Part II—Chapter 5: The Biological Activity of Chondroitin Sulfate Glycosaminoglycans*

General functions of glycosaminoglycans

Proteoglycans are a diverse class of proteins that carry long chains of carbohydrate polymers termed glycosaminoglycans.[1, 2] Glycosaminoglycans (GAGs) are chains of repeating disaccharide units that show tremendous structural diversity with complex patterns of deacetylation, sulfation, length, and epimerization.[3, 4] The GAG chains are covalently bound to proteins via the hydroxyl group of specific serine residues found in the protein core.[5, 6] Proteoglycans are found in the extracellular matrix of all tissues, including cartilage, basement membranes, and connective tissue, as well as on the surface of most cells. The diversity seen among the different proteoglycan families arises from the variety of protein cores available as well as from variations in the length and type of attached GAG chains. Proteoglycans found in the brain are expressed under strict control throughout nervous system development, and they act as regulators of axonal pathfinding, cell migration, and synaptogenesis.[1, 7 -- 9]

Proteoglycans act as scaffold structures constructed to interact with other proteins through noncovalent binding to their GAG chains. In the brain, a variety of proteoglycan families are involved in binding growth factors, cell adhesion molecules, enzymes, and enzyme inhibitors.[1] Both the syndecan and glypican proteoglycan families bind to the neural cell adhesion molecule (NCAM), slit-1 and slit-2, which are involved in the development of midline glia and axon pathways, different members of the fibroblast

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growth factor (FGF) family, and members of the Hedgehog families.[2] These interactions between the syndecan and glypican proteoglycans with their ligands are established through the GAG bound to the protein core.

GAG chains are a family of sulfated polysaccharides involved in diverse biological processes such as neuronal development, tumor growth and metastasis, viral invasion, and spinal cord injury.[10 -- 12] For instance, GAGs modulate key signaling pathways essential for proper cell growth and angiogenesis.[11, 12] They are also important for axon pathfinding in the developing brain and have been linked to the pathology of Alzheimer's disease.[13, 14] Potentially, GAGs and their associated protein cores can recruit protein ligands to the cell surface and mediate the binding of ligands to their corresponding receptors (Figure 5.1).[15, 16] The remarkable ability of GAGs to regulate various processes is only beginning to be understood at a molecular level. Increasing evidence suggests that GAGs encode information in the form of a 'sulfation code.' Namely, discrete sulfation motifs along the carbohydrate backbone carry instructions to direct proteins and regulate complex processes such as neuronal wiring. Deciphering this code and the mechanisms by which it coordinates biological events is critical for understanding diverse aspects of biology and may reveal new therapeutic opportunities.

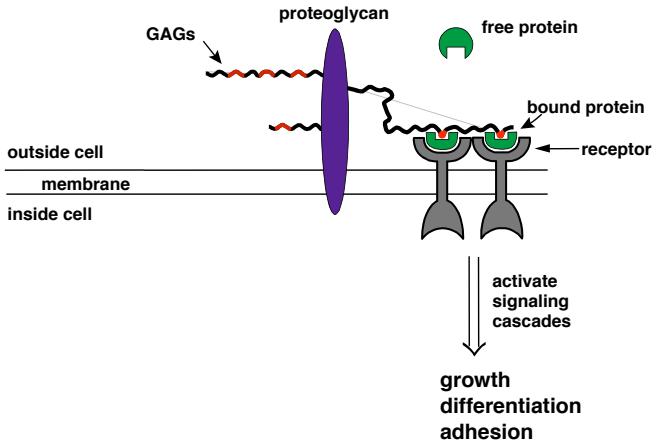

Figure 5.1. One proposed mechanism of GAG function at the cell

Structural diversity of glycosaminoglycans

GAGs are composed of repeating disaccharide subunits that are assembled into linear polysaccharide chains (Figure 5.2). These polysaccharides are often covalently attached to proteins (proteoglycans) at the cell surface or in the extracellular matrix. There are several major classes of GAGs, including heparan sulfate/heparin, chondroitin sulfate, dermatan sulfate, and keratan sulfate, which differ in their core disaccharide subunit. Heparan sulfate (HS) and heparin contain D-glucosamine (GlcN) and either D-glucuronic acid (GlcA) or L-iduronic acid (IdoA) subunits joined via α(1,4) and β(1,4) linkages. Chondroitin sulfate (CS) has *N*-acetylgalactosamine (GalNAc) and GlcA subunits and alternating β(1,3) and β(1,4) linkages.

