Published in final edited form as:

Trends Neurosci. 2018 January ; 41(1): 47–61. doi:10.1016/j.tins.2017.10.005.

Flexible Roles for Proteoglycan Sulfation and Receptor Signaling

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Abstract

Proteoglycans in the extracellular matrix play vital roles in axon growth and navigation, plasticity, and regeneration of injured neurons. Different classes of proteoglycans may support or inhibit cell growth, and their functions are determined in part by highly specific structural features. Amongst these, the pattern of sulfation on the glycosaminoglycan sugar chains is a paramount determinant of a diverse and flexible set of outcomes. Recent studies of proteoglycan sulfation illustrate the challenges of attributing biological actions to specific sulfation patterns, and suggest ways in which highly similar molecules may exert opposing effects on neurons. The receptors for proteoglycans, which have yet to be fully characterized, display a similarly nuanced spectrum of effects. Different classes of proteoglycans function via overlapping families of receptors and signaling pathways. This enables them to control axon growth and guidance with remarkable specificity, but it poses challenges for determining the precise binding interactions and downstream effects of different proteoglycans and their assorted sulfated epitopes. This review examines existing and emerging evidence for the roles of proteoglycan sulfation and receptor interactions in determining how these complex molecules influence neuronal development, growth, and function.

Keywords

proteoglycan; sulfation; chondroitin sulfate; extracellular matrix; axon guidance

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Proteoglycans exhibit diverse sulfation patterns and activate overlapping receptor/signaling pathways

Proteoglycans (PGs) are essential components of the extracellular matrix (ECM), acting as critical mediators of stem cell differentiation, axonal pathfinding, neural plasticity, and regeneration of injured axons. Proteoglycans consist of a protein core decorated by one or more glycosaminoglycan (GAG) chains (see Glossary). Many of the effects attributed to proteoglycans are abrogated by eliminating these GAG chains, and emerging evidence shows that modifying the pattern of sulfation on GAG chains has equivalent effects. The role of sulfation in proteoglycan function has drawn increased interest in recent years, with data showing that minor modifications to GAG chain sulfation produce substantial changes in proteoglycan actions. These results have provoked new investigations into the relationship between proteoglycan structure and function. The binding partners for GAG chains remain largely uncharacterized, and different classes of proteoglycans appear to function through overlapping signaling pathways with diverse outcomes. Identifying the receptors that interact with proteoglycans, and developing a more sophisticated understanding of the signaling pathways they activate, is thus a major priority. Recent advances in glycobiology, biochemistry and molecular biology have provided substantial insights into these two key questions: 1) How does sulfation influence proteoglycan function, and 2) What are the receptors and signaling pathways activated by GAG chains?

Knowledge of the structure and synthesis of proteoglycans is essential to understanding their function (Figure 1). Sulfation of GAG chains is one of the final steps in proteoglycan synthesis, and sulfation patterns can be altered by sulfotransferases and sulfatases in response to homeostatic changes or over the course of development and ageing. It is important to acknowledge that sulfation alone does not determine proteoglycan function: the structure of the core protein and composition of the GAG chains also strongly influence their behavior (Figure 1). Here, we summarize current knowledge on the roles of different classes of GAGs, with a focus on recent developments illuminating how sulfation mediates downstream effects on axon growth and guidance.

Chondroitin Sulfation Regulates Axon Growth and Guidance

Because of the paucity of genetic knockout models, our knowledge of chondroitin sulfate (CS) GAG chain function in the mammalian nervous system derives primarily from studies of the effects of GAG chains on neurite formation and neuronal polarization in culture [1]. In mammals, the disaccharide units of chondroitin are sulfated at discrete locations (Figure 2 and Table 1). Following the demonstration that GAG chains inhibit dorsal root ganglion neurites in culture [2], many groups have used in vitro assays to evaluate how GAG chains with differing sulfate composition influence neurite growth. Unfortunately, it has not been possible to achieve a consistent standard for GAG composition in such experiments. Efforts to synthesize CS GAG chains are still in their infancy, meaning that virtually all data have been collected using tissue-derived GAG chains whose composition varies depending on the source of the tissue; even GAGs from the same tissue exhibit batch-to-batch variation [3]. Therefore, results of experiments studying GAG chain sulfation yield a wide range of results

and interpretations, depending upon both the cell type used and the composition of the GAG chains.

Because of these drawbacks, the overall picture of the role of GAG sulfation gleaned from in vitro experiments is somewhat confusing. For instance, one study showed that cerebral cortical neurons were inhibited by CS-C but not CS-A [4], and another that they were inhibited by CS-E [5]. Dorsal root ganglion (DRG) neurons, on the other hand, were inhibited by CS-E [6], but not CS-A or CS-C, while another study showed that CS-C as well as DS were both inhibitory [7]. Our group has shown that CS-A, but not CS-C, is inhibitory to cerebellar granule cell neurites, and that this inhibition is dependent on 4S GAG [8]. The role of 4S GAG appears to be outsized in mediating the inhibitory actions of CS: an antibody against 4S GAG improves neurite outgrowth on aggrecan [9] and selective removal of 4-sulfation specifically at the non-reducing end of GAG chains is sufficient to reduce CS-mediated inhibition of neuron growth [10]. For retinal neurites, CS-C, -D or -E, but not CS-A, were observed to be inhibitory [11], while trigeminal neurites were inhibited by CS-A, CS-C and dermatan sulfate (DS) [12]. In contrast, hippocampal neurite outgrowth was generally promoted by CS-D and CS-E as well as several different oversulfated DS saccharides [13–16]. The Hsieh-Wilson lab has produced GAG mimetics with pure sulfation patterns. And yet, even among these purified samples, the results are inconsistent, with CS-E mimetics both inhibiting [17] and promoting [18] hippocampal neurite outgrowth.

