC did not result in improved axon recovery compared to either treatment alone (Imagama et al., 2011; Ishikawa et al., 2015). It is unclear whether CS and KS inhibit axon regeneration through common receptors or if KS-specific receptors also exist.

In addition to enzymatic digestion of GAGs, genetic manipulation of CS sugar chains indicates a critical role for the sugars in inhibiting axon regeneration and plasticity after CNS injury. Mice lacking the enzyme

chondroitin sulfate *N*-acetylgalactosaminyltransferase-1 (CSGalNAcT-1), which appends the first GalNAc residue to the core tetrasaccharide linker on CSPGs, synthesized less CS and exhibited reduced scar formation after a spinal cord compression injury (Takeuchi et al., 2013). The mice also displayed enhanced axon growth and a larger area encompassing serotonin-positive (5HT(+)) terminals beyond the lesion site. Notably, CSGalNAcT-1 knockout mice showed improved motor function and more complete, faster recovery in motor function assays compared to mice treated with ChABC. Likewise, genetic deletion of *N*-acetylglucosamine 6-*O*-sulfotransferase-1, an enzyme required for KS chain elongation, resulted in reduction of glial scar formation and promoted axonal regrowth of the corticospinal tract (Ito et al., 2010; Zhang et al., 2006).

Given the importance of the GAG sugars, understanding the functions of different sulfation patterns in the glial scar and characterizing their mechanisms of action may provide new therapeutic strategies for promoting neuronal repair. Several studies indicate that particular CS sulfation patterns are upregulated in response to CNS injury. In addition to enhanced expression of CSPGs, the sulfotransferases C4ST, C6ST, and GalNAc4S-6ST are upregulated at the injury site following cortical lesions in mice and rats (Karumbaiah et al., 2011; Lin et al., 2011; Properzi et al., 2005). Consistent with these observations, disaccharide analysis of CS polysaccharides isolated from injured tissue showed increased levels of the CS-A, CS-C, and CS-E motifs (Gilbert et al., 2005; Wang et al., 2008). For instance, a large increase in total CS and CS-A expression was observed one day after dorsal hemisection injury in mice (Wang et al., 2008). Others studies revealed an increase in CS-C and CS-E levels, but a decrease in CS-A levels, one week and one month after cortical lesion in rats (Properzi et al., 2005). Increased CS-E expression was also observed by immunohistochemistry 2 weeks after an optic nerve crush injury, as well as 24 h after cortical stab and dorsal spinal cord injuries in mice (Brown et al., 2012; Unpublished results). Comparison of these results is confounded by differences in the methods of CS analysis, the timing post injury, and the type of injury model. Nonetheless, these studies all observe an increase in the total amount of CS and, increased CS-E expression is consistently observed. Systematic investigations into the CS patterns in different injury models and at various times post injury are required for a better understanding of the temporal dynamics of CS sulfation following CNS injury.

Studies suggest that specific sulfation motifs within long CS polysaccharide chains enable CSPGs to interact with inhibitory receptors, possibly modulating the clustering and/or activation of those receptors (Fig. 2). Until recently, CSPGs were widely believed to inhibit axon growth through relatively non-specific mechanisms, such as steric blockage of the extracellular space, arrays of negatively charged sulfate, or steric hindrance of adhesive matrix molecules (Gilbert et al., 2005; McKeon et al., 1995; Olson, 2002). However, recent studies suggest that CSPGs can interact directly with cell surface receptors expressed on injured axons and thereby activate growth-inhibitory signaling pathways (Sharma et al., 2012). Several important CSPG receptors have been identified, including the protein tyrosine phosphatases PTP σ and LAR and the Nogo receptors NgR1 and NgR3 (Brown et al., 2012; Coles et al., 2011; Fisher et al., 2011; Shen et al., 2009). DRG neurons from PTP σ -deficient mice crossed CSPG-rich boundaries and exhibited improved neurite outgrowth in response to CSPGs *in vitro* (Brown et al., 2012; Coles et al., 2011; Shen et al., 2009). Axons from PTP σ -deficient mice also extended further from the lesion site following a dorsal column crush injury. In a similar manner, modulating the activity of PTP σ using a peptide mimetic blocked CSPG-mediated inhibition and allowed DRG neurons to cross CSPG-rich barriers (Lang et al., 2015). Systemic treatment with this peptide following contusion spinal cord injury resulted in axon regeneration and recovery of locomotor function. It's worth noting that PTP σ also binds to HS and this interaction promotes neurite outgrowth of cultured DRG neurons (Coles et al., 2011). Like Sema5A, PTP σ is another example where the neuronal response is