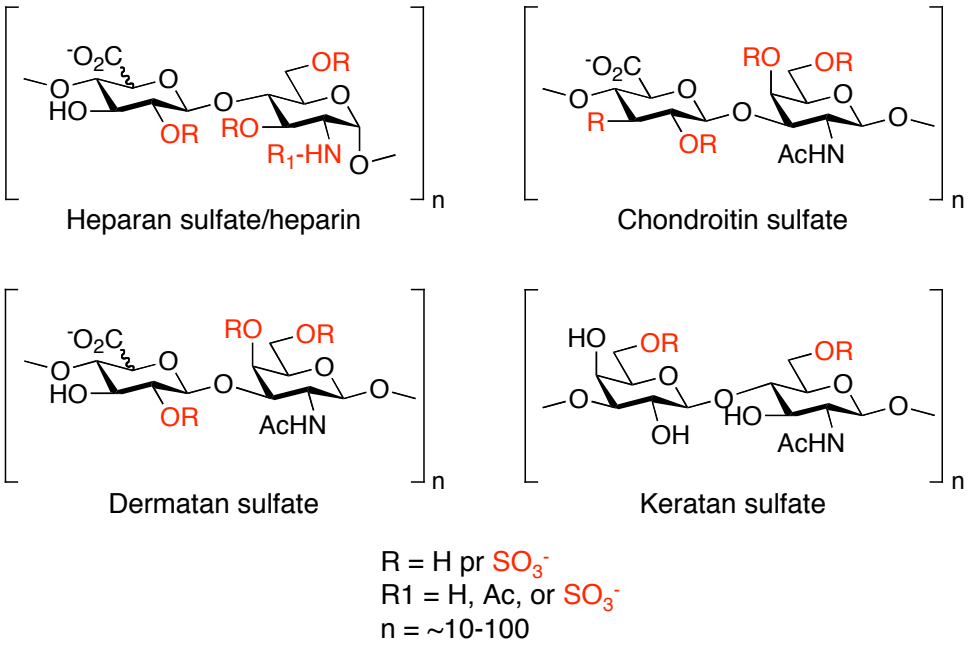

Figure 5.2. Structures of representative classes of GAGs

The biosynthesis of HS and CS occurs in the Golgi apparatus and starts with the generation of the tetrasaccharide linkage region, GlcA-β(1,2)-galactose-β(1,2)-galactose β(1,4)-xylose β-1-*O*-Ser (Figure 5.3). Xylose is first linked to select proteoglycan core protein serines, followed by the addition of two galactose residues and a GlcA moiety.[17, 18] The next residue added determines whether the GAG will be either HS/heparin or CS. Addition of *N*-acetylglucosamine (GlcNAc) commits the biosynthesis to HS/heparin while addition of *N*-acetylgalactosamine (GalNAc) gives rise to CS. The HS/heparin chain is elongated by the EXT1 and 2 polymerases, which add alternating GlcA and GlcNAc residues joined by alternating α(1,4) and β(1,4) linkages.[18] The CS chain, elongated by the CS polymerases, consists of alternating units of GlcA and GalNAc linked through β(1,3) and β(1,4) bonds.[19, 20]

Diverse sulfation patterns are generated *in vivo* through extensive modification of the growing GAG chains (polysaccharides).[10] The polysaccharides are modified in the Golgi apparatus by sulfotransferases, deacetylases, and epimerases.[2] The precise mechanisms for generating such diverse sulfation patterns is not known. However, it is thought that the enzymes may form complexes in the Golgi apparatus and influence each others' activity. The sulfotransferases add sulfate groups from 3' phosphoadenosine-5' phosphosulfate (PAPS). There have been fifteen HS/heparin sulfotransferases identified from mice and humans and the first sulfotransferase to modify HS/heparin chains is *N*-deacetylase-*N*-sulfotransferase (NDST), a bifunctional enzyme which deacetylates and subsequently sulfates the C2 amine of GlcNAc.[4, 21, 22] Following *N*-sulfation, some of the GlcA residues in the HS/heparin chains can be converted to IdoA by the C-5 epimerase. Sulfation can then occur at the C2 hydroxyl of IdoA and GlcA, the C3 hydroxyl of GlcN,