The heterogeneous responses to CS GAG chains in culture yield several possible explanations. One is that each laboratory uses its own strategy for creating substrates, as well as its own tissue source and culture conditions: some studies compare growth on poly-amino acids with growth on CS GAGs, while others evaluate GAG actions on neurons plated on laminin or fibronectin, both of which depend on integrin receptor activation for their growth-promoting activity. Different types of neurons may express specific complements of receptors for the growth promoting substrate or for CS GAG chains, and they may also produce distinct types of ECM molecules that interact with GAGs, altering the outcome. Some of these effects are due to direct interactions with the neurons, while others, especially using the more highly-sulfated GAGs, may be through GAG chain interactions with growth factors such as pleiotrophin and contactin-1, which promote growth [19, 20], and semaphorins, which inhibit growth [21]. Furthermore, methods of measuring effects may fail to detect subtle differences: while both CS-D and CS-E each promoted the outgrowth of hippocampal neurites, there were differences in the morphology of the cells on the different GAGs [16]. The future availability of defined CS GAG chains along with more consistent experimental protocols and molecular probes for different classes of neurons may help sort out these inconsistencies.

The critical role of CS sulfation is also supported by in vivo evidence. For instance, the sulfate composition of CS GAG chains has been shown to change with age. In the cerebellum, the percentage of CS-A units rises from 50% at birth to 85% in the young adult, with a corresponding decrease in CS-C units from 35% to 5%, and O units from 9% to 3% [22]. siRNA-mediated knockdown of sulfotransferases reduced cortical neuronal migration, indicating that sulfation is essential to this developmental process [23]. Other experiments using knockout animals suggest that 6-sulfation on CS-C may promote growth [24], and that

an age-associated increase in the ratio of 4-sulfated GAG to 6-sulfated GAG in perineuronal nets may decrease synaptic plasticity [25]. This is supported by the fact that overexpression of chondroitin 6-O-sulfotransferase-1, which decreases the ratio of 4S to 6S in perineuronal nets, increases seizure susceptibility [26]. These studies emphasize the urgent need for genetic manipulation of other chondroitin sulfotransferases to illuminate their biological functions and generate a clearer picture of the role sulfation plays in development and aging.

Heparan Sulfate Proteoglycans Support Neuron Growth

Unlike chondroitin sulfate, which plays both growth-promoting and -inhibiting roles depending on its sulfation, heparan sulfate (HS) is almost uniformly supportive in its interactions with developing and mature neurons. The neurite-promoting activity of HS GAGs was first recognized in 1982 by Lander, et al. [27], who noted that sympathetic neurons extend neurites on epithelial cells and that adding heparanase inactivated this growth promotion. Following that observation, many groups confirmed the ability of heparanase to reduce the growth of other neuronal types including spinal cord [28] and DRG neurons [29]. This effect extended to sensory neurons growing on laminin [30] or fibronectin fragments [31], suggesting that HS GAG chains on neurons were acting in cis. HS staining was also demonstrated on cultured hippocampal [32], spinal cord [33], and retinal ganglion cell [34] neurons.

The role of HS GAG chains has also been investigated in vivo. Inatani, et al. [35] created a mouse with a brain-specific deletion of EXT1, an enzyme responsible for HS GAG formation. These mutant mice failed to survive beyond one day after birth, and they exhibited malformations in the caudal midbrain-cerebellum region, an abnormally small cerebral cortex, the absence of major commissural tracts, and an absence of the olfactory bulbs. In addition, they found an expansion of the domain for FGF-8, which supports a role for HS chains in maintaining local concentrations of FGF during development. A role for HS chains in the response to netrin1 was established by ablating EXT1 specifically in the dorsal spinal cord, which caused a consistent reduction of axons crossing the ventral midline, similar to *Netrin-1^{-/-}* and *Dcc^{-/-}* mice. Spinal cord explants from these embryos failed to extend axons in response to netrin-1-producing cells [36]. Deletion of EXT1 in neural retina altered intraretinal pathfinding of RGCs, again similar to the netrin1 knockout phenotype [37].