dependent on its GAG binding status. Similarly, DRG and CGN neurons from *LAR*-deficient mice exhibited increased neurite outgrowth when grown on a CSPG substratum compared to neurons from wild-type mice (Fisher et al., 2011). As with *PTP α* , blocking the activity of *LAR* using a peptide reversed the CSPG-mediated inhibition of neurite outgrowth *in vitro* and promoted axon growth and improved locomotor recovery *in vivo* following dorsal transection in mice. Likewise, genetic deletion of *NgR1* and *NgR3* resulted in improved neurite outgrowth of CGN neurons grown on a CSPG substratum and enhanced axon regeneration in mice following an optic nerve crush injury (Dickendesher et al., 2012). However, complete recovery was not observed in either *PTP α ^{-/-}* or *NgR1^{-/-}*; *NgR2^{-/-}*; *NgR3^{-/-}* triple knockout mice, and *NgR1^{-/-}*; *NgR3^{-/-}*; *PTP α ^{-/-}* triple knockout mice displayed increased optic nerve regeneration compared to knockout of *NgR1*, *NgR3*, or *PTP α* alone, suggesting that the inhibitory effects of CSPGs in the glial scar are mediated through multiple different receptors.

Importantly, the disulfated CS-E sulfation motif is critical for engaging these inhibitory CSPG receptors. *PTP α* bound selectively to CS-E, but not to CS-A or CS-C, polysaccharides through its lysine-rich IgG domain (Brown et al., 2012). Moreover, the ability of CS-E polysaccharides to inhibit the outgrowth of DRG neurons was significantly attenuated in neurons from *PTP α* -deficient mice (Brown et al., 2012). Interestingly, however, residual inhibition by CS-E (~22%) remained in *PTP α* -deficient neurons, consistent with the idea that multiple different receptors interact with CSPGs to inhibit neuronal growth. *NgR1* and *NgR3* also interacted preferentially with CS chains containing the CS-E or CS-D motifs, but not those enriched in CS-A or CS-C, suggesting that these receptors also engage the CS-E structure on CSPGs (Dickendesher et al., 2012).

Additional evidence indicates that the CS-E sulfation motif is critical for the inhibitory activity of CSPGs. The CS-E motif has been shown to exert strong inhibitory effects on sensory neurons both *in vitro* and *in vivo* (Brown et al., 2012; Gilbert et al., 2005; Karumbaiah et al., 2011; Shimbo et al., 2013). Studies using CS-E-enriched polysaccharides and pure synthetic polymers containing CS-E showed that the CS-E motif was sufficient to inhibit CGN and DRG neurons in neurite outgrowth, growth cone collapse, and boundary assays (Brown et al., 2012; Gilbert et al., 2005; Shimbo et al., 2013). In contrast, CS-A- and CS-C-enriched polysaccharides and synthetic polymers had no appreciable inhibitory effects, even when used at 10-fold higher concentrations. Notably, CSPGs isolated from mice lacking *GalNAc4S-6ST*, the sulfotransferase that produces CS-E, showed significantly less inhibition of DRG neurite outgrowth (Brown et al., 2012). Similar effects were observed upon knockdown of *C4ST* or *GalNAc4S-6ST* in astrocytes. CSPGs isolated from these astrocytes lacked either CS-A and CS-E or CS-E alone, respectively, and were less inhibitory toward CGN and cortical neurons in neurite outgrowth and boundary assays (Karumbaiah et al., 2011; Wang et al., 2008). In contrast, reduced inhibition by CSPGs was not observed when *G6ST-1*, which produces CS-C, was knocked down (Wang et al., 2008). Importantly, blocking the CS-E, but not the CS-A, motif using a specific monoclonal antibody rescued the CSPG-mediated inhibition of DRG neurite outgrowth *in vitro* and promoted axon regeneration *in vivo* following an optic nerve crush injury (Brown et al., 2012). Interestingly, the CS-E-blocking antibody promoted regeneration in mice to a similar extent as complete digestion of CS chains using ChABC, suggesting that CS-E represents the major inhibitory determinant on CSPGs. The number of regenerating axons and the distance of regeneration was further enhanced by combining the CS-E antibody treatment with a small-molecule cyclic AMP analog to activate intrinsic neuronal growth pathways. Given that CSPGs interact with several different receptors, blocking the CS-E motif may be an effective means to target multiple inhibitory receptors simultaneously.

4. Summary and conclusions

CSPGs are key components of the developing and mature nervous system, where they guide developing axons, restrict synaptic plasticity,

and prevent axon regeneration following CNS injury. The functions of CSPGs are mediated largely by the CS sugar chains that decorate them and the ability of these sugars to engage various protein receptors in a sulfation-dependent manner. The different sulfation patterns on CS chains are tightly regulated, and the spatiotemporal expression of sulfation motifs offers a means to modulate a diverse range of neuronal processes in a cell type- and tissue-specific manner. Highly sulfated motifs, such as CS-E and CS-D, are important for modulating the functions of CSPGs and their interactions with stimulatory growth factors and inhibitory cell surface receptors. Further studies of the spatiotemporal expression of CS sulfation patterns and the identification of their interacting protein partners will provide new insights into many important neural processes. This understanding, coupled with the development of methods to disrupt CS sugars and specific sulfation motifs, such as blocking antibodies or pharmacological agents, should provide novel approaches for promoting neuronal growth, regeneration, and CNS plasticity.

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