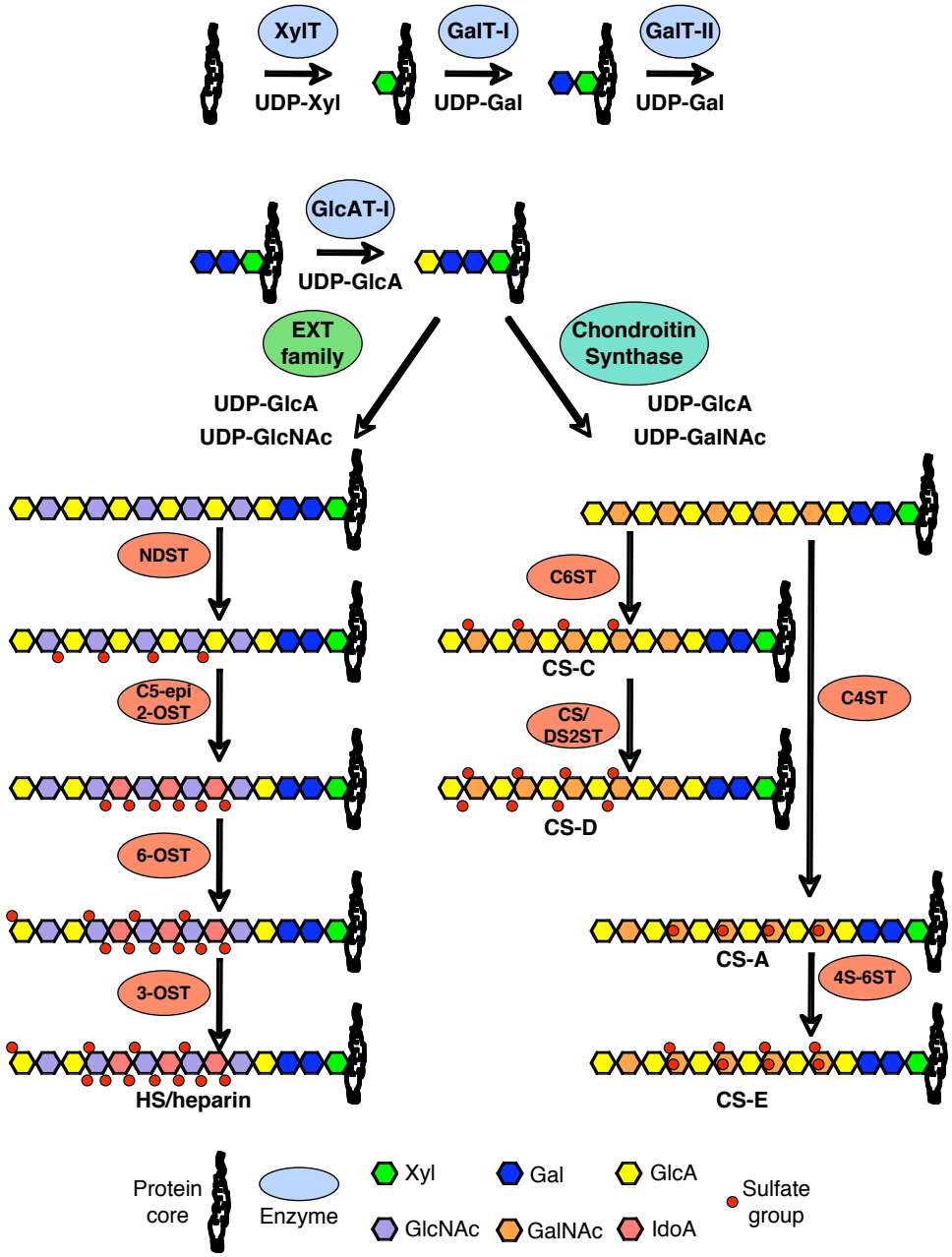

Figure 5.3. Biosynthesis of HS/heparin and CS GAGs takes place in the Golgi apparatus.

and the C6 hydroxyl of GlcN.[23 -- 29] Although HS and heparin are structurally related, HS has greater overall chemical complexity, exhibiting more varied sulfation patterns, lower IdoA content, and longer polysaccharide chains.[3, 10] Furthermore, HS is ubiquitously

expressed *in vivo* and has a broader range of physiological targets than heparin, which is primarily localized to specialized granule cells.