A few studies have investigated the effects of heparan sulfation on development and axonal growth. Mice with a deletion of NDST-1, which catalyzes N-sulfation, die shortly before birth [38]. All *Ndst1^{-/-}* mice displayed some patterning defects, including an absence of the anterior and hippocampal commissures, while a smaller proportion of the mice showed more severe defects, localized to the diencephalon and telencephalon. The phenotype of the severely affected *Ndst1^{-/-}* embryos strongly resembled that of chick embryos deficient in either *Shh* or *FGF-8*; further experiments demonstrated a reduction in signaling through both pathways in the knockout animals. Deletion of both *Ndst1* and *Ndst2* resulted in alteration of retinal ganglion cell axon pathfinding, similar to mouse mutants that have lost either *FGFR1* and *FGFR2* [39]. Knockout of *Hs2st* and *Hs6st1*, responsible for 2-O-sulfation and 6-O-sulfation respectively, causes axon guidance defects: RGC axons in mutants make distinct

errors at the optic chiasm [40, 41] and the corpus callosum [42]. The Slit-Robo system may be involved in these pathfinding defects: the areas in the chiasm coincide with HST and Slit expression domains, and retinal ganglion neurons from Hs6st1 KO growth cones fail to avoid Slit2-expressing cells in vitro [41]. Further evidence for the importance of HS GAG chain sulfation in Slit-Robo signaling comes from in vitro studies of chemically-desulfated heparin oligosaccharides, where different oligosaccharides differentially bound to Slit and Robo, and also differentially affected retinal growth cone collapse. However, there was no consistency between binding and biological activity [43]. Similarly, complete elimination of 6-*O*-sulfation in mice through deletion of Hs6st1 and Hs6st2 caused defective cranial nerve axon extension, while mice with a deletion of Hs2st had neuronal migration defects [44]. Heparan is also modified by 3-*O*-sulfation by several different sulfotransferases. Elimination of one them, Hs3st2, but not another (Hs3st1) altered DRG growth cone collapse to semaphorins, likely through an interaction with neuropilin-2 [45],

Unlike CS, where sulfation occurs only intracellularly, HS GAG chains are also modified extracellularly by two sulfatases, Sulf1 and Sulf2, which have 6-*O*-sulfatase activity [46]. The removal of these sulfates from HS GAG chains alters their ability to interact with proteins, and thus alters signaling. In this way, Sulf activity was found to promote Wnt [47] and GDNF [48] signaling. While both Sulfs have sulfatase activity, Sulf2, but not Sulf1, KO mice have hydrocephalus accompanied by reduced brain size, while Sulf1, but not Sulf2, KO mice have abnormal hippocampal dendritic spines and long-term potentiation (LTP), and the two genotypes differ behaviorally from wild type mice as well as from each other [49]. Other studies showed that both cerebellar and hippocampal neurons from Sulf KO mice have shorter neurites than do wildtype neurons [50]. Blocking Sulf2 activity with antibodies in culture increased DRG neurite outgrowth in response to CSPGs [51]. How these alterations in sulfation relate to signaling pathways is a matter for further investigation.

An Emerging Role for Keratan Sulfate

Compared to CS and HS, relatively little is known about keratan sulfate (KS). KS chains coexist alongside CS chains in several brain proteoglycans, such as aggrecan and phosphacan [52]. When DRG neurons were plated onto chicken CSPGs, neurite outgrowth was inhibited, and this inhibition was reduced when the substrate was treated with either chondroitinase ABC or keratinase [53]. Furthermore, keratinase treatment in vivo promoted recovery after spinal cord injury [54]. This evidence supports a role for KS chains, in addition to CS chains, in neurite inhibition.

Sulfation patterns of KS chains change over the course of development, and these changes have been associated with alterations in plasticity and learning in songbirds [55]. Other evidence supporting a role of KS sulfation comes from studies using a mouse with a deletion of GlcNAc6ST-1, which prevents KS synthesis. While these mice have no gross developmental phenotype, they show a reduction in glial scar formation, leading to better axonal growth, after both cortical stab wound [56] and spinal cord injuries [57]. These mice also have altered ocular dominance plasticity and LTP [58]. While these studies emphasize the importance of KS chains in several nervous system functions, there is limited information about their binding partners or cell biological actions.

Proteoglycans Interact with Multiple Receptors and Binding Partners

The observation that HSPGs and CSPGs, despite their similar structure, exert opposite effects on axonal growth during CNS development and after injury remains a topic of intense interest. Recent experiments have identified two major classes of receptors that bind both HSPGs and CSPGs: the type IIa Receptor protein tyrosine phosphatases (RPTP σ , RPTP δ and LAR) and the Nogo receptors (NgR1 and NgR3). The signaling pathways activated by proteoglycans have also been explored, with HSPGs and CSPGs activating closely related cascades of signaling molecules (Box 2 and Box 3). However, there is incomplete information about how the binding of proteoglycans to their targets initiates signaling. One of the foremost questions in glycobiology therefore concerns the precise mechanisms of proteoglycan-receptor interactions and the signaling processes they initiate.

Type IIa Receptor Protein Tyrosine Phosphatases (RPTPs) Bind CSPGs and HSPGs

Members of vertebrate type-IIa RPTPs include LAR, RPTP σ and RPTP δ (Figure 3). The first evidence that type IIa RPTPs function as receptors for proteoglycans was published by the Stoker group in 2002 [59], where they demonstrated the binding of the HSPGs agrin and collagen XVIII and heparin to RPTP σ using solid-phase binding assays and receptor affinity probe assays. These interactions had affinity in the nanomolar range and were dependent on the HS chains in HSPGs. They also localized a binding site to the first Ig domain of PTP σ [59]. Further functional interactions of HSPGs and RPTPs were evinced by the identification of cell surface HSPGs syndecan and the glypican Dally-like (Dlp) as *in vivo* ligands for LAR in *Drosophila*, where they influence motor axon guidance and regulate synaptic morphogenesis [60, 61]. More recently, RPTP σ was also found to bind glypican-4, acting as a co-receptor for the glypican-4/LRRTM4 complex in presynaptic neurons to maintain excitatory synapse development and function [62].

In addition to binding HSPGs, RPTP σ and LAR were also sequentially identified as functional receptors for CSPGs: RPTP σ and LAR can bind to neurocan, aggrecan or a CSPG mixture through the same cluster of four lysine residues in the first Ig domain responsible for HSPG binding. RPTP σ and LAR bind to CSPGs with an affinity in the nanomolar range. Their binding is disrupted by pre-treating CSPGs with ChABC, indicating that this interaction was dependent on CS-GAGs [63, 64]. Functional involvement of RPTP σ and LAR in mediating CSPG inhibition is further supported by the following series of studies: DRG neurons isolated from RPTP $\sigma^{-/-}$ or LAR $^{-/-}$ mice show reduced sensitivity to the inhibitory effect of either a CSPG mixture or purified neurocan; RPTP $\sigma^{-/-}$ or LAR $^{-/-}$ mice show improved axon regeneration into the lesion area surrounding by inhibitory CSPGs after SCI; and blocking function with peptides selectively binding to RPTP σ or LAR promoted axonal regeneration and functional recovery after SCI [64, 65]. These interactions are sulfation-dependent, as RPTP σ binds to CS-D, CS-E and DS, but not CS-A or CS-C [66]. Together, these studies provide compelling evidence that RPTP σ and LAR function as receptors for both CSPGs and HSPGs. A model for how these receptors mediate opposite effects of CSPGs and HSPGs has been proposed [67], but further experiments to define

specific binding sites and downstream signaling pathways have yet to be undertaken. While it has been demonstrated that HS on muscle cells can interact with LAR on neurons to modulate axonal guidance [60], the fact that both HS and its receptors RPTP σ and LAR are on the surface of neurons raises the issue of whether neuronal HS can also bind and activate these receptors in cis. Another issue is that, although CS-A is strongly inhibitory to neuronal growth [8], it does not bind to type IIA protein tyrosine phosphatases or NgRs [66]. Additionally, it remains an open question whether the third member of the family, RPTP δ , behaves similarly to RPTP σ and LAR.

NgRs Interact with CSPGs to Mediate Growth Inhibition

Before the NgRs were identified as functional receptors for CSPGs, Nogo-66 receptor 1 (NgR1) was shown to act as a functional receptor of myelin-associated axon growth inhibitors (MAIs). NgR1 is a common receptor for three structurally distinct MAIs: Nogo-A, Myelin-associated glycoprotein (MAG) and oligodendrocyte myelin glycoprotein (OMgp). NgR2 and NgR3 are homologues of NgR1 [68]. NgR1 and NgR3, but not NgR2, have since been shown to interact with specific CS-GAG chains, with similar specificity to RPTP σ ; that is, they bind at nanomolar range affinity with heparin, disulfated CS-D and CS-E, and DS, while having no interaction with CS-A and CS-C [66]. Increasing concentrations of RPTP σ compete with NgR1 for binding to CS-E, further indicating that these two classes of proteoglycan receptors interact with overlapping CS-GAG epitopes. Importantly, the GAG-binding motifs of NgR1 and NgR3 are distinct and dissociable from the Nogo-, MAG- and OMgp-binding sequences. While the binding sites for three MAIs in NgR1 are located at the LRR region, GAGs were observed to bind to a highly conserved cluster of basic amino acid residues near the juxtamembrane region of NgR1 and NgR3. This indicates that NgRs may convey inhibitory signals from MAIs and CSPGs simultaneously. More recently, NgR2 was also reported to interact with versican, acting as a local suppressor of axonal plasticity at the dermo-epidermal junction to control density of epidermal sensory fiber innervation. Interestingly, this interaction was not dependent on the CS-GAG side chains, but on the C-terminal G3 domain of the Versican core protein [69].

Given the high affinity binding of NgR1 and NgR3 with heparin detected using the solid-phase binding assay, it is likely that NgR1 and NgR3 also interact with HS-GAGs. However, whether there is a functional interaction between HSPGs and NgRs requires further elucidation. Here again, mechanistic detail for the signaling of NgRs, which are GPI-linked proteins, remains mostly uncertain.

Semaphorins Bind CSPGs and HSPGs with Opposing Downstream Effects

Semaphorins are a large family of secreted and membrane-bound proteins involved in axon guidance, defined by a conserved semaphorin (sema) domain at the N-terminal. The Class 5 Semaphorin Sema5A acts as a bifunctional guidance cue, exerting both attractive and inhibitory effects on axonal extension. This dual role appears to derive from its ability to bind both HSPGs and CSPGs [70]. Sema5A is an integral membrane protein that consists of an extracellular sema-domain followed by a cluster of seven type-1 thrombospondin repeats (TSRs) [71]. This TSR domain mediates direct interactions with HS- and CS-GAGs. HSPGs

mediate the attractive effect of Sema5A, and CSPGs shift Sema5A to a repulsive effect. How this bifunctional behavior is achieved seems to derive from specific structural elements of the binding relationship. While interaction of HSPGs with the TSR domain of Sema5A is necessary and sufficient to exert its permissive effect on axon extension, inhibition requires both CSPG binding to the TSR domain and the oligomerized sema domain. This dual behavior suggests that an actively extending growth cone may respond differently to the same guidance cue at separate locations, depending on the proteoglycan composition of the ECM. This is illustrated by the development of the fasciculus retroflexus (FR), where HSPGs expressed on the surface of the FR mediate axon attraction and CSPGs enriched in the ECM of the prosomere 2 region cause axon repulsion [70].