Diverse sulfation motifs are also found on CS, with sulfation occurring at each of the free hydroxyls.[30] The CS sulfotransferases can be categorized into three major groups: 1) those which add sulfate groups to the C4 hydroxyl of GalNAc, 2) those adding sulfate groups to the C6 hydroxyls of GalNAc, and 3) those adding sulfate groups to the C2 hydroxyl of GlcA.[4] To date, there have been seven CS sulfotransferases identified. Sulfation of the C4 hydroxyl on GalNAc generates the CS-A pattern while sulfation of the C6 hydroxyl on GalNAc affords the CS-C pattern, the two most common motifs found *in vivo*.[31] These two motifs can be further sulfated at the C6 hydroxyl of GalNAc and the C2 hydroxyl of GlcA to generate the CS-E and CS-D pattern, respectively (Figure 5.4).

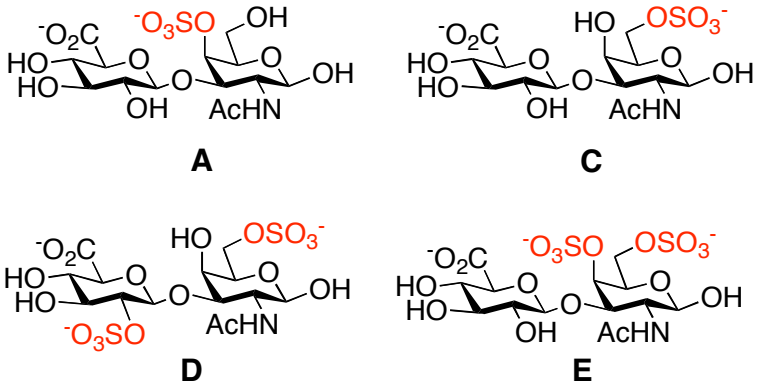

Figure 5.4. Structures of the most common CS sulfation motifs found *in vivo*

The variety of sulfotransferases found *in vivo* produces multiple sulfation motifs and patterns, generating the extraordinary amount of structural complexity observed in HS and CS GAGs. A simple tetrasaccharide of CS has the potential to encode 256 sulfation sequences, while an HS tetrasaccharide, which has greater complexity due to the

presence of IdoA and *N*-sulfation, can display over 2000 sulfation motifs. Although it remains to be seen whether all of these possible sulfation patterns occur *in vivo*, a large number of distinct sulfated structures have been identified to date.[10, 30]

On a macromolecular level, HS and CS polysaccharides exhibit various chain lengths (~ 10 to 100 disaccharide units) and clustered regions of high or low sulfation (Figure 5.5).[10] Structural studies have shown that GAGs adopt helical structures whose pitch may vary with the associated counterion.[32, 33] Moreover, the conformational flexibility of the pyranose ring of IdoA, which exists in equilibrium between different chair and skew-boat conformations when sulfated at the C2 position, has been postulated to enhance the specificity of HS for its protein targets.[10] Thus, the combination of sequence, charge distribution, sugar conformation, and three-dimensional structure endows GAGs with rich structural diversity.