The Class 3 Semaphorin Sema3A is a secreted protein that causes growth cone collapse and repels extending axons. Sema3A displays strong binding to Heparin and CS-E, and to a lesser extent, CS-B and HS, with no binding for CS-A, CS-C and CS-D as determined by ELISA [21]. During development, Sema-3A is expressed in the striatum and overlaps with CS-A to inhibit cortical neuronal migration [72]. In the adult CNS, Sema3A is highly concentrated in the perineuronal nets (PNNs); further experiments demonstrated that CS-E was responsible for Sema3A binding to PNNs [21]. Neuropilin-1 has been identified as a receptor for Sema 3A [73], and it has recently been shown that Neuropilin-1 binds to 3-O-sulfated HS and modulates the response to Sema 3A [45].

LRRTM4 Interaction with HSPGs Modulates Synapse Development and Function

LRRTM4 is a postsynaptic adhesion molecule that belongs to the LRRTM family. It is a membrane protein composed of extracellular leucine-rich repeats, a single transmembrane domain, and a cytoplasmic C-terminal PDZ-binding motif. LRRTM4 can trans-synaptically interact with multiple presynaptic glypicans and syndecans through its ectodomain to promote synapse development [74, 75]. Interactions are dependent on HS GAGs. LRRTM4-Glypican4 interaction occurs in trans and mutually triggers clustering of Glypican4 at presynaptic sites and clustering of LRRTM4 at postsynaptic sites to regulate excitatory synapse development [75]. A recent study showed that presynaptic HS-bound RPTP σ forms an additional complex with postsynaptic LRRTM4 to maintain excitatory synaptic development and transmission [62]. These data indicate that HSPGs are important presynaptic organizers that modulate synapse development and function by forming complexes with various presynaptic and postsynaptic molecules. Because LRRTM4 interacts with HS-bound RPTP σ to regulate synaptic function, and considering that RPTP σ can also bind to CS-GAGs, the question of whether CSPGs play a role in modulating the LRRTM4/HSPGs/RPTP σ complex merits future investigation.

Other Binding Partners for Proteoglycans

Many growth factors (including FGF, HB-EGF, the GDNF family, and others), extracellular matrix proteins or adhesion molecules (NCAM, tenascins, laminin, fibronectin, integrins), and guidance cues (Wnts, Hedgehog family proteins, Ephrins, Robo/Slits) have also been reported to interact with proteoglycans (Box 1 and Box 2). Many of these interactions

depend upon GAG chain sulfation. Interestingly, the HSPGs syndecan-3 and glypican-1 were shown to act as cell-surface receptors for the CSPG neurocan [76]. Two neurocan binding sites for these HSPGs were identified. Interaction with neurocan's C-terminal domain was also shown to promote neurite outgrowth in vitro [76]. Given the structural complexity of both the GAGs and their core proteins, it is to be expected that additional receptors and binding partners of proteoglycans will be identified soon.

Concluding remarks

The functional diversity of proteoglycans in the nervous system is derived from the structural diversity of the molecules themselves. As our understanding of their nuanced roles expands, certain structural features have gained new importance. Emerging research shows that the patterns of sulfation on GAG chains exert outsized effects on their behavior both in vitro and in vivo (Table 2). However, the difficulty of isolating and purifying GAGs with specific sulfation patterns and the lack of a common toolkit for studying neuronal responses to proteoglycans in vitro has led to varying and sometimes contradictory observations. Only with improved methods for GAG purification and standardized selection of cell types and culture substrates will the precise roles of proteoglycan sulfation become clear. Narrow distinctions must also be applied to studies of proteoglycan receptors and binding partners. Recent evidence suggests that CSPGs and HSPGs interact with overlapping families of receptors and signal through similar downstream pathways. This overlap explains the specificity with which proteoglycans control axon growth and guidance during development: the same guidance cue can induce opposing effects on axonal growth cones depending on the composition of the extracellular matrix at discrete locations. Disentangling the receptors and signaling pathways for proteoglycans will be essential for understanding how these complex behaviors are modulated, and whether they can be controlled or modified.

Acknowledgments

This work was supported by National Natural Science Foundation of China (81601066); Program of Introducing Talents of Discipline to Universities (B14036); and the Division of Intramural Research of the National Heart, Lung, and Blood Institute, US National Institutes of Health

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Glossary

Glycosaminoglycan chain

Proteoglycans contain chains of unbranched polysaccharides composed of a repeating disaccharide unit. These chains contain high density of negative charges and are both polar and hydrophilic, attributes which contribute to their functions in the extracellular matrix, cartilage, and elsewhere. In the central nervous system, glycosaminoglycan chains have been found to influence the behavior of neurons.

Sulfation

In the context of proteoglycans, sulfation involves the covalent addition of sulfate groups to carbon atoms on the disaccharide units of the glycosaminoglycan chain. This modification is accomplished by enzymes called sulfotransferases. The addition of sulfate groups according to a discrete set of patterns enhances the functional diversity of proteoglycans, enabling them to perform multiple roles in a variety of contexts.

Trends Box

Sulfation dictates the actions of proteoglycans. Extensive evidence implies that small modifications of sulfation pattern lead to significant alterations in function.

CSPGs and HSPGs share multiple binding partners and activate overlapping signaling pathways, but often produce different outcomes, with CSPGs generally inhibiting neurite growth and HSPGs supporting it.

Proteoglycan actions may be direct, by interacting with receptors, or indirect, by serving as co-receptors for growth factors and cytokines.

Discrete patterning of proteoglycans in the extracellular matrix may provide a mechanism by which growth cones respond differently to the same molecular guidance cue. This suggests a pivotal role for proteoglycans in axon navigation that takes advantage of their overlapping signaling pathways and receptor interactions.

Outstanding Questions Box

Can chondroitin sulfate be synthesized with defined GAG composition to directly investigate the effects of specific sulfation patterns?

What explains the heterogeneous responses of different neuronal cell types to sulfated proteoglycans? Do discrete patterns of chondroitin sulfation exert consistent effects on neurons, or are these effects fundamentally dependent on cell type, substrate, and other variable experimental factors?

What are the precise proteoglycan binding sites for LAR-family members and Nogo receptors? Do CS and HS proteoglycans occupy overlapping binding sites?

What other receptors and binding partners interact with CS and HS proteoglycans?

What are the signaling pathways evoked by interactions of proteoglycans with their receptors?

How does keratan sulfate differ from chondroitin sulfate and heparan sulfate in its function?

What are the primary binding partners and cell biological effects of KS?

What are the in vivo outcomes of eliminating specific sulfations on GAG chains?

Box 1**CSPG signaling****Rho/ROCK**

The Rho GTPase family (Cdc42, Rac1, and RhoA [77]) and their downstream effector ROCK are activated by aggrecan, impeding neurite outgrowth and inducing growth cone collapse [78]. Pharmacologically suppressing ROCK enhances axon growth on an aggrecan substrates [79]. Likewise, directly inhibiting Rho reverses CSPG-mediated inhibition [80]. Inhibiting Rho GTPase family members Cdc42 and Rac1 also overcomes CSPG-dependent inhibition of axon growth [77].

PI3K-Akt-mTOR

Activation of this cell cycle regulatory pathway overcomes CSPG inhibition of axon extension [81]. The CSPG-binding receptors PTP σ and LAR share common signaling pathways, including RhoA, Akt and Erk [82]. An antagonist of the PI3K-Akt-mTOR pathway, GSK-3 β , is activated by CSPGs, and its inactivation leads to neurite growth in vitro and axon sprouting and functional recovery in vivo [83].

EGFR

Suppressing EGFR's kinase function enhances regeneration of neurons [84]. Downstream of EGFR, MAPK signaling mediates CSPG inhibition of neurite growth from cerebellar granule neurons [85]. Blocking EGFR promotes growth and migration of human neural precursor cells [86]. Survival of neural stem cells is promoted by CSPGs acting through EGFR pathways as well as JAK/STAT3 and PI3K/Akt [87].

Integrins

Young embryonic neurons can adapt to inhibitory environments, growing more readily than mature neurons across CSPG surfaces; this may be due to upregulation of integrin [88]. In hostile growth conditions, young neurons express integrin family receptors, and induced expression of alpha-integrin in adult neurons enhanced growth [89]. Aggrecan and Nogo-A both inactivate integrins. Aggrecan decreases levels of phosphorylated FAK and pSrc without directly affecting surface integrins. Activating integrins directly reverses the inhibitory effects [90]. In melanoma cells, CSPGs bind alpha-4-beta-1 integrin to inhibit cell adhesion, mediated by a CS-GAG binding site on alpha-4 integrin [91]. Neuronal precursor cells respond to cleavage of CSPGs by ChABC with enhanced proliferation, differentiation, and migration, mediated by integrin signaling [92].

Calcium

Intracellular calcium regulates growth cone dynamics during axon extension [93]. In culture, neurons encountering a CSPG substrate display a rise in intracellular calcium, dependent on influx through non-voltage-gated calcium channels [94]. However, growth cone avoidance of CSPG surfaces occurs regardless of a transient rise in intracellular calcium, suggesting that this behavior is not dependent on elevated intracellular calcium [94]. The transient calcium influx provoked by CSPGs is similar to that elicited by AMPA and kainate, and antagonizing AMPA and kainate receptors blocked CSPG-

mediated calcium influx [95]. This suggests that CSPGs activate AMPA and kainate receptors to elevate intracellular calcium.

PKC

Blocking PKC activity reduces inhibition from CSPGs, and inhibiting PKC in vivo led to enhanced axon regeneration after spinal cord injury in rats [96]. Downregulating or inhibiting PKC increased neurite crossing on nonpermissive astrocytes, suggesting that astrocyte-derived matrix molecules such as CSPGs signal through PKC to influence neurite growth [97].