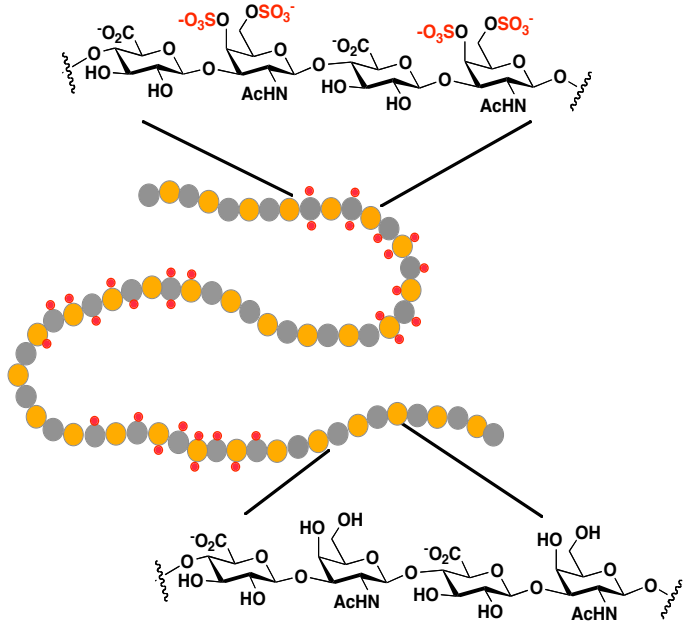

Figure 5.5. Heterogeneous GAG polysaccharides exhibit areas of low and high sulfation.

The potential sulfation code of HS and CS glycosaminoglycans

Evidence suggests that the chemical diversity of GAGs serves essential functions *in vivo*. Genetic studies have established the importance of GAGs and the various sulfotransferases that decorate the carbohydrate backbone. For instance, conditional knock-out mutants of the HS polymerizing enzyme EXT1 display severe CNS defects with a complete loss of olfactory bulbs, abnormally small cerebral cortices, and an absence of major commissural tracts.[34] Similarly, reducing the levels of the CS polymerizing enzyme in *C. elegans* resulted in decreased levels of cell surface CS and caused a reversion of cytokinesis, where cells were unable to divide and eventually died.[35] Deletion of 2-*O*-sulfotransferase activity in worms caused cell migration defects[36] while 2-*O*-sulfotransferase-deficient mice displayed a significant decrease in cell proliferation in the developing cerebral cortex[37]. Furthermore, mutation of the *N*-deacetylase/*N*-sulfotransferase gene in *Drosophila* inhibited growth factor signaling and disrupted embryonic development.[38] Together, these studies indicate an essential requirement for GAGs and their modifying enzymes during development.

Consistent with their essential roles, the sulfation patterns of GAGs are tightly regulated *in vivo*. Distinct sulfated forms are associated with particular tissues, developmental stages, and disease states. For instance, 2-*O*-sulfotransferase null mice present complete failure of kidney development[24, 39] while 6-*O*-sulfotransferase deficient flies exhibit embryonic lethality and disruption in initial branching of the tracheal system.[40] Additionally, differentially sulfated CS motifs are localized to specific brain regions and found along axonal growth tracts.[41, 42] The sulfation patterns of HS and CS are also altered during embryonic brain development, as are specific sulfotransferase

activities.[43, 44] Moreover, distinct HS motifs have been linked to the development of several diseases, including the pathology of Alzheimer's disease and cancer metastasis.[12, 14]

The molecular mechanisms by which GAGs contribute to these biological events are only beginning to be understood. However, many studies suggest that GAGs coordinate complex processes by regulating the activities of growth factors and other target proteins. The four main HS proteoglycan core proteins found in the nervous system are syndecans, glypicans, perlecan, and agrin.[1] In addition to modifying the above proteins, HS can bind a large variety of proteins found on the cell surface and in the extracellular matrix as well as potentiate the activity of secreted proteins.

One of the most studied examples is the binding of HS to the fibroblast growth factors (FGFs). The FGFs comprise a large family of growth factors (23 members to date) and have been shown to play critical roles in morphogenesis, development, angiogenesis and wound healing.[45] They activate signaling pathways by inducing the dimerization of tyrosine kinase receptors (FGFRs). Two distinct models have been proposed to explain the essential contribution of HS to FGF-FGFR signaling. A crystal structure of the HS-FGF2-FGFR1 complex obtained by Schlessinger, Mohammadi, and co-workers suggests that two ternary complexes of HS-FGF-FGFR come together with the non-reducing ends of each sugar chain facing one another upon activation (Figure 5.6A).[33] In contrast, the HS-FGF1-FGFR2 structure of Pellegrini, Blundell, and co-workers suggests that a single HS chain initiates the assembly of two FGF-FGFR complexes (Figure 5.6B).[46] Although these models propose distinct roles for HS in

coordinating receptor dimerization, both agree on the formation of an activated FGF-FGFR-HS complex.