Local protein synthesis

Depletion of intra-axonal RhoA synthesis enhanced growth of neurons in CSPG-rich media [98]. Increased protein translation was confirmed by an increase in phosphorylated 4E-BP1 levels [98]. Sema3A, a negative guidance cue, also stimulates local translation of RhoA mRNA in axons [99].

Cytoskeleton

ROCK pathway activation acts through downstream effectors related to cytoskeletal dynamics, including cofilin, which disassembles actin filaments [100]. Inhibition of nonmuscle myosin II causes actin and microtubule reorganization, which accelerates axon extension and enables axons to cross boundaries with inhibitory CSPG substrates [101, 102]. When actin filament formation was inhibited in DRGs, microtubule realignment upon contact with a CSPG boundary was limited and growth cone turning prevented [103]. Suppressing microtubule dynamics produced a similar effect, with limited growth cone turning at a CSPG boundary [103].

Box 2**HSPG signaling****Growth factors**

HSPGs and FGFs interact throughout development and during neuronal growth and differentiation. HS is required for FGF signaling, and binding of HSPGs to FGFs is dependent on the tissue source, with distinct and specific HS affinities for different FGFs [104]. In many cell types, neurite outgrowth is mediated by binding of FGF-2 to the HSPG agrin. The ability of FGF-2 to stimulate this outgrowth is potentiated by agrin, and inhibiting the FGF receptor abolishes the effect [105]. Agrin enhances ERK phosphorylation and both augments and sustains FGF-2 mediated c-fos phosphorylation. In cerebellar granule neurons, removal of the HS sulfatases Sulf1 and Sulf2 in mice led to reduced neurite length and cell survival, as well as reduced migration capacity in the case of Sulf1 [50]. These impairments were correlated with Sulf-specific interference with FGF-2 signaling, among other pathways. Deletion of Hs2ST reduces the interaction of FGF-2 with its receptor FgfR1 [106]. Syndecan-3 is a receptor for ECM-localized heparin-binding growth-associated molecule (HB-GAM) [107]. HB-GAM binding to HS chains of Syndecan-3 activates Src kinases and promotes hippocampal neurite outgrowth [108]. Syndecan-3 also acts as a receptor for GDNF family ligands (GFLs) including GDNF, neurturin, and artemin [109]. Binding of GFLs with HS chain triggers rapid syndecan-3 oligomerization and mediates cell spreading and neurite outgrowth. Sdc3 modulation of GDNF in the lateral hypothalamus was shown to control cocaine motivation in mice [110]. GDNF signaling was also correlated with impairments affecting neurite outgrowth and cell survival following removal of Sulf1 and Sulf2 in mouse cerebellar granule neurons [50].

Wnt/PCP

Vangl2, an effector in Wnt/PCP signaling, colocalizes with the HSPG syndecan 4 (Sdc4) and negatively regulates Sdc4 protein levels in HEK293 cells [111]. Overexpression of Vangl2 reduced Sdc4 levels while knockdown elevated them, implicating the Wnt/PCP pathway in the regulation of HSPG steady-state levels [111]. HSPG sulfation plays a key role in regulating synaptic development and neurotransmission via the bidirectional control of HS 6-O-sulfotransferase (hs6st) and HS sulfatase (sulf1). In mutant mice, hs6st knockout decreased—and sulf1 knockout increased—neurotransmission strength via differential activation of Wnt and BMP signaling pathways [112]. Genetic correction of these pathways restored normal synaptic development. Likewise, embryonic development relies on HS sulfation, as the presence of a sulfation inhibitor prevented neural tube closure in mice [111].

UNC-6/netrin

The HSPG LON-2/glypican controls axon guidance by regulating the axonal response to UNC-6/netrin [113]. This may occur via its association with UNC-40/DCC receptor-expressing cells. LON-2/glypican knockdown led to axon misguidance, and LON-2/glypican contributes both to attractive and repulsive UNC-6/netrin signaling pathways.

The HSPG core proteins syndecan (SDN-1) and glypican (LON-2) are essential for dorsal guidance of D-type motor axons, a process regulated by UNC-6/Netrin [114].

MAPK/ERK

Agrin enhances ERK phosphorylation downstream of FGF-2 [115]. Additionally, MAPK/ERK signaling influences the downstream effects of HS sulfation, which regulates optic disc and stalk morphogenesis. Disrupting HS sulfation in mice with mutations to several HS sulfotransferase genes led to developmental defects that were linked to MAPK/ERK signaling [39, 106, 116].

Trends in Neurosciences

Figure 1. Structural diversity of proteoglycans

Sugar residues are added to the protein cores by several cooperative enzymes, whose regulation remains a matter of intense investigation. The number of sugar residues added to the protein core may vary in length up to 100. Further processing occurs through families of sulfotransferase enzymes, which place sulfate groups on one of several positions on the sugars, and epimerases which convert GlcA to IdoA. The factors which regulate the activity of these enzymes are unknown. Chain extension and sulfation are not template-driven, leading to enormous diversity in combinations of chain length, epimerization and sulfation. Chondroitin Sulfate (CS) and Heparin Sulfate (HS) share a common linkage region of Xylose (Xyl)-Galactose (Gal)-Gal. Specialized enzymes then either add disaccharides of Glucuronic Acid (GlcA)/N-Acetylgalactosamine (GalNAc) for chondroitin. When some GlcA residues are epimerized to Iduronic Acid (IdoA), the chondroitin molecule is termed Dermatan sulfate.

For Heparan sulfate, another series of enzymes adds disaccharides consisting of GlcA and N-Acetylglucosamine (GlcNAc). Here again, some GlcA residues are epimerized to IdoA. Keratan sulfate is broadly classified into either KSI or KSII. KSI is N-linked to the protein, while KSII is linked to either Ser or Thr. Both are biantennary structures, with a disaccharide composition of Gal and GlcNAc, with additional sugar modifications of Fuc or Neu5Ac. The addition of sulfate groups to the GAG chains of proteoglycans enhances their functional diversity, but this sulfation does follow a discrete set of common patterns. The modal

patterns for CS include only one sulfate per disaccharide. Less common in are “over-sulfated” disaccharides containing multiple sulfate groups (Figure 2). Heparin sulfation may occur in any of several different patterns, while keratan GAG chains are only sulfated on the 6-position of either Gal or GlcNAc.

Trends in Neurosciences

Fig. 2. Mammalian chondroitin disaccharides

Chondroitin chains are comprised of disaccharides of Glucuronic Acid and N-Acetyl-Galactosamine. Each disaccharide may be unsulfated or sulfated on one or more groups as indicated in the structure. Each sulfation pattern is named in Table 1.

Trends in Neurosciences

Fig. 3. Putative mechanisms of CSPG-mediated neurite growth inhibition

Several mechanisms have been proposed for CSPG-mediated neurite growth inhibition. CSPGs may inhibit neurite growth through binding to the receptors including the type IIa RPTPs (RPTP α , LAR, RPTP δ), the Nogo receptors (NgR1 and NgR3) or other unknown receptors. CSPGs may suppress neurite growth through blocking or interfering other pathways regulating neurite growth, such as integrin pathway, semaphorin pathway, EGFR pathway or others. Several intracellular events have been reported to convey CSPG functions, including calcium, RhoA/ROCK, Akt, PKC, and MAPK signaling, which inhibit neurite growth by affecting microtubule or actin cytoskeleton organization, or by altering gene expression and protein synthesis. However, there is still lack of information pertaining to how binding of CSPGs to these receptors triggers those downstream signaling pathways. For instance, how do the CSPG receptor RPTPs, as tyrosine phosphatases, inhibit the serine threonine protein kinase Akt pathway?

Table 1

Chondroitin disaccharide nomenclature

Sulfation	Position	Unit Name
No sulfation	0S	CS-O
Chondroitin-4- <i>O</i> -sulfate	4S (R2)	CS-A
Chondroitin-6- <i>O</i> -sulfate	6S (R3)	CS-C
Chondroitin-2,4- <i>O</i> -sulfate	2S, 4S (R1, R2)	CS-B
Chondroitin-2,6- <i>O</i> -sulfate	2S, 6S (R1, R3)	CS-D
Chondroitin-4,6- <i>O</i> -sulfate	4S, 6S (R2, R3)	CS-E
Chondroitin-2,4,6- <i>O</i> -sulfate	2S, 4S, 6S (R1, R2, R3)	CS-T

Dermatan sulfate disaccharides have the glucuronic acid epimerized to iduronic acid. Dermatan-4-*O*-sulfate is sometimes also termed CS-B [117], or alternately iA [118].

Table 2

Summary of nervous system effects of proteoglycan sulfations

Sulfation Pattern	Biological Action	Refs
Chondroitin-4S	Inhibition of neurite outgrowth	[8]
Chondroitin-6S	Promotion of nigrostriatal axon regeneration	[24]
	Increased percentage in PNNs increases synaptic plasticity	[119]
Chondroitin-4,6S	Binding of heparin-binding growth factors	[120]
	Promotion of hippocampal neurite outgrowth	[14]
	Promotion of neurite outgrowth through pleiotrophin	[121]
	Inhibition of DRG neurite outgrowth	[122]
	Promotion of neurite outgrowth through contactin-1	[20]
	Reduction of excitatory amino acid neurotoxicity	[123]
	Repulsion of retinal growth cones	[11]
	Binds Sema-3A in perineuronal nets	[21]
	Growth cone collapse of DRG neurons	[6]
Chondroitin-2,6S	Promotion of hippocampal neurite outgrowth	[13, 124]
Chondroitin-2,4,6S	Binding to midline	[125]
Dermatan-4,6S	Promotion of hippocampal neurite outgrowth	[16]
	Binding of heparin-binding growth factors	[126, 127]
Keratan-6S	Inhibition of neural regeneration after injury	[57]
Heparin-2S	Binding of GDNF	[128]
	Retinal ganglion pathfinding at chiasm through slit	[41]
	Facial neuronal migration through FGF modulation	[44]
	Reduced cortical neuroblast proliferation and migration	[129]
Heparin-3S	Modulation of DRG growth cone response to semaphorins	[45]
Heparin-6S	Muscle development in zebrafish	[130]
	Retinal ganglion pathfinding at chiasm through slit	[41]
	Cranial nerve extension	[44]
Heparin-NS	Brain development through modulation of Shh binding	[38]