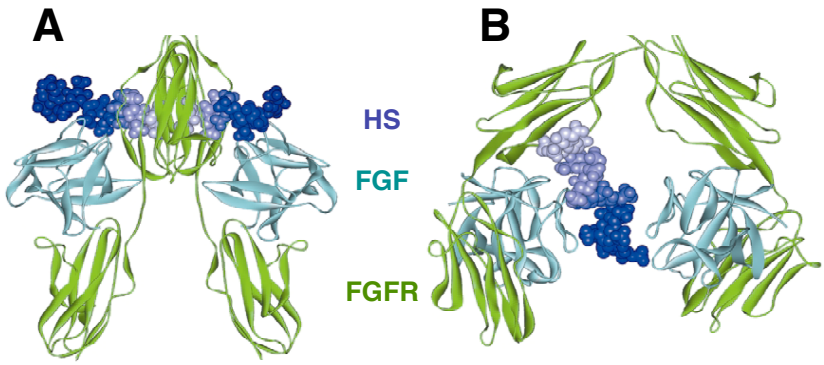

Figure 5.6. Crystallographic models of the FGF-FGFR-HS complex. A) The crystal structure of an FGF2-FGFR1-heparin complex has a 2:2:2 stoichiometry with two chains enabling complex formation. B) The crystal structure of an FGF1-FGFR2-heparin complex has a 2:2:1 stoichiometry with a single chain initiating complex formation.

Importantly, the specific sulfation pattern of HS appears to be critical for binding to FGF and assembly of the complex. Structural analyses have shown that many of the interactions between HS/heparin and the FGFs involve salt bridges and hydrogen-bonding contacts between the sulfate and carboxylate groups of the oligosaccharide with polar residues on FGF.[47] Optimal van der Waals contacts and the flexibility of HS chains may further enhance the interaction.[48] Interestingly, none of the residues in the heparin-binding region, including the polar side chains, are completely conserved throughout the FGF family.[47] This raises the intriguing possibility that variations in HS sequence or sulfation pattern might specify the binding of particular FGFs, allowing for the selective activation of signaling pathways. Consistent with this view, biochemical studies have shown that FGF-2 requires 2-*O*-sulfation, but not 6-*O*-sulfation, for HS binding, whereas FGF-10 has the reverse preference, and FGF-1 requires both 2-*O*-sulfation and 6-*O*-sulfation.[49] Distinct sulfation preferences are also exhibited by the FGFRs; for instance,

6-*O*-sulfation was required for FGFR2 IIIb but not FGFR1 activation.[50] Unfortunately, the heterogeneity of biochemical HS preparations has made it difficult to examine specific sulfation sequences. It is anticipated that homogeneous oligosaccharide libraries of defined sequence will provide additional insights into the importance of the sulfation code. Resolution of the code and precise activation mechanism will be critical for understanding growth-factor signaling and may reveal common mechanistic themes utilized by both HS and CS GAGs.

Recent studies have also revealed striking roles for GAGs and their sulfation patterns during neuronal development. Axons are guided to their target locations by diffusible and cell-surface-bound cues that either attract or repel the growing tip of the axon. One such cue is the cheomotropic axon guidance molecule Slit. Slit proteins can bind several different receptors and act as a chemorepellent signal for appropriate axon growth. Several studies have shown that HS is essential for Slit binding to its receptors. Removal of HS by heparinase treatment abolished Slit binding to glypican-1 and Robo.[51, 52] Absence of cell surface HS also altered Slit activity and prevented repulsion of migrating olfactory neurons and growing olfactory bulb axons.[51] Further evidence for the importance of HS in axonal guidance comes from mutants completely lacking HS in the developing brain. Conditional knock-out mutants of the HS polymerizing enzyme EXT1 displayed severe CNS defects.[34] Similar mutants in *C. elegans* also display severe developmental defects.[53]

In related studies, Bülow and Hobert used genetic approaches to probe the role of HS sulfation in axon guidance.[13] Abolishing the activity of three HS modifying enzymes, C-5 epimerase, 2-*O*-sulfotransferase, and 6-*O*-sulfotransferase, in *C. elegans* revealed

that particular neuron types require specific HS motifs for normal growth. Some axons required all three modifying enzymes, others required either C-5 epimerase or 2-*O*-sulfotransferase activity, and still other neuron types did not require any of the enzymes. These studies support the idea that distinct modifications to HS structure are essential for neuronal development and may encode instructions that guide neurons to their proper targets *in vivo*.

Along with HS, CS has been shown to be an important factor for neuronal development and axon guidance. Reducing levels of the CS polymerizing enzyme in *C. elegans* resulted in decreased levels of cell surface CS and caused a reversion of cytokinesis, where cells were unable to divide and eventually died.[35] Treatment of normal embryonic cells with chondroitinase ABC also caused incomplete cytokinesis, thus indicating a crucial role for CS in embryonic development.[54]

In the central nervous system, CS mainly acts as a barrier-forming molecule and there are many examples of such boundaries to growing axons. In the notochord, there are high levels of CS preventing axon extension.[55] Removal of CS with chondroitinase leads to misguided growth of embryonic motor nerves and dorsal root ganglion axons. In the optic pathway, CS prevents retinal axons from growing to the outer parts of the retina and guides them directly towards the optic nerve.[56] Retinal axons will grow randomly, however, upon treatment with chondroitinase. High levels of CS are also found in glial scars formed after injury to the CNS and are one of the main obstructions to axon regeneration. As detailed in the above studies, removal of CS abolishes the axon-growth barrier and thus can be a useful strategy to treat axon growth inhibition following CNS injury *in vivo*. Indeed, several studies have used chondroitinase treatment to regenerate

growth of dopaminergic neurons following nigrostriatal tract lesion as well as regrowth of sensory and motor axons after dorsal column lesions.[57, 58]

Despite the studies demonstrating inhibitory roles of CS on axon growth, there are contrasting studies showing stimulatory roles of CS on neurite growth. CS molecules with particular oversulfated structures (E and D motifs) enhance the outgrowth of hippocampal neurons as well as enable binding of particular growth-promoting receptors to their ligands (PTPζ or phosphacan to midkine and pleitrophin).[59 -- 62] CS polysaccharides enriched in the CS-C and CS-D motif have also been shown to promote the growth of mesencephalic dopaminergic neurons.[59, 63, 64] Moreover, CS has been associated with axonal growth tracts in the developing brain and thalamic neurons.[41, 65, 66]

In all, the above studies underscore the importance of GAG structure in regulating critical biological processes. The molecular diversity of GAGs may provide a powerful means to influence complex signaling pathways *in vivo*. The spatial and temporal regulation of HS and CS modifications could facilitate or inhibit ligand-receptor interactions in a highly localized manner. With the considerable diversity that exists in HS and CS chains, the sulfation code would represent an elegant means of molecular level control. It will be exciting to discover the extent to which Nature utilizes this potential.

Deciphering the sulfation code using chemistry

Deciphering the sulfation code will require the development of new strategies for manipulating and evaluating specific GAG structures. At present, there are no methods for the rapid identification of biologically active sulfation motifs. Genetic and

biochemical approaches have established critical roles for GAGs in particular biological contexts. However, deletion of a sulfotransferase gene leads to global changes throughout the carbohydrate chain, making it difficult to pinpoint the impact of a specific structural motif. GAGs have also been isolated from natural sources, but their structural complexity and heterogeneity are a significant limitation. The presence of multiple sulfation motifs in biochemical preparations complicates efforts to attribute a biological function to a specific sulfation motif. Moreover, studies with purified natural GAGs are biased toward abundant, readily isolable sequences. As such, it can be difficult to study physiologically important sulfation patterns that are present in low cellular abundance.

Chemical approaches provide a powerful solution to these challenges. Virtually any desired GAG structure can be generated using synthetic chemistry, with exquisite control over stereochemistry, length, and pattern of sulfation. Access to homogeneous, well-characterized structures facilitates the identification of biologically active sequences and enables systematic investigations into structure-activity relationships. As such, our research combines synthetic chemistry with neurobiology to enable the elucidation of the sulfation code of CS in the brain. We seek to generate the first synthetic library of well-defined CS oligosaccharides containing various sulfation sequences in order to evaluate and assign specific functions to distinct CS motifs. The ability to obtain defined GAG structures and related analogues should also accelerate investigations into the therapeutic potential of GAGs, in areas such as cancer biology, neurobiology, and virology.

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